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ADDENDUM

SUMMARY

p X para 1 line 10: After "MDM" insert "(high multiplicity of infection)"
p X para 2 line 12: After "monocytes" insert "(infrequently infected with HIV-1)"

CHAPTER 1

p 12 para 2 line 6: Delete "including" and insert "that include"
p 12 para 2 line 7 Before "altered metabolism" insert "and"
p 20, end of para 1: Insert: "eg via abnormal production of cytokines and chemokines produced by other peripheral blood cells infected with HIV-1"
p 31 para 2 line 5: Delete "Kupffer cells and microglial cells"

CHAPTER 3

p 72 para 2 line 6: Delete "Croydon, Australia" and insert " La Jolla, CA, USA "
p 73, last line: Delete "Kilsyth, Australia" and insert "Dorset, UK"
p 77 para 1 line 1: Delete "mediated by" and insert "associated with".
p 80, end para 1: Insert "It would be of interest to investigate the tyrosine phosphorylation pattern downstream of GM-CSF R and FcγR"
Fig.3.4, legend, line 6: Delete "cytoplasmic fraction" and insert "non-ionic detergent soluble fraction; described in section 2.8.3.3"

CHAPTER 4

p 92 last para line 4: Delete "Upstream of Hck and Syk"
p 94 line 3: Delete "any" and insert "many"
p94 para 2 line 6: After "associate with γ chain" insert "of FcγR"

CHAPTER 5

p 97, title: Delete "in vivo" and insert "ex vivo"
p 99 para1 line 6: After "since the latter" insert "studies"
p 102 para 2 line 3: After "GST" insert "(unrelated protein available in the laboratory)".
p 105 para 1 line 2: Delete "if" and insert "of"
Fig.5.1, legend, line 1: After "C54" insert "(see clinical details in Table 5.1, time-point IV)"

CHAPTER 6

p 120 para 1 line 7: Delete "NSW, Australia" and insert "Kenilworth, NJ, USA"
p125, para 4, line 8: Change "and" to "but"; also after "*in vivo*" insert "in this individual"
p 128, end para 1: Insert "Minimal but insignificant effects of E21R GM-CSF on HIV-1 replication in MDM may be due to its signalling via the α/β heterodimers of the GM-CSF receptor".
p 125 para 2 line5: Insert at end of para: "The reason for the increased bacteraemia on day 38 of GM-CSF therapy is unknown"

MECHANISMS UNDERLYING DEFECTIVE PHAGOCYTOSIS BY HUMAN MONOCYTES AND MACROPHAGES FOLLOWING HIV-1 INFECTION

A thesis submitted for the degree of

Doctor of Philosophy at Monash University

by

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SUMMARY

Cells of the macrophage lineage including peripheral blood monocytes and tissue macrophages play an important role in the pathogenesis of human immunodeficiency virus type 1 (HIV-1) infection. Following HIV-1 infection, a number of effector functions carried out by monocytes and macrophages are impaired, including phagocytosis of a variety of pathogens. Defective immunological function of these cells contributes to development of opportunistic infections in HIV-infected individuals. Although the treatment of HIV-infected individuals has improved with the introduction of highly active antiviral therapy (HAART), opportunistic infections are the major problem for patients failing HAART and their incidence is likely to increase, as the resistance to antiretroviral drugs becomes more widespread. Our understanding of the mechanisms by which HIV-1 impairs cell-mediated immunity leading to the development of opportunistic infections is critical for the full control of HIV-1 disease progression.

This study aimed to investigate the mechanisms underlying defective monocyte and macrophage function following HIV-1 infection. As monocytes and macrophages are distinct cell populations that differ in their susceptibility to HIV-1 infection (monocytes being refractory, whereas macrophages are fully permissive to HIV-1), impairment of phagocytosis in monocytes and macrophages was examined independently. The mechanism underlying defective phagocytosis mediated via Fcγ receptors (FcγR) by HIV-infected monocyte-derived macrophages (MDM) was the main focus of my doctoral studies. The results presented in this thesis show that the early signalling events during FcγR-mediated phagocytosis in human uninfected MDM are similar to those previously characterised in murine macrophages. In both species, FcγR-mediated phagocytosis is mediated via tyrosine kinase-dependent pathways involving the key tyrosine kinases Hck, Syk and Pyk2, and the actin-binding protein, paxillin (Kedzierska *et al.*, 2001b). The effect of HIV-1 on these signalling pathways was

investigated. Using various specifically-opsonised, defined targets it was shown that FcγR-mediated phagocytosis was inhibited in MDM infected with HIV-1 *in vitro*. The observed inhibition was not accompanied by altered surface levels of FcγRs, but was associated with the strong inhibition of tyrosine phosphorylation of cellular proteins stimulated during phagocytosis. Further results demonstrated impaired phosphorylation of tyrosine kinases Hck and Syk, defective formation of Syk complexes with other tyrosine-phosphorylated proteins and inhibition of paxillin activation. Down-modulation of protein expression but not mRNA of the γ signalling subunit of FcγR (a docking site for Syk) was observed in HIV-infected MDM. Having established that inhibition was independent of Nef, infection of MDM with a construct of HIV-1 in which *nef* was replaced with the gene for the γ signalling subunit augmented FcγR-mediated phagocytosis, consistent with the interpretation that downmodulation of γ-chain protein expression in HIV-infected MDM caused the defective FcγR-mediated signalling and impairment of phagocytosis (Kedzierska *et al.*, submitted to *J Immunol*).

Phagocytosis of the common opportunistic pathogens *Mycobacterium avium* complex (MAC) and *Toxoplasma gondii* by monocytes obtained from HIV-infected individuals and from the Sydney Blood Bank Cohort (SBBC) members was investigated. MAC phagocytic capacity of monocytes from individuals infected with wild type HIV-1 was impaired when compared to that of uninfected controls. Defective phagocytosis was irrespective of CD4 counts and HIV-1 viral load, but was associated with defective actin rearrangement during FcγR-mediated phagocytosis. Monocytes obtained from blood from SBBC members infected with an attenuated strain of HIV-1 containing deletions within the *nef* gene as well as rearrangements and deletions within the overlapping long terminal repeat displayed phagocytic efficiency equivalent to that of cells from uninfected individuals. These results suggest the importance of nef/LTR region in inhibiting phagocytosis by monocytes following HIV-1 infection *in vivo* (Kedzierska *et al.*, 2001a).

The final aim of my doctoral studies was to assess the effect of the immunomodulator and growth factor, granulocyte-macrophage colony-stimulating factor (GM-CSF) on phagocytosis by monocytes and macrophages following HIV-1 infection. GM-CSF was found to markedly augment the ability of HIV-infected MDM to phagocytose MAC *in vitro* (Kedzierska *et al.*, 2000b). Phagocytosis of MAC was not altered by exposure to a mutant form of GM-CSF (E21R) binding only to the alpha chain of the GM-CSF receptor, suggesting that augmentation of phagocytosis by GM-CSF requires binding to the αβ heterodimeric form of the receptor. A pilot clinical study confirmed the beneficial effect of GM-CSF in a patient with AIDS and disseminated multi-drug resistant MAC infection. GM-CSF therapy improved phagocytosis of MAC by peripheral blood monocytes obtained from this patient. GM-CSF treatment was accompanied by a reduction in MAC bacteremia as measured by the length of time required for blood cultures to become positive (Kedzierska *et al.*, 2000b). GM-CSF also consistently suppressed HIV-1 replication in MDM *in vitro*, as assessed by measurement of supernatant RT activity and intracellular p24 antigen. This effect was reversed by the addition of the neutralising anti-GM-CSF monoclonal antibody (4D4). Analysis of intracellular HIV-1 DNA and mRNA suggests that HIV-1 replication is inhibited at or before transcription (Kedzierska *et al.*, 2000a). These results imply that adjunctive GM-CSF therapy may be useful to augment monocyte and macrophage function and to restore antimycobacterial activity in HIV-1 infected patients with opportunistic infections. Therefore, this thesis combines basic research with some supporting clinical evidence to provide a scientific rationale for using GM-CSF treatment in the setting of HIV-1 infection.

LIST OF FIGURES

LIST OF TABLES

LIST OF PUBLICATIONS

The following publications have arisen from the research presented in this thesis:

Kedzierska, K., Rainbird, M.A., Lopez, A.F. and Crowe, S.M. (1998) Effect of GM-CSF on HIV-1 replication in monocytes/macrophages in vivo and in vitro: a review. *Vet Immunol Immunopathol*, **63:**111-121.

Kedzierska, K., Mak, J., Mijch, A., Cooke, I., Rainbird, M., Roberts, S., Paukovics, G., Jolley, D., Lopez, A. and Crowe, S.M. (2000) Granulocyte-macrophage colony-stimulating factor augments phagocytosis of *Mycobacterium avium* complex by HIV-1 infected monocyte/macrophages in vitro and in vivo. *J Inf Dis*, **181**:390-394.

Kedzierska, K., Maerz, A., Warby, T., Jaworowski, A., Chan, H., Mak, J., Sonza, S., Lopez, A. and Crowe, S.M. (2000) Granulocyte-macrophage colony-stimulating factor inhibits HIV-1 replication in monocyte-derived macrophages. *AIDS*, **14**:1739-1748.

Kedzierska, K., Vardaxis, N., Jaworowski, A. and Crowe, S.M. (2001) FcγR-mediated phagocytosis by human macrophages involves Hck, Syk and Pyk2, and is augmented by GM-CSF. *J Leukoc Biol*, **70**:322-328.

Kedzierska, K., Mak, J., Jawarowski, A., Greenway, A., Violo A, Chan, H., Hocking, J., Purcell, D., Sullivan, J., Mills, J. and Crowe, S.M. (2001) *nef*-deleted HIV-1 inhibits phagocytosis by monocyte-derived macrophages *in vitro* but not by peripheral blood monocytes *in vivo*. *AIDS*, **15**:945-955.

Kedzierska, K. and Crowe, S.M. (2001) Cytokines and HIV-1: interactions and clinical implications. *Antivir Chem & Chemother*, **12**:133-145.

Kedzierska, K. and Crowe, S.M. (2001) Culture of HIV in monocytes and macrophages. *Current Protocols in Immunology.* John Wiley & Sons, Inc. **2**:12.4.1-12.4.11.

Chan, H., **Kedzierska, K.,** O'Mullane, J., Crowe, S.M. and Jaworowski, A. (2001) Quantifying complement-mediated phagocytosis by human monocyte-derived macrophages. *Immunol Cell Biol.* **79**:429-435.

Kedzierska, K., Ellery, P., Mak, J., Jaworowski, A. and Crowe, S.M. (2002) Mechanisms underlying defective phagocytosis by human monocytes and macrophages following HIV-1 infection. *J Clin Virol* (in press).

Kedzierska K, Ellery P, Mak J, Lewin S, Crowe SM and Jaworowski A. HIV-1 downmodulates γ signalling chain of Fcγ receptor in human macrophages: a possible mechanism for inhibition of phagocytosis (*J Immunol,* responses to the reviewers' comments/questions submitted).

Kedzierska, K., Paukovics, G., Azzam, R., Crowe, S.M. and Jaworowski, A. An intracellular pool of CD16A in human monocytes and monocyte-derived macrophages is mobilised during Fcγ receptor-mediated phagocytosis (submitted to *J Leukoc Biol*).

DECLARATION OF AUTHENTICITY

This thesis contains no material that has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of this thesis. All research procedures reported in this thesis were approved by the relevant Ethics Committee.

Katarzyna Kedzierska

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ABBREVIATIONS

A	adenine
ADCC	antibody-dependent cellular cytotoxicity
AIDS	acquired immunodeficiency syndrome
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AZT	zidovudine
bDNA	branched DNA
bp	base pair
BSA	bovine serum albumin
C	cytidine
CA	capsid protein (p24)
cAMP	cyclic AMP
CD	cluster differentiation
cDNA	complimentary DNA
CFU	colony-forming unit
cpm	counts per minute
C'R	complement receptor
CTL	cytotoxic T lymphocytes
DC-SIGN	dendritic cell-specific ICAM-3-grabbing nonintegrin
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol

E	sheep red blood cells
E-IgG	IgG-opsonised sheep red blood cells
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
EthBr	ethidium bromide
F-actin	filamentous (polymerised) actin
FACS	fluorescence activated cell sorter
FAK	focal adhesion kinase
FcγR	Fcγ receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
fMLP	*formyl*-methionyl-leucyl-phenylamine
G	guanine
g	gram
G-actin	monomeric actin
GDP	guanosine diphosphate
GM-CSF	granulocyte-macrophage colony-stimulating factor
GST	glutathione S-transferase
GTP	guanosine triphosphate
HAART	highly active antiretroviral therapy
HIS	heat-inactivated serum
HIV	human immunodeficiency virus

HLA	human leukocyte antigen
hr	hours
HRPO	horseradish peroxidase
IB	immunoblotting
IFN	interferon
Ig	immunoglobulin
IgG	immunoglobulin G
IL	interleukin
IN	integrase
IP	immunoprecipitation
ITAMs	immunoreceptor tyrosine-based activation motifs
kb	kilobase
kDa	kilodalton
LPS	lipopolysaccharide
LTR	long terminal repeats
M	molar
M-tropic	macrophage-tropic
MA	matrix protein (p17)
Mab	monoclonal antibody
MAC	*Mycobacterium avium* complex
MAC-FITC	fluoresceinated *Mycobacterium avium* complex
M-CSF	macrophage-colony stimulating factor
MDM	monocyte-derived macrophages
MESF	molecules of equivalent soluble fluorochrome

mg	milligram
MHC	major histocompatibility complex
min	minutes
MIP	macrophage inflammatory protein
ml	millilitre
MLCK	myosin light chain kinase
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger RNA
MW	molecular weight
NAK	Nef-associated kinase
NC	nucleocapsid (p7)
ng	nanogram
NO	nitric oxide
NM40	Nonited-40
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAK1	p21-activated kinase
PBMC	peripheral blood mononuclear cells
pbs	primer binding site
PBS	phosphate-buffered saline
PBS-CMF	calcium- and magnesium-free PBS
PCR	polymerase chain reaction
PE	phycoerythrin

PHA	phytohemagglutinin
PI3-kinase	phosphatidyl inositide 3-kinase
PMA	phorbol-12-myristate-13-acetate
PR	protease
PY	phosphotyrosine
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
RT-PCR	reverse transcriptase PCR
SBBC	Sydney Blood Bank Cohort
SD	standard deviation
SDF	stromal-derived factor
SDS	sodium dodecyl sulphate
sec	seconds
SEM	standard error of the mean
SSC	sodium chloride/sodium citrate buffer
SU	surface glycoprotein (gp120)
T	thymidine
T-tropic	T cell-tropic
TAE	tris, sodium citrate buffer
TBS	tris-buffered saline
$TCID_{50}$	50% tissue culture infectivity dose
TE	Tris-EDTA
TEMED	N,N,N'N'-tetramethyl-ethylenediamine

TGA	Therapeutic Goods Administration
T.gondi	*Toxoplasma gondii*
T.gondi-FITC	fluoresceinated *Toxoplasma gondii*
Th	T helper
TLB	Triton-X 100 lysis buffer
TM	transmembrane glycoprotein (gp41)
TNF	tumour necrosis factor
Tris	tris[hydroxymethyl]aminomethane
UV	ultraviolet
µg	microgram
µl	microliltre
µM	micromolar
V	volts
VL	viral load
× *g*	times gravitational constant
Y	tyrosine

CHAPTER 1

GENERAL INTRODUCTION

1.1 OVERVIEW.

The acquired immunodeficiency syndrome (AIDS) was originally recognised in 1981 following observations of increasing prevalence of *Pneumocystis carinii* pneumonia, Kaposi's sarcoma and chronic herpes simplex lesions in previously healthy homosexual men (Gottlieb *et al.*, 1981; Masur *et al.*, 1981; Siegal *et al.*, 1981). These clinical observations were associated with low CD4 T cell counts, a reversed ratio of CD4 T cells to CD8 T cells and impaired T cell function (Masur *et al.*, 1981). The causative virus was isolated from individuals with AIDS in 1983 (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983; Levy *et al.*, 1984) and eventually named the human immunodeficiency virus type 1 (HIV-1). A second, related virus was isolated from AIDS patients in West Africa in 1986 and named the human immunodeficiency virus type 2 (HIV-2) (Clavel *et al.*, 1986). Although both viruses cause AIDS, the pathogenicity and transmissibility of HIV-2 are much lower than those of HIV-1 (reviewed in Barre-Sinoussi, 1996). HIV-2 is prevalent in West Africa, whereas HIV-1 is present in most parts of the world, including Australia, and will be the focus of this review.

Since the identification of AIDS two decades ago, the epidemic has resulted in 21.8 million deaths, with an estimated 36.1 million people currently living with HIV. In 2000 alone, 5.3 million people became infected with the virus, of whom 600 thousand were children (UNAIDS website, accessed in Aug 2001). Although the therapeutic efficacy of antiretroviral drugs has improved considerably over the past six years, AIDS remains incurable. A more complete understanding of the biology of HIV-1 and the pathogenesis of AIDS will facilitate the development of novel drug therapies and effective vaccines for the full control of HIV-1 infection.

1.2 ETIOLOGY OF AIDS.

HIV, the etiologic agent of AIDS, is a member of the lentivirus subfamily of retroviruses. Lentiviruses differ from other retroviruses by their complex genomes and cone-shaped capsid core particles. They infect cells of haemopoietic and nervous systems, display long latent periods before the onset of disease and lead to immunosuppression (Coffin, 1992). Other examples of lentiviruses include the simian immunodeficiency virus, bovine immunodeficiency virus, feline immunodefiicency virus, equine infectious anaemia virus, visna maedi virus and caprine arthritis encephalitis virus. All retroviruses, including HIV-1, use RNA to encode their genome, which is reverse-transcribed to DNA by viral reverse transcriptase (RT) before integration into the host cell's chromosomal DNA. The retroviruses have three major genes in common, viz *gag, pol* and *env* and may contain additional regulatory and accessory genes. In the case of HIV-1 these are *tat, rev, nef, vif, vpu* and *vpr.*

1.2.1 Virion structure.

The HIV-1 virion is spherically shaped and approximately 110nm in diameter (Evans and Levy, 1989). Similar to other lentiviruses, HIV-1 is enveloped by a lipid bilayer derived from the host cell membrane and traversed by 72 glycoprotein spikes (Figure 1.1). Surface glycoprotein (SU, gp120) is anchored to the virus via its noncovalent interaction with the transmembrane glycoprotein (TM, gp41). Under the lipid bilayer a matrix shell comprising approximately 2000 copies of the matrix protein (MA, p17) lines the inner surface of the viral membrane, whereas approximately 2000 copies of the capsid protein (CA, p24) form a conical capsid core. The core of the virion is composed of two copies of the unspliced viral genomic RNA complexed with approximately 2000 copies of the nucleocapsid protein (NC, p7) and 70 to 100 copies of the viral enzymes:

protease (PR), reverse transcriptase (RT) and integrase (IN) (reviewed in Turner and Summers, 1999).

1.2.2 HIV-1 genomic organisation.

The HIV-1 genome is approximately 9.5 kb in length and is composed of two identical, positive sense, single-stranded RNAs. The HIV-1 provirus contains open-reading frames encoding three structural virus-associated polyproteins (Gag, Pol and Env), flanked by two long terminal repeats (LTR), and sequences encoding six regulatory and accessory proteins (Figure 1.2). Each LTR is organised into three domains: U3 (untranslated 3'), R (the repeated) and U5 (the untranslated 5'). The Gag and Pol polyproteins are encoded by overlapping reading frames. The Gag 55 kDa precursor is synthesised from unspliced mRNA. The Gag-Pol 160 kDa precursor does not have its own initiation codon, and its synthesis results from a -1 translational frameshift event with respect to *gag* (Jacks *et al.*, 1988). Gag polyprotein is proteolytically cleaved during virus maturation into three structural proteins: MA, CA and NC, and three additional cleavage products p1, p2 and p6. Pol is cleaved into three enzymes PR, RT and IN, whereas the Env 160 kDa precursor is cleaved into two envelope proteins, surface glycoprotein gp120 and transmembrane glycoprotein gp41 (reviewed in Wang *et al.*, 2000).

Regulatory and accessory proteins are products of spliced mRNA. The HIV-1 genome encodes three regulatory proteins: Tat (transactivator of transcription), Rev (regulator of expression of virion proteins) and Nef (negative factor) as well as three accessory proteins: Vif (virion infectivity factor), Vpr (viral protein r) and Vpu (viral protein u). These proteins play important roles at multiple stages of the HIV-1 replication cycle, including reverse transcription, transcription, assembly and budding (summarised in Table 1.1).

1.2.3 Cellular targets for HIV-1.

The major targets for HIV-1 are cells expressing CD4 molecules on their surface, mainly the CD4 subset of T cells and cells of macrophage lineage. Both cell types express chemokine co-receptors for HIV-1 entry, which are important for the cellular tropism of HIV-1. The G protein-coupled seven-transmembrane chemokine receptors have been identified as co-receptors for HIV-1 entry. The β-chemokine co-receptor, CCR5, is the major co-receptor for macrophage (M)-tropic (R5) strains of HIV-1 (Alkhatib *et al.*, 1996; Dragic *et al.*, 1996), whereas the α-chemokine co-receptor, CXCR4, is the main co-receptor for T-tropic (X4) strains of HIV-1 (Feng *et al.*, 1996). A restricted subset of M-tropic strains of HIV-1 can also use the chemokine co-receptors CCR2 and CCR3 for the viral entry (Choe *et al.*, 1996). M-tropic strains of HIV-1 infect cells of macrophage lineage, primary CD4 T cells, but not T cell lines *in vitro* (Collman *et al.*, 1989; Gartner and Popovic, 1990). These strains of HIV-1 do not induce syncytium formation, replicate slowly in comparison with syncytium-inducing strains (reviewed in Fenyo *et al.*, 1989) and can be isolated at all stages of disease (Schuitemaker *et al.*, 1991). T-tropic strains of HIV-1 infect CD4 T cells and T cell lines *in vitro*, but not primary monocytes or macrophages (Collman *et al.*, 1989). These strains have syncytium-inducing phenotypes, are more cytopathic than M-tropic strains and generally emerge at late stages of HIV-1 infection (Connor *et al.*, 1993).

Activated CD4 T cells are readily infected with HIV-1, resulting in cytopathology and cell death (Barre-Sinoussi *et al.*, 1983; Popovic *et al.*, 1984; Zagury *et al.*, 1986) via syncytium formation or apoptosis (Sodroski *et al.*, 1986; Stevenson *et al.*, 1988). Although resting CD4 T cells can also be infected with HIV-1, the viral genome is integrated into chromosomal DNA and remains unexpressed in the absence of activation (Stevenson *et al.*, 1990; Finzi *et al.*, 1997; Wong *et al.*, 1997).

Cells of macrophage lineage, including both blood monocytes and tissue macrophages are important targets for HIV-1 infection *in vivo* and *in vitro* (Ho *et al.*, 1986; Crowe *et al.*, 1987) and are described in more detail in Section 1.4.1.

Dendritic cells are, to a lesser degree, susceptible to HIV-1 infection and can support HIV-1 replication (Pope *et al.*, 1995; Canque *et al.*, 1999). These cells play also an important role in the initial mucosal infection by capturing HIV-1 in peripheral tissue and transporting it to lymph nodes, where it infects activated CD4 T cells (Cameron *et al.*, 1992; Pope *et al.*, 1994). Dendritic cells express the C-type lectin "dendritic cell-specific ICAM-3-grabbing nonintegrin" (DC-SIGN) on their surface, which binds HIV-1 envelope glycoprotein and facilitates the contact with CD4 T cells (Geijtenbeek *et al.*, 2000).

1.2.4 Viral replication.

1.2.4.1 Viral entry.

HIV-1 infection of CD4-expressing cells is initiated by high affinity binding between envelope glycoprotein gp120 and the amino-terminal extracellular domain of CD4 molecules (Landau *et al.*, 1988; Arthos *et al.*, 1989) (Figure 1.3a). This interaction results in conformational changes in gp120, exposes its chemokine receptor-binding domain and facilitates the interaction with chemokine co-receptors (Trkola *et al.*, 1996). Subsequent conformational changes in gp41 allow the fusion between the lipid envelope of the virion and the cell membrane, followed by the entry of the virion into the cytoplasm (Dimitrov, 1997) (Figure 1.3b).

1.2.4.2 Reverse transcription.

Following viral entry, the cone-shaped capsid core is uncoated within the cytoplasm prior to reverse transcription. The process of reverse transcription is mediated by

virion-associated RT (Baltimore, 1970; Temin and Mizutani, 1970) and primed by cellular transfer RNA specific for lysine ($tRNA^{lys}$). The cellular derived $tRNA^{lys}$ is packaged into HIV-1 during virion assembly via Gag-Pol precursor protein (Mak *et al.*, 1994). The primer $tRNA^{lys}$ is annealed to the primer-binding site positioned immediately downstream of the 5'R sequence of the HIV-1 genome. The synthesis of viral cDNA includes two template switching events and the degradation of the viral genomic RNA template by HIV-1 RT-associated RNase H activity (reviewed in Mak and Kleiman, 1997). Completion of cDNA synthesis results in a linear double-stranded molecule with an LTR at each end (Goff, 1990) (Figure 1.3c).

1.2.4.3 Integration.

The viral cDNA is transported to the host cell's nucleus together with IN, MA, RT and Vpr as a preintegration complex (Miller *et al.*, 1997). This process is directed by Vpr (Vodicka *et al.*, 1998) and is followed by random integration of the viral cDNA into the host chromosomal DNA by the catalytic activity of IN (Levy, 1993b). IN removes two nucleosides from the 3' end of both viral DNA strands. This is followed by insertion of viral DNA into chromosomal DNA that has been cleaved asymmetrically (Pauza, 1990) (Figure 1.3d). Depending on the state of activation, the proviral cDNA may remain latent in the target cell or produce viral mRNA.

1.2.4.4 Transcription.

Transcription is initiated at the U3-R interface of the 5' LTR by cellular RNA polymerase II and results in unspliced (9kb), singly spliced (4kb) and multiply spliced (2kb) mRNA transcripts. The multiply spliced mRNAs encoding the regulatory proteins Tat, Rev, and Nef are transported out of the nucleus and subsequently synthesised. Nef is the first protein that accumulates in HIV-infected cells. It plays an important role in downregulation of CD4 receptors (Garcia *et al.*, 1993; Rhee and Marsh, 1994) and

therefore protects cells from superinfection. Tat is the essential regulator of HIV-1 transcription as it increases transcriptional initiation and improves the processivity of RNA polymerase II (Wei *et al.*, 1998). Rev mediates the export of unspliced (encoding Gag and Gag-Pol proteins) and single spliced (encoding Env, Vpu, Vif and Vpr) cellular mRNAs from nucleus to the cytoplasm. This is achieved by binding of Rev to the Rev Response Element (RRE).

Binding of Rev to the RRE present in unspliced and single spliced mRNAs results in the recruitment of the cellular nuclear shuttling protein exportin-1 (Ohno *et al.*, 1998) and the GTP-bound form of the nuclear export factor Ran guanosine triphosphatase (Pollard and Malim, 1998). This complex is transported through the nuclear pore to the cytosol, where it dissociates following hydrolysis of GTP to GDP. Rev is then imported back to the nucleus via its amino-terminal nuclear localisation signal (NLS) (Emerman and Malim, 1998). The Env precursor, gp160, is synthesised from the single spliced *env* mRNA. It oligomerises to a trimeric structure in the endoplasmic reticulum (ER) where it is glycosylated (Chan *et al.*, 1997; Wyatt and Sodroski, 1998), cleaved into the non-covalently associated gp120-gp41 trimeric glycoprotein complex and transported to the cell membrane for virus assembly. Gag and Gag-Pol are synthesised from unspliced mRNA and subsequently directed to the cellular membrane by the amino-terminally myristoylated MA domain of the polyproteins (Bryant and Ratner, 1990).

1.2.4.5 Viral assembly and budding.

Viral proteins and RNA are assembled at the cell membrane in CD4 T cells or predominantly within intracytoplasmic vesicles in macrophages (Gendelman *et al.*, 1988). The formation of the virion particle is initiated by multimerisation of the 55 kDa Gag precursor and incorporation of the 160 kDa Gag-Pol precursor and $tRNA^{Lys}$. Proteolytic cleavage of $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ results in maturation of the virion. The accessory proteins Vif, Vpr and Nef are also packaged into the virion. The viral capsid

buds through the cell membrane and incorporates the viral envelope glycoprotein present at the cell surface (Levy, 1993a) (Figure 1.3).

1.3 PATHOGENESIS OF HIV-1 INFECTION.

1.3.1 The clinical course of HIV-1 infection in untreated individuals.

HIV-1 is transmitted by exposure to blood and blood products, genital sexual contact or perinatal transmission. Primary HIV-1 infection is symptomatic in 40 to 60% of individuals, causing an acute mononucleosis-like syndrome within 2 to 6 weeks after infection. Symptoms of this acute HIV-1 seroconversion illness include fever, sweating, malaise, myalgia, headache, ulcers, nausea, and diarrhoea (Cooper *et al.*, 1985). During this early period after infection plasma concentrations of HIV-1 RNA (viral load) are very high, often reaching more than one million of copies of HIV-1 RNA per ml (Figure 1.4). Such a high viral load is accompanied by a transient decline in the CD4 T cell count and extensive viral dissemination to lymphoid organs (Pantaleo *et al.*, 1993). Within 1 to 4 weeks after initial infection, humoral and cellular immune responses are initiated, leading to a decline in the viral load and restoration of the CD4 T cell count (Clark *et al.*, 1991). Cytotoxic T lymphocytes (CTL; specialised CD8 T cells which eliminate cells expressing viral proteins in association with MHC class I) play a critical role in the control of HIV-1 infection. The appearance of virus-specific CTL correlates with viral decline (McKinney *et al.*, 1999), and higher CTL numbers correspond to a lower plasma viral load (Koup *et al.*, 1994). An equilibrium between HIV-1 and host immunity is usually established between 6 to 9 months after infection. At that time, the steady-state plasma viral load ("virologic set point") (Ho, 1996) predicts the long-term clinical outcome of infection in untreated patients (Figure 1.4). A low virologic set point correlates with longer clinical latency, whereas a high set point correlates with more rapid progression to AIDS (Mellors *et al.*, 1996).

The acute phase of HIV-1 infection is followed by a period of clinical latency with no overt signs of disease, but a steady state of viral replication (Embretson *et al.*, 1993). During that period the high rate of HIV-1 production of up to 10^9 virions per day (Ho *et al.*, 1995; Wei *et al.*, 1995) and destruction of HIV-infected CD4 T cells with a half-life of less than two days (Ho *et al.*, 1995; Perelson *et al.*, 1997) is efficiently controlled by the immune system. The duration of clinical latency varies considerably among HIV-infected patients and may be affected by genetic, environmental and viral factors (discussed in Section 1.3.2). Eventually, without antiretroviral treatment the immune system fails and CD4 T cell levels steadily decline as the viral load steadily increases. The progressive degeneration of the immune system is associated with defective immunological functioning of CD4 T cells (Gruters *et al.*, 1990; Shearer and Clerici, 1991), CD8 T cells (Trimble and J, 1998), monocytes (Bravo-Cuellar *et al.*, 1992) and macrophages (Musher *et al.*, 1990) (discussed in Section 1.4.3) as well as dysregulation of cytokine production (Clerici and Shearer, 1993) (discussed in Section 1.3.4). The later stages of HIV-1 infection are characterised by a sudden depletion of CD4 T cells, which partly results from the appearance of T-tropic strains of HIV-1, which are responsible for syncytium formation and CD4 T cell destruction (Collman *et al.*, 1992; Connor *et al.*, 1993).

The immune dysfunction caused by HIV-1 infection results in immunosuppression and development of HIV-related symptoms. As the CD4 T cell count declines, patients may develop a number of HIV-related complications including recurrent mucocutaneous herpes simplex, herpes zoster, oral candidiasis and oral hairy leukoplakia (reviewed in McCullough *et al.*, 1997). From cohort studies in the absence of antiretroviral treatment, the onset of AIDS-defining opportunistic infections and malignancies has generally been shown to occur in individuals with CD4 counts of <200 cells/μl and high plasma viral load about 9 to 10 years after infection. The most common opportunistic pathogens are *Pneumocystis carinii*, *Mycobacterium avium* complex, *Mycobacterium*

tuberculosis, Toxoplasma gondii, Candida albicans, and cytomegalovirus, whereas AIDS-related malignancies include Kaposi's sarcoma, non-Hodgkin's lymphoma, intraepithelial cervical carcinoma and anal neoplasia. HIV-infected patients may also develop a subacute dementia and a wasting syndrome.

1.3.2 Long-term non-progressors.

A small proportion of HIV-infected individuals (<5%) remains asymptomatic with normal CD4 T cell counts and low viral load for more than 10 years in the absence of antiretroviral therapy (Buchbinder *et al.*, 1994). Several genetic or viral factors may prolong the duration of the asymptomatic phase. The majority of long-term non-progressor or slow-progressor cases have been due to host factors. One of the most important genetic factors is the expression of the chemokine co-receptor for HIV-1, CCR5. Long term non-progression has been associated with a deletion in the CCR5 allele, named CCR5-Δ32, which encodes for a non-functional truncated CCR5 receptor (Samson *et al.*, 1996). Individuals homozygous for CCR5-Δ32 are highly resistant to HIV-1 infection, whereas heterozygosity for CCR5-Δ32 delays the progression to disease by an average of 2 years (Dean *et al.*, 1996). Specific alleles of HLA loci including class I B27, B52 and B57, and class II DR1, DR4, DR7 have been also associated with slower progression to AIDS (Kaslow *et al.*, 1996; Klein *et al.*, 1998). Long-term non-progressors may also display a broad and potent HIV-specific CTL response for effective control of HIV-1 replication and associated with stable CD4 T cell counts (Rinaldo *et al.*, 1995; Cao *et al.*, 1995; Munoz *et al.*, 1995; Shankarappa *et al.*, 1999).

In a minority of cases, long term non-progression is due to viral factors. Clinical studies of the Sydney Blood Bank Cohort (SBBC), consisting of a blood donor and eight transfusion recipients infected with a strain of HIV-1 containing deletions within the *nef*

gene, as well as duplications and deletions within the overlapping LTR (Deacon *et al.*, 1995), suggest the importance of Nef/LTR region for HIV-1 pathogenesis and disease progression. Whilst there is evidence of recent clinical progression in two of the cohort members after 17 years of infection (Learmont *et al.*, 1992; Learmont *et al.*, 1999), the virus is clearly attenuated in comparison to wild type HIV-1 strains, indicating that viral factors may delay disease progression.

1.3.3 The course of HIV-1 infection in the era of HAART.

With the introduction of antiretroviral drugs and the development of sensitive assays to monitor plasma viral loads, treatment of HIV-infected individuals has improved considerably (Carpenter *et al.*, 2000). Currently, there are sixteen antiretroviral drugs approved by the Australian Therapeutic Goods Administration (TGA) for treatment of AIDS. They are nine RT inhibitors (six nucleoside inhibitors and three non-nucleoside inhibitors) and seven PR inhibitors (Dr. Anne Mijch, personal communications). Highly active antiretroviral therapy (HAART), consisting of combinations of three or more of these drugs has been very successful in delaying the progression of AIDS and reducing plasma viral loads in many cases to undetectable levels (Hammer *et al.*, 1996; Hammer *et al.*, 1997; Gulick *et al.*, 1997). The viral load in lymphoid tissues is reduced to a lesser extent (Cavert *et al.*, 1997). HAART also improves the CD4 T cell count and hence decreases the occurrence of opportunistic infections and AIDS-related morbidity and mortality (Palella *et al.*, 1998) (Figure 1.5).

The persistence of latent reservoirs of HIV-1 is a problem and prevents eradication of HIV-1 even by prolonged HAART (Ho, 1998; Saag and Kilby, 1999). In patients receiving HAART and characterised by long-term undetectable viral load, replication-competent HIV-1 can be recovered from resting CD4 T cells (Finzi *et al.*, 1997; Chun *et al.*, 1997; Wong *et al.*, 1997) or peripheral blood monocytes (Crowe and Sonza, 2000;

Sonza *et al.*, 2001). Viral reservoirs therefore represent a major obstacle to curing HIV-1 infection with HAART. They are established during primary infection: initiation of HAART as early as 10 days after development of symptoms of acute infection does not prevent their establishment (Chun *et al.*, 1998). As the half-life of the resting CD4 T cell reservoir is between 6 to 44 months and such reservoirs can be replenished by small burst of viremia, it is estimated that the time to eradication would take up to 60 years of continuous HAART with maximal suppression of HIV-1 replication (Zhang *et al.*, 1999; Pierson *et al.*, 2000). CTL cannot eliminate latently infected cells containing HIV-1 proviral DNA, but not expressing viral proteins. In summary, viral reservoirs represent a life long persistence of replication-competent forms of HIV-1, and although current antiretroviral regimens are associated with clinical improvement, they are unlikely to eradicate HIV-1.

HAART is also associated with the development of HIV-1 strains resistant to antiretroviral drugs. Multiple drug resistance has been observed (Hecht *et al.*, 1998) and raises a great concern. Opportunistic infections are a major problem for patients failing HAART and are likely to increase, as the resistance to antiretroviral drugs becomes more widespread. Resistance develops as a result of low plasma drug levels due to factors including poor adherence, reduced absorption (eg due to diarrhoea), altered metabolism (eg. drug interactions). The adverse effects and toxicities of antiretroviral drugs, including nausea, diarrhoea, myopathy, neuropathy, abdominal pain and hyperglycemia are common and often lead to cessation of the treatment and/or poor compliance (reviewed in Landor, 1998).

1.3.4 Abnormal cytokine profile in HIV-1 infected individuals.

Cytokines play an important role in controlling the homeostasis of the immune system. HIV-1 infection at all stages of the disease is associated with chronic immune activation

and dysfunctional cytokine production *in vivo*. Decreased secretion of specific cytokines and increased production of others contributes to the progression of HIV-associated immune deficiency. Using different experimental approaches, numerous investigators have examined cytokine production by cells from HIV-infected individuals as well as within cultures infected with HIV-1 *in vitro* (summarised in Table 1.2). Several groups have demonstrated that HIV-1 infection is associated with a switch from a predominantly T helper (Th) type 1 to Th type 2 response. During the course of HIV-1 infection, secretion of Th type 1 cytokines such as interleukin (IL)-2, and interferon gamma (IFN)-γ, is generally decreased, whereas production of Th type 2 cytokines, IL-4, IL-10 is increased (Clerici and Shearer, 1993). Increased production of pro-inflammatory cytokines, ie. IL-1, IL-6, IL-8 and tumor necrosis factor (TNF)-α (Esser *et al.*, 1991; Esser *et al.*, 1996) is thought to activate HIV-1 replication and maintain active HIV-1 expression via binding of NF-κB to LTR (Granowitz *et al.*, 1995; Munoz-Fernandez *et al.*, 1997). HIV-1 infection is also associated with increased secretion of the α-chemokine, stromal-derived factor (SDF)-1 (Ohashi *et al.*, 1998), and the β-chemokines, macrophage-inflammatory protein (MIP)-1α, MIP-1β, RANTES (Garzino-Demo *et al.*, 1999). Such abnormal cytokine production contributes to the pathogenesis of HIV-1 infection by impairing cell-mediated immunity. Dysregulated cytokine profiles can be partially reversed with HAART resulting in increases in IL-2 and IFN-γ secretion (but not decreased IL-4 and IL-10 levels), paralleled by suppression of HIV-1 replication and restored CD4 T cell counts (Imami *et al.*, 1999).

A number of cytokines have been shown to subsequently modulate HIV-1 infection and replication *in vitro* in both CD4 T lymphocytes and cells of macrophage lineage. The effects of cytokines on HIV-1 can be either inhibitory, stimulatory or bifunctional (ie. both inhibitory and stimulatory) (summarised in Table 1.3), indicating that HIV-1 infection and replication *in vivo* and *in vitro* is under the continuous regulation of a complex cytokine network produced by a variety of cells. HIV-inductive cytokines

include TNF-α and TNF-β (Folks *et al.*, 1989; Novak *et al.*, 1990), IL-1 (Poli *et al.*, 1994) and IL-6 (Poli *et al.*, 1990; Poli *et al.*, 1992), which stimulate HIV-1 replication in T cells and macrophages; IL-2 (Foli *et al.*, 1995), IL-7 (Moran *et al.*, 1993) and IL-15 (Lucey *et al.*, 1997), which up-regulate HIV-1 in T cells, and macrophage-colony stimulating factor (M-CSF) (Perno *et al.*, 1992), which stimulates HIV-1 in macrophages. HIV-suppressive cytokines include IFN-α and IFN-β (Kornbluth *et al.*, 1989; Poli *et al.*, 1989) and IL-16 (Maciaszek *et al.*, 1997), which inhibit HIV-1 replication in T cells and monocyte-derived macrophages (MDM); IL-10 (Weissman *et al.*, 1994) and IL-13 (Montaner *et al.*, 1993) which inhibit HIV-1 in MDM. Bifunctional cytokines such as IFN-γ (Kornbluth *et al.*, 1989; Vyakarnam *et al.*, 1990), IL-4 (Schuitemaker *et al.*, 1992; Naif *et al.*, 1994) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Koyanagi *et al.*, 1988; Kornbluth *et al.*, 1989; Matsuda *et al.*, 1995), have been shown to have both inhibitory and stimulatory effects on HIV-1. Bifunctional cytokines may exert suppressive or inductive effects on HIV-1 infection and replication, depending on the state of differentiation of monocytes and the experimental protocols used by various laboratories (Kazazi *et al.*, 1992; Schuitemaker *et al.*, 1992b; Naif *et al.*, 1997). The β-chemokines, MIP-1α, MIP-1β and RANTES are important inhibitors of macrophage-tropic strains of HIV-1 (Cocchi *et al.*, 1995) by binding to CCR5 and preventing HIV-1 binding. Similarly, the α-chemokine SDF-1 suppresses infection of T cells by T-tropic strains of HIV-1 (Oberlin *et al.*, 1998). Therefore, there is a complex and dysregulated interplay between cytokines and HIV-1 *in vivo* (Figure 1.6). The modulation of infection by exposure of cells to specific cytokines *in vitro* is a reductionist approach which may oversimplify the biologic situation.

1.4 ROLE OF MONOCYTE/MACROPHAGES IN HIV-1 PATHOGENESIS.

1.4.1 Cells of macrophage lineage as a target and reservoir for HIV-1.

Cells of the macrophage lineage contribute to the pathogenesis of HIV-1 infection throughout the course of the disease (reviewed in Crowe, 1995). They express CD4 receptor (Gartner *et al.*, 1986; Ho *et al.*, 1986; Crowe *et al.*, 1987) and CCR5 co-receptor (Alkhatib *et al.*, 1996) for HIV-1 entry, and hence are targets for M-tropic strains of HIV-1. Both blood monocytes and tissue macrophages can be infected with HIV-1 *in vitro* and *in vivo* and persist in the circulation or tissues as viral reservoirs (Gartner *et al.*, 1986; Salahuddin *et al.*, 1986; Crowe *et al.*, 1987; McElrath *et al.*, 1989).

1.4.1.1 Tissue macrophages.

Tissue macrophages are major targets for HIV-1 infection (reviewed in Crowe, 1995). Resident tissue macrophages such as alveolar macrophages (Rich *et al.*, 1992; Lewin *et al.*, 1996), peritoneal macrophages (Olafsson *et al.*, 1991) and placental macrophages (Kesson *et al.*, 1993) are readily susceptible to HIV-1 infection *in vitro* on the day of isolation. Other specialised tissue macrophages including Kupffer cells (Schmitt *et al.*, 1990) and microglia (Jordan *et al.*, 1991) can also be productively infected with HIV-1. The proportion of HIV-infected macrophages within tissues is relatively high and ranges from 1 to 50% depending on the site of infection (Clarke *et al.*, 1990; Plata *et al.*, 1987; Stoler *et al.*, 1986; McElrath *et al.*, 1989; Embretson *et al.*, 1993; Hufert *et al.*, 1993; McIlroy *et al.*, 1996; Orenstein *et al.*, 1997; Lewin *et al.*, 1998). Although tissue macrophages are highly susceptible to HIV-1 infection, they are responsible for producing only a small proportion of the total viral load in HIV-infected individuals when compared to CD4 T cells (Meltzer *et al.*, 1990). Tissue macrophages infected with HIV-1 are resistant to cytopathic effects and persist throughout the course

of infection (Gartner *et al.*, 1986). Their half-life is very long, depending on the cell type and the anatomical location, and ranges from as long as about two months for alveolar macrophages to decades for microglial cells (Lassmann *et al.*, 1993). Hence, tissue macrophages can potentially act as long-term stable reservoirs for HIV-1 capable of disseminating the virus to other tissues (Orenstein *et al.*, 1997; Ghorpade *et al.*, 1998) and contributing to progression to AIDS.

As discussed above (Section 1.3.3), HIV-1 persists in viral reservoirs during HAART in patients with undetectable viral load (reviewed in Pierson et al., 2000). The potential existence of sanctuary sites within tissue compartments where drug concentrations are sub-optimal as a result of blood-tissue barriers may allow HIV-1 infection and replication in cells present within those sites. Differences in viral load detected in plasma and cerebrospinal fluid (Garcia *et al.*, 1999) or semen (Liuzzi *et al.*, 1996) indicate the ongoing HIV-1 replication in tissue macrophages present within such sanctuary sites.

1.4.1.2 Monocyte-derived macrophages.

Similar to tissue macrophages, monocyte-derived macrophages (MDM) derived from peripheral blood monocytes are susceptible to HIV-1 infection *in vitro*. They can produce HIV-1 for weeks to months, with a lack of significant cytopathic effects (Crowe *et al.*, 1987; Sonza *et al.*, 1996). Therefore, MDM provide a very useful model for the assessment of HIV-1 infection of tissue macrophages. As immune activation affects HIV-1 infection and replication in MDM (Sonza *et al.*, 1996), cytokines and growth factors responsible for monocyte differentiation (eg. M-CSF) augment HIV-1 replication within cells of this lineage *in vitro* (Perno *et al.*, 1992; Matsuda *et al.*, 1995).

1.4.1.3 Peripheral blood monocytes.

Peripheral blood monocytes are infrequently infected with HIV-1 *in vivo* (Schuitemaker *et al.*, 1992b) and only a very small proportion of blood monocytes (0.001 to 1%) harbour HIV-1, at any time throughout the course of infection (McElrath *et al.*, 1989; Lewin *et al.*, 1998). Freshly isolated monocytes are also much less permissive to HIV-1 infection *in vitro* compared to MDM cultured for a few days prior to *in vitro* infection (Rich *et al.*, 1992; Sonza *et al.*, 1995; Sonza *et al.*, 1996). HIV-1 replication in freshly isolated monocytes is blocked prior to reverse transcription and integration (Sonza *et al.*, 1996). Susceptibility of monocytes to HIV-1 infection is independent of the level of CD4 expression (Sonza *et al.*, 1995), but correlates with the surface expression of CCR5 level (Fear *et al.*, 1998; Naif *et al.*, 1998; Tuttle *et al.*, 1998).

Despite the low proportion of peripheral blood monocytes infected with HIV-1 *in vivo*, HAART is not able to eradicate the virus from those cells. Replication-competent HIV-1 can be recovered from monocytes purified from peripheral blood of patients effectively treated with HAART (plasma viral load below 50 copies/ml) and co-cultured with CD8-depleted, PHA-activated peripheral blood mononuclear cells (PBMC) (Crowe and Sonza, 2000; Sonza *et al.*, 2001). Peripheral blood monocyte reservoirs may be established shortly after infection prior to the commencement of HAART or may become infected with HIV-1 at any stage of disease via trafficking in and out of the central nervous system or other sanctuary sites (Thomas and Segal, 1998), and therefore reflect ongoing HIV-1 replication within cellular sanctuary sites in the body.

1.4.2 Role of macrophages in AIDS-related dementia.

HIV-infected individuals are susceptible to neurological disorders such as subacute dementia, sensory neuropathy and vacuolar myelopathy. HIV-associated dementia characterised by cognitive, motor and behavioural abnormalities, is the most severe

manifestation of HIV-1 infection in the brain. Although the prevalence of neurological disorders has declined since the introduction of HAART, HIV-associated dementia still affects a significant number of HIV-infected individuals at late stages of infection.

Within the brain of HIV-infected individuals, HIV-1 replicates productively in blood-derived macrophages and microglia (resident brain macrophages responsible for removing dead neurons), since HIV-1 DNA, RNA and proteins can be readily detected within those cells (Gabuzda *et al.*, 1986; Wiley *et al.*, 1986; Johnson *et al.*, 1988; Price *et al.*, 1988). Although astrocytes have been reported to contain HIV-1 DNA (Watkins *et al.*, 1990; Tornatore *et al.*, 1991), there is no evidence for productive HIV-1 infection of those cells (Price *et al.*, 1988; Gorry *et al.*, 1999), possibly due to inefficient translation of HIV-1 structural proteins (Gorry *et al.*, 1999). Other cells within the brain such as neurones, oligodendrocytes or epithelial cells have not been found to be productively infected with HIV-1 (Wiley *et al.*, 1986; Vazeux *et al.*, 1987), suggesting that cells of macrophage lineage play a central role in the neuropathogenesis of HIV-1 infection.

HIV-associated dementia is associated with massive infiltration of blood-derived macrophages to the brain through the disrupted blood-brain barrier, and the formation of multinucleated giant cells and microglial nodules (Price *et al.*, 1988). Both HIV-infected macrophages and microglia are highly activated and produce a number of neurotoxins contributing to disease progression (Glass *et al.*, 1993). Those include pro-inflammatory cytokines (TNF-α, IL-1β), chemokines (SDF-1), excitotoxins (quinolinate and glutamate), reactive oxygen intermediates (nitric oxide and superoxide) or lipid mediators (arachidonate metabolites) (reviewed in Wesselingh and Thompson, 2001), and may result in the neuropathological changes via neuronal damage and death (Genis *et al.*, 1992; Achim *et al.*, 1993; Yoshioka *et al.*, 1995). The number of activated macrophages and their products within the brain strongly correlates with the clinical

status of the HIV-infected individuals (Wesselingh *et al.*, 1994), confirming the contribution of those cells to HIV-related encephalopathy and HIV-related dementia.

1.4.3 Altered monocyte/macrophage functions following HIV-1 infection.

Cells of macrophage lineage including peripheral blood monocytes and tissue macrophages provide critical effector functions within the immune system. Following HIV-1 infection a number of these functions are impaired, including phagocytosis, intracellular killing and cytokine production. These defects contribute to the pathogenesis of AIDS by allowing reactivation and development of opportunistic infections and resulting in significant morbidity and mortality (reviewed in Crowe, 1995).

1.4.3.1 Impaired phagocytosis.

Cells of macrophage lineage including peripheral blood monocytes and tissue macrophages provide critical functions in the cell-mediated response to a variety of pathogens such as bacteria (*Mycobacterium avium* complex (MAC), *Mycobacterium tuberculosis, Rhodococcus equi*), parasites (*Toxoplasma gondii,* microsporidia) and fungi (*Candida albicans, Cryptococcus neoformans, Pneumocystis carinii*) (Drancourt *et al.*, 1992; Wehle *et al.*, 1993; Doultree *et al.*, 1995). Defective monocyte/macrophage function leading to inefficient control of opportunistic pathogens contributes to the development of opportunistic infections due to these pathogens in HIV-infected individuals at late stages of disease.

Defective phagocytic capacity for opportunistic pathogens by cells of macrophage lineage following HIV-1 infection has been well documented by our group and others. A number of investigators have reported impairment of phagocytosis of *Toxoplasma gondii, Candida pseudotropicalis, Porphyromonas gingivalis, Fusobacterium nucleatum, Cryptococcus neoformans* by peripheral monocytes obtained from HIV-

infected individuals at different stages of disease (Bravo-Cuellar *et al.*, 1992; Cameron *et al.*, 1993; Delemarre *et al.*, 1995; Estevez *et al.*, 1986; Dobmeyer *et al.*, 1995; Baqui *et al.*, 1999). Decreased phagocytosis has been found in monocytes obtained from patients with both undetectable (<400 copies/ml) and high viral loads (>10,000 copies/ml) (Baqui *et al.*, 1999) as well as over a wide range of CD4 count (Roilides *et al.*, 1993). However, a correlation between defective phagocytosis and the CD4 counts or disease stage has been also reported (Pittis *et al.*, 1993; Dobmeyer *et al.*, 1995). As only a small proportion of blood monocytes (0.001% to 1%) is infected with HIV-1, a high level of phagocytic inhibition by monocytes from HIV-infected individuals may reflect an indirect consequence of infection.

The inhibition of phagocytosis of a variety of opportunistic pathogens such as *Pneumocystis carinii, Toxoplasma gondii, Aspergillus fumigatus, Histoplasma capsulatum* and *Staphylococcus aureus* has also been observed in alveolar macrophages obtained from HIV-infected individuals (Musher *et al.*, 1990; Wehle *et al.*, 1993) as well as MDM cultured *in vitro* from monocytes obtained from patients with HIV-1 infection (Capsoni *et al.*, 1992; Roilides et al., 1993; Capsoni *et al.*, 1994; Chaturvedi *et al.*, 1995; Delemarre *et al.*, 1995). Similarly, MDM infected with HIV-1 *in vitro* displayed impaired phagocytic capacity against *Toxoplasma gondii* and *Candida albicans* (Crowe *et al.*, 1994; Biggs et al., 1995). Since in these experiments a high proportion of MDM (20 to 70%) were infected with HIV-1, impaired phagocytosis by those cells may reflect a direct effect of HIV-1 on macrophage function. Such an effect may be significant in HIV-infected individuals since a high proportion of tissue macrophages is also infected with HIV-1 (Section 1.4.1.1).

Despite numerous studies demonstrating defective phagocytosis by either monocytes or macrophages following HIV-1 infection *in vivo* and *in vitro,* a few reports have shown normal monocyte (Nielsen *et al.*, 1986; Eversole *et al.*, 1994) and macrophage (Nottet

et al., 1993) function after HIV-1 infection. These discordant results may be, at least partially, explained by substantial variation in methods used by investigators, including the stage of maturation of monocytes and macrophages at the time of functional assessment, the specific intracellular pathogens used as targets, type of phagocytosis assay and in particular the failure of some groups to control for endotoxin contamination.

The mechanism whereby HIV-1 impairs phagocytosis remains unknown. A number of phagocytic receptors (Section 1.6.1) can mediate engulfment of specific opportunistic pathogens. These include complement receptors (C'R) which are generally responsible for MAC uptake (Schlesinger and Horwitz, 1991) and Fcγ receptors (FcγR) which mediate *Toxoplasma gondii* phagocytosis (Joiner *et al.*, 1990). The effect of HIV-1 on the surface expression of these phagocytic receptors has been investigated. The majority of reports show that HIV-1 infection results in either elevated or unchanged surface expression of FcγRs or C'Rs (Petit *et al.*, 1988; Capsoni *et al.*, 1992; Nottet *et al.*, 1993; Capsoni et al., 1994; Stent *et al.*, 1995; Dunne *et al.*, 1996). Those studies suggest that the inhibition of phagocytosis by HIV-1 occurs during or after receptor binding.

Defective phagocytosis might also be caused by binding of viral proteins present in the circulation *in vivo* or in the culture supernatant *in vitro* to CD4 or other receptors on the surface of cells of macrophage lineage. Exposure of bronchoalveolar macrophages to HIV-1 glycoprotein gp120 has been demonstrated to inhibit phagocytosis of *Cryptococcus neoformans* (Wagner *et al.*, 1992).

1.4.3.2 Defective intracellular killing.

Our group has previously reported impaired intracellular killing of *Toxoplasma gondii* leading to increased intracellular growth of the pathogen in MDM infected with HIV-1 *in*

vitro (Biggs *et al.*, 1995). Other investigators have also shown impaired intracellular killing of Candida species in HIV-infected MDM when compared to uninfected macrophages although normal function has also been reported (Baldwin *et al.*, 1990; Nottet *et al.*, 1993). Similarly, MDM cultured from monocytes obtained from asymptomatic HIV-infected individuals are less efficient in killing *Toxoplasma gondii* than cells from uninfected individuals (Eales *et al.*, 1987; Delemarre *et al.*, 1995), and MDM cultured from monocytes obtained from patients with advanced HIV-1 infection display increased growth of opportunistic pathogens such as MAC and *Leishmania donovani* (Crowle *et al.*, 1992; Ghassemi *et al.*, 1995; Kallenius *et al.*, 1992; Newman *et al.*, 1993).

Phagosome-lysosome fusion is critical for killing of ingested pathogens and is significantly reduced in HIV-1 infected MDM when compared to mock-infected control MDM (Moorjani *et al.*, 1996). Phagosome-lysosome fusion is also impaired in peripheral blood monocytes obtained from HIV-infected individuals, and correlates with the stage of disease progression (Pittis *et al.*, 1997).

1.4.3.3 Impaired chemotaxis.

HIV-1 infection also impairs chemotactic activity of cells of macrophage lineage. Monocytes obtained from patients with AIDS display defective migratory response to chemotactic stimuli (Wahl *et al.*, 1989) and are associated with downmodulation of surface expression of receptors for the chemotactic ligands, C5a and *formyl*-methionyl-leucyl-phenylamine (fMLP; an analogue of bacterial N-terminal peptide). Impaired chemotaxis (assessed as fMLP-induced polarisation) by peripheral blood monocytes of HIV-infected men has been also reported (Tas *et al.*, 1988).

1.4.3.4 Altered cytokine and chemokine profile.

As described in Section 1.3.4, HIV-1 infection at all stages of the disease is associated with chronic immune activation and dysfunctional cytokine production. Both monocytes and macrophages contribute considerably to the altered cytokine and chemokine profiles, and hence play a major role in the immunopathogenesis of the disease. Several groups have demonstrated that HIV-1 infection is associated with increased production of pro-inflammatory cytokines by cells of macrophage lineage. Infection of MDM with HIV-1 *in vitro* results in upregulation and constitutive secretion of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α in culture supernatants (Esser *et al.*, 1991; Esser *et al.*, 1996). Similarly, increased levels of IL-1 (Sadeghi *et al.*, 1995), IL-6 (Birx *et al.*, 1990), IL-8 (Matsumoto *et al.*, 1993) and TNF-α (Lahdevirta *et al.*, 1988; Baqui *et al.*, 2000) are found in the sera of HIV-infected individuals and monocytes from HIV-infected subjects. Increased production of pro-inflammatory cytokines is thought to activate HIV-1 replication and maintain active HIV-1 expression via binding of NF-κB to LTR.

HIV-1 infection is also associated with a switch from a predominantly Th type 1 to Th type 2 response (Section 1.3.4) (Clerici and Shearer, 1993). Cells of macrophage lineage contribute to that process by producing elevated levels of IL-10, a Th type 2 cytokine, which inhibits T cell proliferation and macrophage activation (reviewed in Moore *et al.*, 2001). *In vitro* HIV-1 infection or gp120 stimulation of monocytes and MDM significantly increase IL-10 secretion and IL-10 mRNA levels in comparison to uninfected or untreated cells (Borghi *et al.*, 1995; Gessani *et al.*, 1997).

HIV-1 infection also leads to the altered secretion of interferons and colony-stimulating factors by cells of macrophage lineage. Productive HIV-infection of monocytes *in vitro* results in reduced production of IFN-α (Gendelman *et al.*, 1990), although binding of CD4 on monocytes with HIV-1 envelope glycoprotein, gp120, has been reported to

induce IFN-α production (Capobianchi, 1996). HIV-1 infection *in vitro* or gp120 treatment of monocytes or MDM results in induction of low levels of IFN-β in comparison to uninfected/untreated cells (Gessani *et al.*, 1997). MDM infected with HIV-1 *in vitro* produce decreased amounts of GM-CSF (Esser *et al.*, 1996), but 5- to 24-fold higher levels of M-CSF in comparison to uninfected cells (Gruber *et al.*, 1995; Kutza *et al.*, 2000). Increased levels of M-CSF have been detected in the cerebrospinal fluid of HIV-infected patients, presumably synthesised by cells of macrophage lineage within the brain (Gallo *et al.*, 1994).

HIV-1 infection induces MDM to secrete the β-chemokines MIP-1α, MIP-1β and RANTES, which have been identified as natural ligands for CCR5, a major co-receptor for M-tropic strains of HIV-1 (Cocchi *et al.*, 1995). HIV-infected MDM produce increased levels of MIP-1α and MIP-1β, which subsequently enhance chemotaxis and activation of resting T cells (Schmidtmayerova *et al.*, 1996; Swingler *et al.*, 1999). Exposure of monocytes and MDM to gp120 or interactions between soluble CD40 ligand and CD40 on MDM also stimulate the production of MIP-1α, MIP-1β and RANTES in a dose-dependent manner (Kornbluth *et al.*, 1998; Fantuzzi *et al.*, 2001).

1.5 BIOLOGY OF MONOCYTES AND MACROPHAGES.

1.5.1 Monocyte differentiation and activation.

Monocytes originate from a self-renewing population of CD34-expressing, CD38-negative pluripotent haemopoietic stem cells, located mainly in the bone marrow. These haemopoietic stem cells generate progenitor cells committed irreversibly to either the myeloid or lymphoid lineage. The earliest committed progenitor cells of myeloid lineage are the colony-forming unit (CFU-GEMM), which give rise to granulocytes, erythrocytes, monocytes and megakaryocytes. The coordinated

proliferation and differentiation of these cells is controlled by several haemopoietic growth factors and cytokines including stem cell factor (SCF), thrombopoietin (TPO), IL-3, IL-5, IL-6, GM-CSF and M-CSF (Lydyard and Grossi, 1993) (Figure 1.7).

Cells of the macrophage lineage share a common progenitor, granulocyte-macrophage colony forming unit (CFU-GM) with neutrophils (reviewed in Auger and Ross, 1992). Once IL-3-responsive CFU-GM progenitor cells express receptors for GM-CSF and M-CSF, they become responsive to these cytokines, proliferate and mature into monocytes. Upon stimulation with GM-CSF and IL-3, the CFU-GM proliferates and differentiates into the first monocyte precursor, the monoblast. These differentiate into the larger promonocyte and then upon stimulation with GM-CSF, M-CSF and IL-3 into mature monocytes. After spending less than 24 hr in the bone marrow, monocytes enter the blood stream and circulate in peripheral blood with a half-life of about three days. Monocytes subsequently adhere to endothelium and migrate into various extravascular tissues, where they further differentiate into morphologically and phenotypically distinct sub-populations of tissue macrophages such as alveolar macrophages in lungs, the synovial macrophages in the joint capsule, the hepatic Kupffer cells or peritoneal macrophages (Table 1.4). The life span of tissue macrophages varies from two months to years depending on the anatomical site (reviewed in Ralph, 1989). Tissue macrophages display a spectrum of specialised functions depending on diverse microenvironmental signals. These functionally and phenotypically distinct macrophage populations increase the responsiveness of the non-specific immune system to a wide variety of pathogens.

Differentiation of monocytes into macrophages is associated with many morphological changes such as an increase in cell size, the cytoplasm to nucleus ratio and cytoplasmic granularity (van Furth *et al.*, 1979; Musson, 1983; Kreutz *et al.*, 1992). The surface expression of a number of proteins also alters as myeloid cells differentiate into

macrophages. Mature cells of macrophage lineage lose the CD34 surface marker, gain the receptor for LPS (CD14), but maintain expression of MHC class II molecule (Figure 1.8). They have increased surface expression of phagocytic receptors FcγRs and the complement receptor CR3 (CD11b/CD18) (reviewed in Rutherford *et al.*, 1993). Similarly, functions including phagocytosis and intracellular levels of lysosomal enzymes increase during differentiation (Auger and Ross, 1992). Monocytes purified from peripheral blood can be differentiated to MDM *in vitro.* In the presence of human serum they undergo similar morphological and phenotypical changes as tissue macrophages including cell size, cytoplasm to nucleus ratio, cytoplasmic granularity and surface markers expression (Dougherty and McBride, 1989; Akiyama *et al.*, 1988; Andreesen *et al.*, 1988; van Furth *et al.*, 1979; Kaplan and Gaudermack, 1982; Musson, 1983). Macrophages are considered terminally differentiated cells, although modest proliferation of human MDM has been reported (Schuitemaker *et al.*, 1992b; Schuitemaker *et al.*, 1994).

Resident tissue macrophages are immunologically quiescent. They have low levels of MHC II surface expression required for antigen presentation, low oxygen consumption and little cytokine production, although they display phagocytic and chemotactic activities. Resident macrophages can achieve maximal functional capacity following activation with cytokines or endotoxin. In contrast to macrophage differentiation (normal cellular development associated with permanently altered expression of genes), macrophage activation involves acquisition of an increased ability to perform effector functions and is associated with reversibly-altered expression of a set of genes (Rutherford, 1993). Macrophage activation leads to an alteration in cellular size, cellular metabolism and surface expression of HLA-DR, FcγRs and C'Rs required for their maximal functional capacity (Ezekowitz and Gordon, 1982; Guyre *et al.*, 1983; Basham and Mengan, 1983). Activated macrophages are the major effectors for cell-mediated immunity against a broad spectrum of pathogens.

Activation of macrophages is a two-step process. The first stage involves "priming" for their full activation. Cytokines produced by stimulated T cells such as IFN-γ, and to a lesser extent IFN-α, IFN-β and TNF-α are the major macrophage "priming" agents (Ruco and Meltzer, 1978; Gordon, 1986; Peck, 1991; Knight *et al.*, 1992; Rutherford *et al.*, 1993). Those "primed" macrophages display enhanced bactericidal and tumouricidal activities, increased secretory and phagocytic capacities, MHC class II expression, antigen presentation and oxygen consumption as well as release of cytokines such as TNF-α (Adams and Hamilton, 1984; Adams and Hamilton, 1987). Following stimulation with GM-CSF, LPS or phorbol esters, those "primed" macrophages become fully activated (Adams and Hamilton, 1987; Belosevic *et al.*, 1988). The activation state is associated with production of pro-inflammatory cytokines including TNF-α, IL-1 and IL-6 as well as prostaglandins (PGE_2), increased secretion of catalytic enzymes (acid hydrolyses and proteases), reactive oxygen products and nitric oxide (Pantalone and Page, 1975; Gordon, 1978; MacMicking *et al.*, 1997). Activated macrophages also display high oxygen consumption, increased antimicrobial activity, killing of parasites, tumor cell lysis, augmented phagocytic capacity and the ability to phagocytose C'-opsonised pathogens (Rutherford *et al.*, 1993).

1.5.2 Monocyte/macrophage function.

Cells of macrophage lineage play a pivotal role within the immune system. They participate in host defences against infection by bacteria, viruses and protozoa as well as immune surveillance against malignancies. They generate many important immunological and inflammatory responses, affect most of the cells of the immune system and contribute to humoral and cellular immunity. Their ability to act as antigen presenting cells and major producers of cytokines enables macrophages to induce adaptive immune responses and stimulate both T cells and B cells (reviewed in Medzhitov and Janeway, 1997). They regulate both the local and systemic environment

through their large and diverse secretory potential as well as very potent phagocytic capacity. Tissue macrophages are ubiquitously distributed throughout the body and hence provide an immediate defence against pathogens prior to induction of antibody production and T cell responses. As discussed in Section 1.5.1, the function of macrophages is influenced by their state of activation.

Macrophages play an important role in the innate immune responses against bacteria, viruses, fungi and parasites. They recognise invading pathogens at the site of entry including the submucosae, spleen, liver and lymph nodes. They decrease pathogen load and clear infectious agents via phagocytosis and subsequent intracellular killing. Splenic macrophages and Kupffer cells are important in the clearance of bloodborne infections, whereas macrophages at the submucosae (eg. gastrointestinal tract) and alveolar macrophages clear pathogens that have passed through the anatomical barriers. The effectiveness of macrophages to limit the pathogen load depends on the nature of inoculum and the susceptibility of macrophages to infection by invading pathogens.

Macrophages mediate their phagocytic function via a number of surface receptors for a variety of bacteria, fungi and parasites (eg. FcγRs, C'Rs and mannose receptors; described in Section 1.6.1). Apart from the phagocytic receptors, they also express a surface receptor for LPS, CD14, which is important for eliminating Gram-negative bacteria from the peripheral blood and interstitial fluids (Ziegler-Heitbrock and Ulevitch, 1993). Apart from removing a variety of pathogens, macrophages also recognise and ingest infected, senescent and otherwise abnormal cells. They engulf and remove apoptotic bodies, which can be readily detected within the cytoplasm of activated macrophages (Bofill *et al.*, 1995). In this context, infectious HIV-1 has been recovered from macrophages that have phagocytosed infected cellular debris (Kornbluth *et al.*, 1993).

Cells of macrophage lineage kill pathogens and infected cells via oxygen-dependent and oxygen-independent mechanisms, respectively. The former mechanism depends on a massive increase in oxygen consumption (respiratory burst) following the ingestion of the pathogen and leads to generation of superoxides, halogenated anions and other toxic molecules, whereas the oxygen-independent mechanism eliminates infected cells via perforin cytotoxicity (reviewed in Mazzarella *et al.*, 1998). Nitric oxide (NO) is also important for intracellular killing of a broad range of pathogens including bacteria, viruses, parasites and fungi (DeGroote and Fang, 1995). NO production can be stimulated by microbial products (LPS, lipoteichoic acid) or pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1) (Nicholson *et al.*, 1996) and correlates with the ability of macrophages to inhibit microbial proliferation and thus with a better clinical outcome (Anstey *et al.*, 1996).

Macrophages play an important role as antigen presenting cells and therefore are involved in initiating specific immune responses. They process exogenous antigens in vesicles derived from phagosomes and present the processed antigens to CD4 T cells in association with MHC class II molecules. A small subset of macrophages is able to present exogenous antigens to CD8 T cells in association with MHC I molecules (Pfeifer *et al.*, 1993).

Cells of macrophage lineage also eliminate antibody-coated cells via antibody-dependent cellular cytotoxicity (ADCC). This process is mediated by FcγRs on the macrophage surface and leads to macrophage activation and subsequent death of the target cell.

Monocytes and macrophages secrete numerous cytokines including IFN-α, IFN-β, TNF-α, IL-1, IL-6, IL-8, IL-10, IL-12, IL-15, growth factors including GM-CSF, G-CSF, and chemokines such as MIP-1α, MIP-1β, RANTES, SDF-1. Production of pro-

inflammatory cytokines, IL-1, IL-6, IL-8 and TNF-α (which act as adjuvants of immune responses) by macrophages contributes to the initiation and broadening of host defences against a variety of pathogens.

1.5.3 GM-CSF and cells of macrophage lineage.

The function of cells of macrophage lineage is influenced by their state of activation, which in turn is initiated by exposure to growth factors and cytokines. GM-CSF is one of the major growth factors which regulate macrophage function. It stimulates both the proliferation and differentiation of cells of macrophage lineage as well as a number of their functions including antimicrobial and antitumoricidal activity, synthesis of cytokines and antibody-dependent cell-mediated cytotoxicity (reviewed in Armitage, 1998).

1.5.3.1 The structure of GM-CSF and its receptor.

GM-CSF is a glycoprotein of 127 amino acids with a molecular weight of 23 to 28 kDa and is produced by a variety of cell types including activated T cells, endothelial cells, macrophages and fibroblasts (Hercus *et al.*, 1994). The structure of GM-CSF determined by x-ray crystallography reveals the presence of four alpha helices with a novel fold combining a two-stranded antiparallel beta sheet (Diederichs *et al.*, 1991). The regions between amino acid residues 14 to 28 (Clark-Lewis *et al.*, 1988) and 88 to 96 in the first alpha helix (Brown *et al.*, 1990) are essential for GM-CSF biological activity. In contrast, the elimination of the 14 most N-terminal amino acids and 6 C-terminal amino acids does not affect its bioactivity. Using site-directed mutagenesis, two amino acids Glu21 in helix A (Lopez *et al.*, 1992) and Asp101 in helix D (Hercus *et al.*, 1994) have been determined to be critical for the full biological activity of GM-CSF. These residues are thought to bind to the separate subunits of the GM-CSF receptor (Hercus *et al.*, 1994).

The human GM-CSF receptor has been cloned and comprises a low affinity α chain (K_D=2.6nM) and a β chain which provides a high affinity binding when co-expressed with the α chain. The affinity of GM-CSF for the αβ heterodimer is in the pM range (Gearing *et al.*, 1989; Hayashida *et al.*, 1990). The β chain of the GM-CSF receptor is shared with IL-3 and IL-5 receptors and does not appear to bind GM-CSF by itself (reviewed in Crowe and Lopez, 1997). A mutant form of GM-CSF, E21R, binding selectively to the α chain of GM-CSF receptor and behaving as a competitive GM-CSF antagonist, induces apoptosis of primary haematopoietic cells (Iversen *et al.*, 1996). These results suggest that engaging the β chain and high-affinity binding is important for cell survival.

1.5.3.2 Function of GM-CSF.

In addition to its role in activating macrophage function, as discussed in Section 1.5.1, GM-CSF is a multipotential haematopoietic growth factor regulating cells of eosinophilic, neutrophilic, monocytic and megakaryocytic lineages (Figure 1.7) (reviewed by Metcalf, 1985). Low concentrations of GM-CSF (pM ranges) have been found to enhance monocyte survival (Elliott *et al.*, 1989; Eischen *et al.*, 1991). GM-CSF induces the differentiation of circulating monocytes into specialised macrophages such as alveolar macrophages, Kupffer cells or microglial cells upon their migration into the tissues. *In vitro,* GM-CSF has been reported to enhance the effector function of mature macrophages including increasing their phagocytic capacity (Smith *et al.*, 1990) cytotoxicity against tumor cells (Parhar *et al.*, 1992), antiparasitic and antimycobacterial activity (Bermudez *et al.*, 1994; Laursen *et al.*, 1994) as well as antibody-dependent cellular cytotoxicity (Keler *et al.*, 2000). GM-CSF also stimulates the effector function of circulating neutrophils and eosinophils (Vadas *et al.*, 1983).

Stimulation with GM-CSF significantly increases FcγR-mediated phagocytosis by both human (Capsoni *et al.*, 1992) and murine macrophages (Coleman *et al.*, 1988). GM-CSF also augments C'R-mediated phagocytosis of *Mycobacterium avium* complex (Schlesinger and Horwitz, 1991) by human (Bermudez and Young, 1990) and murine macrophages (Bermudez *et al.*, 1994). GM-CSF augments antimicrobial activity against a number of other opportunistic pathogens including *Candida albicans* (Smith *et al.*, 1990), *Aspergillus fumigatus* (Roilides *et al.*, 1996), *Trypanosoma cruzi* (Reed *et al.*, 1987), *Histoplasma capsulatum* (Newman and Gootee, 1992) and *Cryptococcus neoformans* (Collins and Bancroft, 1992). Additionally, this cytokine increases surface expression of the phagocytic receptors such as FcγR II, which subsequently correlates with the enhanced binding of IgG-opsonised erythrocytes (Rossman *et al.*, 1993).

1.5.3.3 Clinical applications of GM-CSF.

GM-CSF is used in numerous clinical applications including cancer treatment and bone marrow repopulation after chemotherapy or irradiation-depletion. Clinical trials have indicated that recombinant human GM-CSF can shorten the duration of chemotherapy-induced neutropenia in persons receiving chemotherapy for various malignancies (Antman *et al.*, 1988; Brandt *et al.*, 1988; Nemunaitis *et al.*, 1988). Administration of GM-CSF increases the number of circulating peripheral blood monocytes as well as the *in vitro* effector functions of cells of macrophage lineage obtained from patients receiving GM-CSF including FcγR-mediated phagocytosis, oxidative burst, cytotoxicity, antigen presentation and secretion of TNF-α and IFN-γ (Coleman *et al.*, 1988; Wing *et al.*, 1989; Wiltschke *et al.*, 1995). Treatment with GM-CSF also increases the number of circulating monocytes and neutrophils in AIDS patients (Groopman *et al.*, 1987).

GM-CSF is potentially useful in combating opportunistic infections in AIDS patients by stimulating monocyte/macrophage number and function. Currently GM-CSF is rarely used for the treatment of HIV-infected patients due to concerns regarding potential

activation of HIV-1 replication, with adverse effects on viral load. Early studies investigating the effect of GM-CSF on HIV-1 replication *in vitro* reported upregulation of HIV-1 production in both MDM (Koyanagi *et al.*, 1988; Perno *et al.*, 1989; Schuitemaker *et al.*, 1990; Perno et al., 1992; Wang *et al.*, 1998) as well as in the promonocytic cell line U937 and its subclone U1 (Folks *et al.*, 1987; Pomerantz *et al.*, 1990). However, other investigators have found either inconsistent (Kornbluth *et al.*, 1989; Hammer *et al.*, 1990) or inhibitory effects of GM-CSF on HIV-1 entry or replication in MDM (Matsuda *et al.*, 1995; Di Marzio *et al.*, 1998). A number of laboratory variables could contribute to the conflicting data, including source/concentration of GM-CSF, timing of incubation with cytokine, maturity (monocyte versus MDM) and strain of HIV-1 used for infection.

Early clinical trials showed that GM-CSF treatment of HIV-infected patients in the absence of antiretroviral therapy results in increased serum p24 antigen levels and plasma HIV RNA titres (Kaplan *et al.*, 1991; Lafeuillade *et al.*, 1996). However, when used in combination with effective antiretroviral therapy, GM-CSF has been safely administered to patients without any significant increase in viral load (Krown *et al.*, 1992; Yarchoan *et al.*, 1990; Angel *et al.*, 2000). In fact GM-CSF has been found to increase the activity of antiretroviral drugs (Perno *et al.*, 1992). Data from studies of HIV-infected patients receiving antiretroviral therapy combined with GM-CSF have shown that patients experienced a decrease in viral load and an increase in CD4 counts (Brites *et al.*, 2000). Clinical improvement and augmented monocyte phagocytic function against mycobacteria and fungal infections, again without an increase in viral load, has also been reported in patients with advanced HIV-1 infection and drug-resistant oropharyngeal candidiasis treated with GM-CSF (Vazquez *et al.*, 1998). These studies suggest that GM-CSF might be useful adjunctive therapy in augmenting macrophage function in HIV-infected patients with opportunistic infections provided that effective antiretroviral therapy can be given concurrently.

1.6 MECHANISM OF PHAGOCYTOSIS.

1.6.1 Phagocytic receptors.

Phagocytosis is mediated via a range of receptors promoting microbial binding and uptake. They include opsonin-dependent receptors, which interact with target particles by recognising opsonins supplied by the host, and opsonin-independent receptors (pattern recognition receptors), which interact directly with pathogens by recognising structural determinants present on their surface (Table 1.5) (reviewed in Greenberg, 1995). The best-characterised opsonin-dependent receptors are FcγRs for the crystallisable fragment (Fc) of IgG and C'Rs for the complement protein C3bi. Opsonin-independent receptors include mannose receptors as well as other receptors of less characterised specificity.

Fcγ receptors for the constant Fc region of IgG are widely expressed on cells of haematopoietic lineage and play an important role in host defences. FcγRs (FcγR I, FcγR II, FcγR IIIA) are the major means by which monocytes and macrophages recognise IgG-opsonised particles and promote phagocytosis, antibody-dependent cellular cytotoxicity, cytokines and chemokines secretion. The major Fcγ receptors expressed on monocytes are the high affinity FcγRI (CD64) and a low affinity FcγRII (CD32), while macrophages also express FcγRIIIA (CD16) (reviewed in Daeron, 1997). FcγRs share a highly homologous extracellular IgG binding domain. The receptors differ, however, in their cytoplasmic domains. FcγR I and FcγR IIIA have relatively short domains, whereas FcγRII has a large cytoplasmic domain containing tyrosine residues arranged into specific signalling motifs $YxxLx_{5-12}Yx_{2-3}L/I$, named ITAMs (immunoreceptor tyrosine-based activation motifs). FcγR I and FcγR IIIA signal via γ γ homodimers, which contain the ITAMs.

Complement receptors promoting phagocytosis of complement-opsonised particles are C'R1 (CD35), C'R3 (CD11b/CD18) and C'R4 (CD11c/CD18). C'R1 is a single chain transmembrane receptor containing an extracellular domain which binds complement proteins present in serum; C3b, C4b and C3bi (Fearon, 1980). C'R3 and C'R4 are β_2 integrin heterodimers comprising a common β subunit (β_2, CD18) and distinct α chains (α_M, CD11b and α_L, CD11c respectively) (Wright *et al.*, 1983; Myones *et al.*, 1988). C'R3 plays also an important role as an opsonin-independent receptor by binding LPS (Wright *et al.*, 1989). Apart from their roles in phagocytosis, C'R3 and C'R4 are also involved in transendothelial movement of macrophages and cell adhesion to endothelium (Chatila *et al.*, 1989).

Opsonin-independent receptors, with broad pathogen specificity, recognise conserved motifs on pathogens not found in higher eukaryotes, and have important functions in immune responses against infections as indicated by gene-knockout studies (Thomas *et al.*, 2000). The best-characterised opsonin-independent receptor is the mannose fucose receptor, which recognises mannans on, predominantly, yeast cell walls and subsequently mediates phagocytosis of those pathogens (Stahl and Ezekowitz, 1998). The mannose receptor is a single chain type 1 membrane protein with an extracellular domain containing 8 lectin-like carbohydrate-binding domains and a short cytoplasmic tail crucial for endocytic and phagocytic functions (Aderem and Underhill, 1999). Recently identified toll receptors (Medzhitov *et al.*, 1997) are thought to transduce the intracellular signals initiated by binding yeast to mannose receptors.

1.6.2 Mechanism of phagocytosis.

Phagocytosis is a process utilised by cells of macrophage lineage and neutrophils to engulf and clear particles larger than 0.5μm such as pathogens, senescent cells, apoptotic and necrotic bodies. Phagocytosis is initiated by the interaction of

phagocytosis-promoting receptors on the surface of macrophages with specific ligands on the surface of the target particles. This is followed by a cascade of signalling events resulting in protrusion of membrane extension, pseudopodia formation, actin polymerisation at the site of ingestion, reorganisation of the actin-based cytoskeleton, formation of the phagosome enclosing the phagocytosed particle and its engulfment into the cytoplasm (Figure 1.9).

After internalisation of the engulfed particle, actin fibres are depolymerised and the phagosome matures via multiple transient and incomplete membrane fusion and fission events, which have been termed "kiss and run" mechanism of phagosome maturation (Desjardins *et al.*, 1994). The phagosome fuses with lysosomes and forms the mature phagolysososme, where degradation of the ingested particle occurs. Phagocytosis mediated via a number of different receptors induces actin rearrangement and subsequent particle ingestion, suggesting that signalling mechanisms activated by distinct phagocytic receptors are likely to converge at some stage in the cell. The details of the above events are described in Section 1.6.3.

1.6.3 Fcγ receptor-mediated phagocytosis - signal transduction.

Most studies delineating the specific signalling events during FcγR-mediated phagocytosis have been performed using murine macrophages (Greenberg *et al.*, 1993; Greenberg *et al.*, 1994; Aderem, 1995; Crowley *et al.*, 1997; Strzelecka *et al.*, 1997; Kiefer *et al.*, 1998), or cell lines transfected with FcγR (Indik *et al.*, 1994; Cox *et al.*, 1996; Greenberg *et al.*, 1996). FcγR-mediated phagocytosis is initiated by the interaction of Fcγ receptors with the Fc portion of IgG antibody on the surface of the opsonised particle. This is followed by a cascade of signalling events resulting in internalisation of the particle via an actin-based mechanism, as evidenced by confocal microscopy and treatment with cytochalasins D (specific inhibitors of actin

polymerisation) (Greenberg *et al.*, 1990; Greenberg *et al.*, 1994). There are two studies reported using human macrophages (Kusner *et al.*, 1999) or monocytes (Zheleznyak and Brown, 1992). Several signal transduction pathways utilised by murine macrophages activated during FcγR-mediated phagocytosis have been described (reviewed in Aderem and Underhill, 1999), including requirements for Src family kinases (Crowley *et al.*, 1997; Fitzer-Attas *et al.*, 2000), Syk kinase (Indik *et al.*, 1995c; Crowley *et al.*, 1997; Bonnerot *et al.*, 1998), phosphatidylinositol 3-kinase (PI3-kinase) (Kanakaraj *et al.*, 1994; Ninomiya *et al.*, 1994) and the Rho family of GTPases (Hackam *et al.*, 1997; Cox *et al.*, 1997) (summarised in Table 1.4).

1.6.3.1 Involvement of Src kinases.

The above-mentioned signalling events are initiated following clustering of FcγRs. This leads to activation of tyrosine kinases of the Src family which associate with the γ chain (signalling subunit) of FcγR I and IIIA or the cytoplasmic domain of FcγR II (Jouvin *et al.*, 1994; Sarmay *et al.*, 1994). Several kinases of the Src family are involved in FcγR-mediated phagocytosis. Both Hck and Lyn have been also shown to co-immunoprecipitate with FcγR following cross-linking of receptors on THP-1 monocytic cell lines (Ghazizadeh *et al.*, 1994) and human monocytes (Wang *et al.*, 1994). A role for Hck, Fgr and Fyn in FcγR signalling has been suggested based on data showing that macrophages prepared from mice deficient in these three Src family tyrosine kinases exhibit poor signalling downstream of FcγR (ie. Syk activation) as well as reduced levels of FcγR-induced functional responses such as phagocytosis, actin cup formation and respiratory burst (Crowley *et al.*, 1997; Fitzer-Attas *et al.*, 2000). A specific requirement for Hck in phagocytosis has been demonstrated using Hck knock-out mice, which fail to internalise IgG-opsonised erythrocytes, while other haematopoietic functions remain intact (Lowell *et al.*, 1994). This study also showed

that while some functions of Hck are compensated for by increases in the activity of another Src kinase, Lyn, phagocytosis still remained defective.

1.6.3.2 Phosphorylation of ITAMs.

The activation of Src kinases results in the rapid and transient phosphorylation of ITAMs on either the ligand binding subunit in the cytoplasmic domain of Fcγ RII or on the associated γ chain of Fcγ RI and IIIA (Park *et al.*, 1993; Indik *et al.*, 1994; Indik *et al.*, 1995a). In fact, this phosphorylation is absolutely required for FcγR-mediated phagocytosis as replacement of tyrosine residues within ITAMs with phenylalanine abolishes the phagocytic signal and subsequent internalisation of target particles (Park *et al.*, 1993). Two γ chain cytoplasmic YXXL sequences are required for FcγR-mediated phagocytosis to occur (Indik *et al.*, 1995c). A single class of human FcγR can induce phagocytosis in the absence of other FcγRs (Indik *et al.*, 1995b). The high affinity FcγR I does not require its cytoplasmic domain to mediate a phagocytic signal as it may interact with the γ chain of FcγR IIIA to mediate phagocytosis (Indik *et al.*, 1994). However, deletion of the intracellular γ chain of FcγR I, in the absence of the cytoplasmic domain of FcγR IIIA, markedly impairs phagocytosis despite unchanged surface receptor level (Edberg *et al.*, 1999). The cytoplasmic γ signalling subunit of FcγRs is critical for subsequent Syk activation (Indik *et al.*, 1995b).

1.6.3.3 Critical role of Syk.

Phosphorylated ITAMs create docking sites for Syk at the cell membrane, allowing its subsequent activation coupled to receptor clustering (Indik *et al.*, 1995c; Bonnerot *et al.*, 1998). Syk has also been found to be a part of the activated FcγR complex following cross-linking of receptors in U937 and THP-1 cell lines (Darby *et al.*, 1994; Ghazizadeh *et al.*, 1995). A requirement for Syk in FcγR-mediated phagocytosis was initially demonstrated in human monocytes by using anti-sense oligodeoxynucleotides

to inactivate Syk mRNA (Matsuda *et al.*, 1996). Gene knockout studies subsequently confirmed that FcγR-mediated phagocytosis by murine macrophages has an absolute and specific requirement for Syk (Crowley *et al.*, 1997). This requirement does not necessary extend to other phagocytic receptors, since macrophages derived from the fetal livers of Syk-deficient mice showed unimpaired phagocytosis of yeast (phagocytosed by mannose receptors) or latex particles (phagocytosed via non-specific receptors) (Crowley *et al.*, 1997). Transfection studies using COS and DT40 cell lines have demonstrated that Syk activation is absolutely required for coupling phagocytosis-promoting FcγRs to the actin-based cytoskeleton and mediates actin assembly and FcγR-mediated transport to lysosomes (Cox *et al.*, 1996; Kiefer *et al.*, 1998; Bonnerot *et al.*, 1998).

1.6.3.4 Downstream effectors of Syk.

1.6.3.4.1 PI3-kinase.

Activated Syk promotes phosphorylation and/or activation of a number of downstream substrates including phosphatidyl inositide 3-kinase (PI3-kinase) (Crowley *et al.*, 1997) (Figure 1.10a), GTP-binding proteins (Massol *et al.*, 1998) (Figure 1.10b), and actin-binding proteins (Greenberg *et al.*, 1994) (Figure 1.10c). PI3-kinase, an enzyme that catalyses phosphorylation of phosphoinositides in the D3 position of the inositol ring, has been shown to be a downstream effector of Syk during FcγR-mediated phagocytosis (Crowley *et al.*, 1997). Crowley and colleagues have shown that tyrosine phosphorylation of the p85 regulatory subunit of PI3-kinase, stimulated by FcγR engagement, is inhibited in Syk knockout mice. A requirement for PI3-kinase in FcγR-mediated phagocytosis has been demonstrated in a study showing that cross-linking of FcγRs increases the activity of PI3-kinase, and wortmannin (a tight-binding specific inhibitor of PI3-kinase) inhibits both PI3-K activity and ingestion of IgG-opsonised particles (Ninomiya *et al.*, 1994). A role for PI3-kinase in FcγR-mediated phagocytosis

has been suggested by studies in which wortmannin prevents either pseudopod extension or phagosome closure and engulfment of IgG-opsonised targets, but does not affect the actin-based formation of the phagocytic cup (Araki *et al.*, 1996; Crowley *et al.*, 1997; Cox *et al.*, 1999).

1.6.3.4.2 Rho family of GTPases.

The members of the Rho family of GTPases, including Rac1, RhoA and Cdc42, provide a critical link between the initial signalling events and the subsequent cytoskeletal rearrangements. Distinct cytoskeletal processes have different requirements for the GTP-binding proteins, for example RhoA promotes elongation of the stress fibres, Rac1 is involved in the formation of focal complexes, whereas Cdc42 mediates filipodia extension (Hall, 1998).

FcγR-mediated signalling activates two proteins of the Rho family, Rac1 and Cdc42. Both proteins accumulate at the phagosome when exposed to IgG-opsonised target particles and promote phagocytic signalling and membrane ruffling (Cox *et al.*, 1997; Caron and Hall, 1998; Lee *et al.*, 2000). Rac1 is involved in the closure of the phagosome and particle internalisation, whereas Cdc42 is involved in the appearance of the membrane around the IgG-opsonised particles (Massol *et al.*, 1998). FcγR-mediated phagocytosis differs in its requirements for Rho proteins from C'-mediated phagocytosis, as the latter requires the activation of RhoA for internalisation of the phagocytosed particle to occur (Cox *et al.*, 1997; Caron and Hall, 1998).

Rac1 can be activated by either PI3-kinase (Massol *et al.*, 1998) or by proteins promoting its GDP/GTP exchange activity (Yaku *et al.*, 1994), in particular the guanine nucleotide exchange factor, Vav (Bustelo *et al.*, 1994). Vav can be stimulated by either products of the PI3-kinase reaction (Crespo *et al.*, 1997; Han *et al.*, 1998) or by its phosphorylation catalysed by Syk (Kiener *et al.*, 1993; Xu and Chong, 1996).

Phosphorylation of Syk has been linked to the activation of Vav in a study showing that inactivation of the *syk* gene in the DT40 B cell line abolishes Vav phosphorylation following cross-linking of B cell receptor (Wu *et al.*, 1997). Therefore, Vav is thought to link the initial FcγR-signalling to cytoskeletal pathways regulated by proteins of the Rho family (reviewed in Bustelo, 2000). The GDP-GTP exchange activity of phosphorylated Vav, however, also depends on two products of PI 3-kinase, PIP_3 or PI-3,4-P_2 (Han *et al.*, 1998), suggesting that those two pathways converge to activate the Rho family G proteins.

Activated Rac1 subsequently signals to its downstream effectors ROCK and p21-activated kinase (PAK1). The GTP-bound form of ROCK is thought to inactivate myosin phosphatase resulting in increased myosin light chain phosphorylation and actomyosin contractility (Maekawa *et al.*, 1999). Activated ROCK also phosphorylates its downstream substrate LIM-kinase (LIMK), which in turn phosphorylates and inactivates cofilin, a protein which catalyses actin depolymerisation (Arber *et al.*, 1998). Activated Rac1 may also result in cofilin phosphorylation via activation of PAK1, a serine/threonine kinase (Arber *et al.*, 1998; Bubeck Wardenburg *et al.*, 1998). PAK1 co-localises in focal adhesions with F-actin adjacent to the phagosome and is associated with remodelling of the actin-based cytoskeleton (Manser *et al.*, 1997). It has been shown to localise in pseudopodia, membrane ruffles and phagocytic cups during FcγR-mediated phagocytosis in activated human neutrophils (Dharmawardhane *et al.*, 1999).

Recently, PAK1 has been identified as Nef-associated kinase (NAK), essential for cytoskeletal rearrangements and cellular signalling induced by this HIV-1 accessory protein (Fackler *et al.*, 2000). Nef also interacts with Vav and the G proteins Rac1 and Cdc42. Nef inhibits the function of Vav and subsequent cytoskeletal rearrangements by induction of trichopodia formation (Fackler *et al.*, 1999). The role of Nef on the

cytoskeletal processes involved in FcγR-mediated phagocytosis has not yet been elucidated.

1.6.3.4.3 Actin-binding proteins.

Activation of Syk also results in the phosphorylation and localisation of various cytoskeletal proteins including the actin-binding proteins paxillin, vinculin, talin and alpha-actinin (Greenberg *et al.*, 1990; Greenberg *et al.*, 1994; Allen and Aderem, 1996). During phagocytosis, these proteins are localised in the submembranous region beneath phagocytosed particles and are required for actin polymerisation and cytoskeletal rearrangement (Cox *et al.*, 1996). Paxillin is a potential downstream effector of Syk, as this protein has been shown to interact with a variety of proteins involved in growth control and cytoskeletal reorganisation (reviewed in Turner, 1998). The abundance of binding motifs in paxillin for structural and regulatory proteins has led to a suggestion that it is important in recruiting signalling molecules at sites of actin rearrangement and to facilitate their interaction during phagocytosis (Turner *et al.*, 1999). Paxillin has been previously shown to be tyrosine phosphorylated in murine peritoneal macrophages during FcγR-mediated phagocytosis (Greenberg *et al.*, 1994) and to co-localise with phagosomes (Greenberg *et al.*, 1994; Allen and Aderem, 1996).

Paxillin interacts with a variety of proteins, such as FAK (focal adhesion kinase), Pyk2 and Vav, and in this way it organises focal adhesion complexes and cytoskeletal rearrangement (reviewed in Turner, 1998). Paxillin phosphorylation following cell adhesion has been postulated to involve the kinase p125 FAK, shown previously to bind to paxillin LD motifs (Turner, 1998). The involvement of FAK in FcγR-mediated phagocytosis remains controversial, with one study showing no enhancement of tyrosine phosphorylation of FAK during phagocytosis in murine macrophages (Greenberg *et al.*, 1994), and another report demonstrating the expression and phosphorylation of FAK as a result of FcγR cross-linking in human monocytes (Pan *et*

al., 1999). However, it has been reported that human monocytes/macrophages isolated under stringent conditions and free of platelet contamination do not express FAK (Li *et al.*, 1998), but they activate and phosphorylate another member of the FAK family, Pyk2. This kinase is 45% identical in amino acid sequence to FAK (Avraham *et al.*, 1995), and is involved in reorganisation of the cytoskeleton, such as locomotion and adhesion and subsequent calcium or PKC co-stimulatory activation in human monocytes (Astier *et al.*, 1997; Li *et al.*, 1998; Duong *et al.*, 1998).

1.6.3.5 Rearrangement of actin-based cytoskeleton.

Actin assembly and disassembly plays a crucial role in a number of cellular functions such as cell motility and cell shape changes including phagocytosis. The actin cytoskeleton is a dynamic network composed of actin polymers and actin-binding proteins. The polymeric form of actin assembles via interactions of monomeric actin (G-actin) with polymerised actin (F-actin) at barbed ends. The first step of actin polymerisation is uncapping of the barbed ends of existing actin filaments by barbed-end-capping proteins such as gelsolin or capG (reviewed in Schmidt and Hall, 1998). Subsequent binding of actin monomers onto pre-existing actin filaments is controlled by actin filament capping proteins such as profilin or heterodimeric capping protein preventing addition and loss of actin, and severing proteins (gelsolin, villinin, fragmin, adseverin, scinderin) allowing actin polymerisation in response to a phagocytic signal (Schmidt and Hall, 1998). Cofilin is an actin depolymerisation protein and promotes disassembly of actin filaments by binding to actin monomers and polymers, and removing monomers from the filaments (Hayden *et al.*, 1993; Hawkins *et al.*, 1993). Phosphorylation of cofilin by LIM-K inactivates cofilin actin-binding and depolymerising activities leading to accumulation of actin filaments (Arber *et al.*, 1998).

Organisation of actin polymers is mediated by actin binding proteins such as α-actinin, filamin, fimbrin and villin (which are responsible for the formation of high-angle

branches between actin filaments or actin bundles generated by linking parallel actin filaments) (Schmidt and Hall, 1998). Actin filaments are formed at distinct sites in the cell called focal adhesions. During FcγR-mediated phagocytosis focal adhesions in the submembranous region beneath phagocytosed particles are associated with protein complexes including paxillin, talin, α-actinin, vinculin and FAK (Burridge and Fath, 1989; Greenberg *et al.*, 1990; Burridge *et al.*, 1990; Turner and Burridge, 1991). Pseudopodial extension occurs via recruitment of actin-binding proteins and protrusion of the F-actin meshwork against the plasma membrane (Greenberg, 1995). A specific inhibitor of F-actin, cytochalasin D, inhibits both actin polymerisation and FcγR-mediated phagocytosis, suggesting that F-actin assembly is critical for phagocytosis of IgG-opsonised particles (Greenberg *et al.*, 1994). Myosins are motor proteins interacting with actin polymers and controlling the movement of actin filaments (Wells *et al.*, 1999). Three types of myosin, I, V, IX have been demonstrated to localise adjacent to the phagosome and participate in the phagosome closure behind the phagocytosed particle (Swanson *et al.*, 1999). The myosin ATPase activity is augmented by myosin light chain kinase (MLCK), which in turn is required for ingestion of IgG-opsonised particles (Mansfield *et al.*, 2000).

1.7 OBJECTIVES AND RATIONALE.

Although the treatment of HIV-infected individuals has improved considerably with the introduction of HAART, research into the pathogenesis of AIDS-related opportunistic infections is still extremely important and relevant. HAART dramatically reduces the plasma viral load, improves CD4 T cell counts, thereby delaying the progression of the disease and decreasing the occurrence of opportunistic infections (Hammer *et al.*, 1996; Hammer *et al.*, 1997; Gulick *et al.*, 1997; Palella *et al.*, 1998). Despite the clinical improvement associated with HAART, current antiviral drugs are not able to eradicate HIV-1 due to the persistence of the latent reservoirs of the virus (Ho, 1998; Saag and

Kilby, 1999). In patients receiving HAART and characterised by long-term undetectable viral load, replication-competent HIV-1 can be recovered from resting CD4 T cells (Finzi *et al.*, 1997; Chun *et al.*, 1997; Wong *et al.*, 1997) or peripheral blood monocytes (Crowe and Sonza, 2000; Sonza et al., 2001). Viral reservoirs represent therefore a potentially life long persistence of replication-competent forms of HIV-1 that can not be suppressed by current antiretroviral treatment. Strains of HIV-1 that are resistant to reverse transcriptase and protease inhibitors arise in the majority of healed patients who are not adhered to treatment or have other reasons for low plasma drug levels (Hecht *et al.*, 1998). Viral load rises as a result of emergence of drug resistant HIV-1. Opportunistic infections are the major problem for patients failing HAART and are likely to increase, as the resistance to antiretroviral drugs becomes more widespread.

Development of novel adjunctive immunotherapy able to control opportunistic pathogens is urgently needed. Although current drugs used for treatment of opportunistic infections are usually effective, drug resistance leading to persistent clinical symptoms is increasingly being recognised. Furthermore, some of the antibiotics used for the treatment of opportunistic infections (eg. rifamycin) lower the blood levels of antiviral drugs, and therefore can not be used in combination with HAART.

Our understanding of the mechanisms by which HIV-1 impairs cell-mediated immunity leading to the development of opportunistic infections is critical for the full control of HIV-1 disease progression. Cells of macrophage lineage provide critical immune responses against a variety of opportunistic pathogens in immunocompetent individuals. Following HIV-1 infection, a number of those functions are impaired, including phagocytosis and intracellular killing of a variety of pathogens including *Mycobacterium avium* complex, *Toxoplasma gondii, Candida albicans* (Crowe *et al.*, 1994; Biggs *et al.*, 1995). Therefore, defective immunological function of monocytes

and macrophages leading to inefficient control of opportunistic pathogens contributes to the development of opportunistic infections and results in significant morbidity and mortality.

The main aim of this study was to investigate the mechanisms underlying defective phagocytosis by cells of macrophage lineage following HIV-1 infection. As monocytes and macrophages are distinct cell populations that differ in their susceptibility to HIV-1 infection (monocytes being highly refractory, whereas macrophages are fully permissive to HIV-1) (Sonza et al., 1996), the mechanism of defective phagocytosis by monocytes and macrophages was examined independently. Phagocytosis of pathogens is mediated by different receptors (eg FcγRs and C'Rs) utilising distinct signalling pathways to promote cytoskeletal rearrangements and engulfment (Allen and Aderem, 1996). The mechanism underlying defective phagocytosis mediated via FcγRs by HIV-infected MDM was investigated in this study. Since most reports which delineate specific signalling events during FcγR-mediated phagocytosis have been performed using murine macrophages, the initial aim was to examine the signalling pathways required for FcγR-mediated phagocytosis in uninfected human MDM before studying the impact of HIV-1 on these pathways. Phagocytosis of the common opportunistic pathogens such as *Mycobacterium avium* complex (utilising C'Rs) and *Toxoplasma gondii* (utilising FcγRs) by monocytes obtained from HIV-infected individuals, as well as from the Sydney Blood Bank Cohort (SBBC) members infected with an attenuated strain of HIV-1 was also examined. In this work, mechanistic studies were limited by the amount of blood available, therefore flow cytometric assays were developed to determine potential causes for inhibition of phagocytosis. The final aim of this thesis was to assess the effect of the immunomodulator GM-CSF on phagocytosis by both monocytes and macrophages following HIV-1 infection, in order to determine whether it is feasible to use GM-CSF in adjunctive therapy for the treatment of opportunistic infections in HIV-infected individuals.

1.8 RELEVANT PUBLICATIONS.

The following publications have arisen from the work presented in this chapter:

Kedzierska, K., Rainbird, M.A., Lopez, A.F. and Crowe, S.M. (1998) Effect of GM-CSF on HIV-1 replication in monocytes/macrophages in vivo and in vitro: a review. *Vet Immunol Immunopathol* (Invited Review), **63:**111-121.

Kedzierska, K. and Crowe, S.M. (2001) Cytokines and HIV-1: interactions and clinical implications. *Antivir Chem & Chemother* (Invited Review), **12:**138-145.

Kedzierska, K., Ellery, P., Mak, J., Jaworowski, A. and Crowe, S.M. (2002) The mechanisms underlying defective phagocytosis by human monocytes and macrophages following HIV-1 infection. *J Clin Virol* (Invited Review; in press).

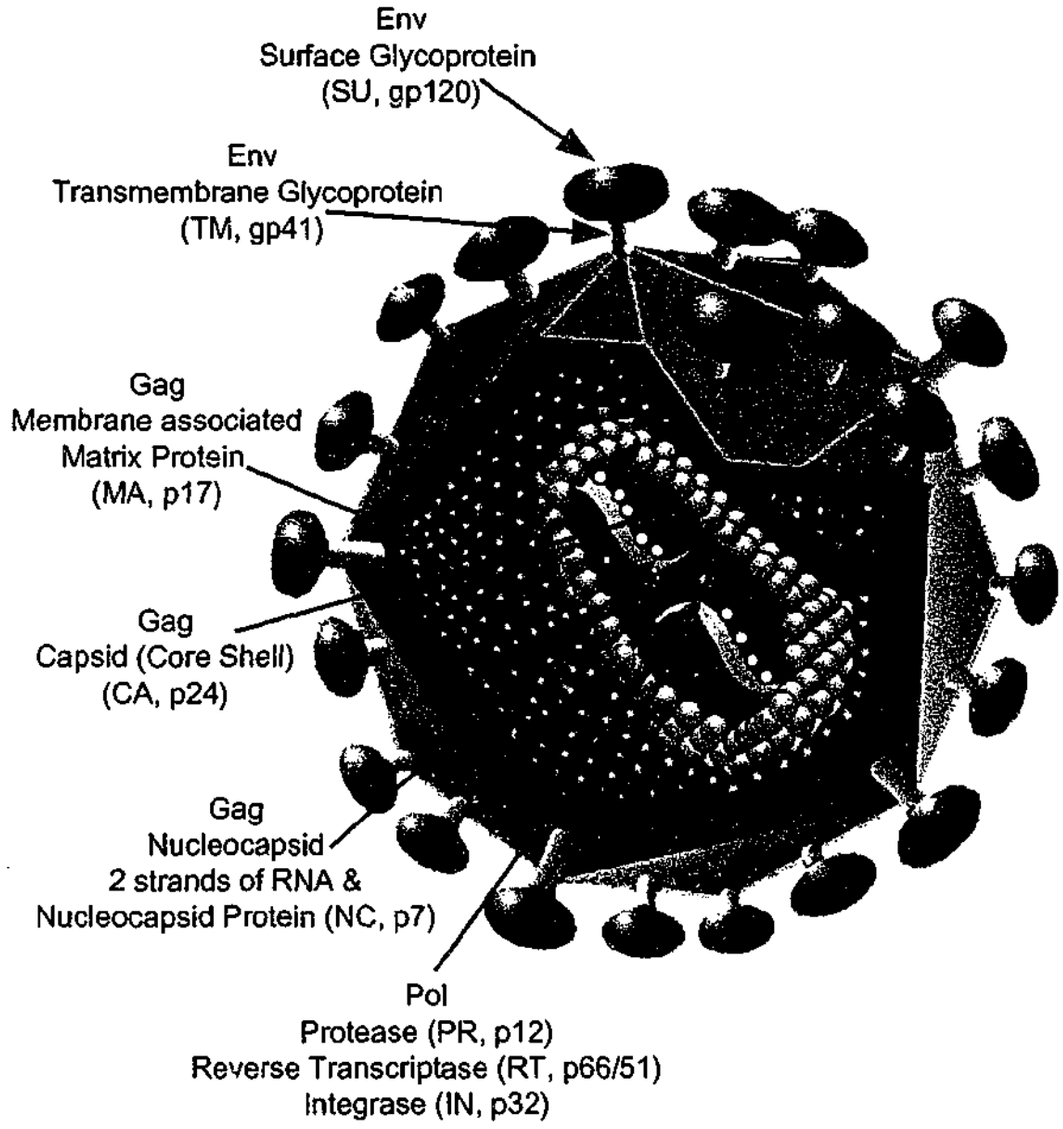

Figure 1.1 Structure of the HIV-1 virion.

HIV-1 is enveloped by a lipid bilayer containing 72 glycoprotein spikes consisting of the externally expressed glycoprotein (SU, gp120) and the membrane embedded transmembrane glycoprotein (TM, gp41). The cone shaped capsid core is composed of the capsid protein (CA, p24), which contains two identical RNA strands complexed with the nucleocapsid protein (NC, p7) and viral enzymes: protease (PR, p12), reverse transcriptase (RT, p66/p51) and integrase (IN, p32). A matrix shell between the lipid bilayer and the capsid is formed by the membrane-associated matrix protein (MA, p17) (modified from the Critical Path AIDS Project; www.critpath.org).

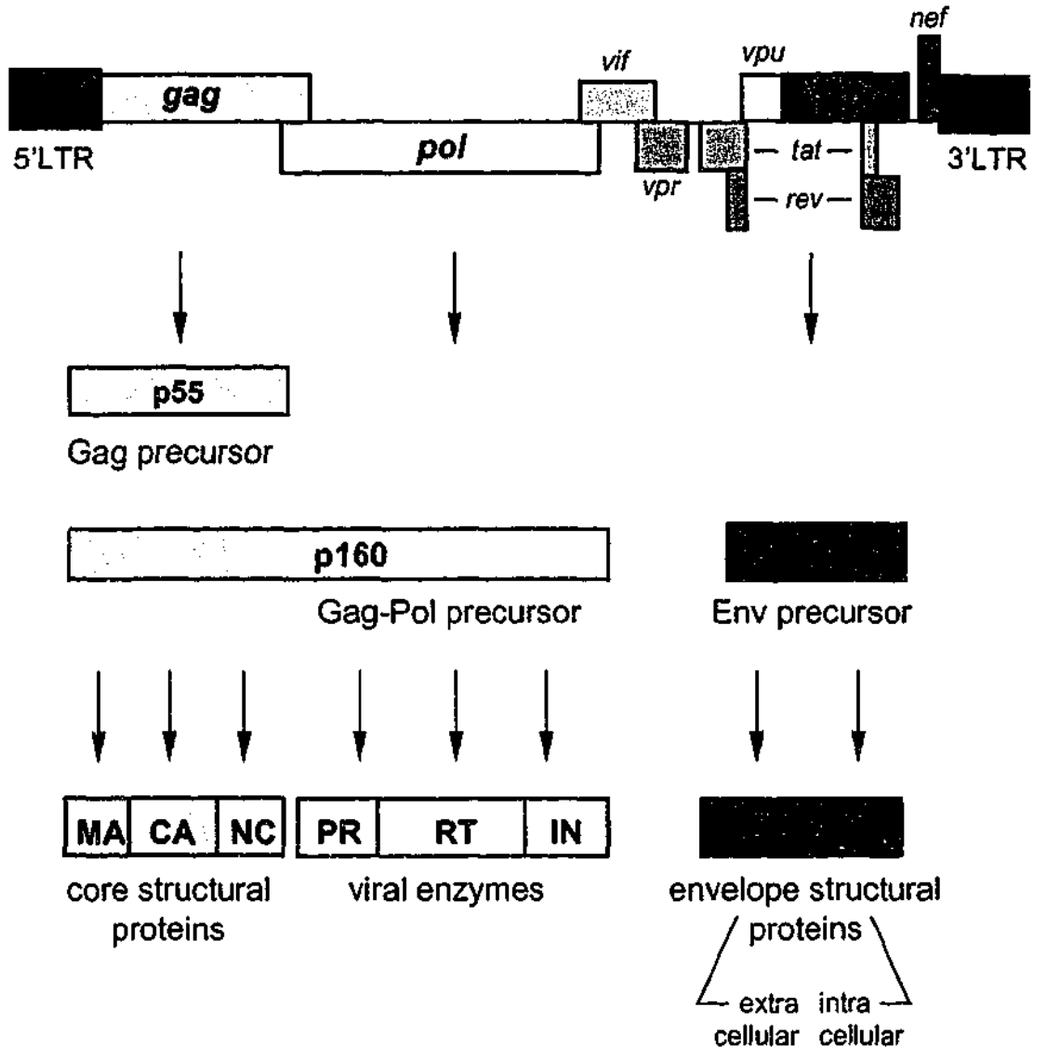

Figure 1.2 Organisation of the HIV-1 genome.

The HIV-1 provirus encodes three structural proteins Gag, Pol and Env, flanked by two long terminal repeats (LTR). Gag precursor is cleaved into three core structural proteins: matrix protein (MA), capsid (CA) and nucleocapsid protein (NC). Pol polyprotein is cleaved into three enzymes: protease (PR), reverse transcriptase (RT) and integrase (IN), whereas Env precursor is cleaved into two envelope proteins gp120 and gp41. The three regulatory proteins are Tat, Rev and Nef, and the three accessory proteins are Vif, Vpr and Vpu (modified from www.yale.edu/bio243/HIV/genome.htlm).

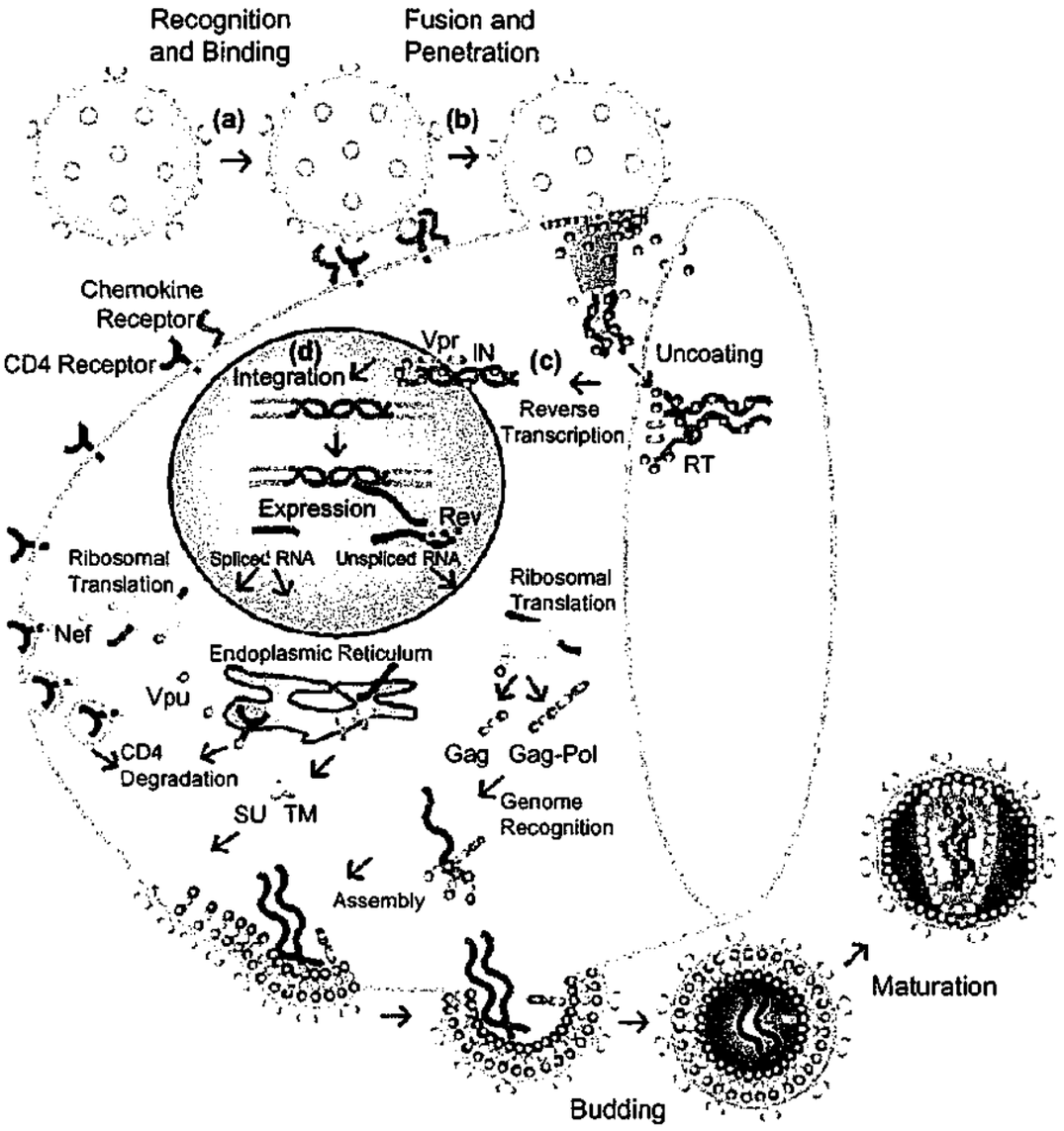

Figure 1.3 The HIV-1 replication cycle.

Following (a) HIV-1 binding to the CD4 receptor and the chemokine co-receptor, (b) the virion fuses with the cell membrane and enters the cytoplasm. After the virion is uncoated, (c) the viral RNA is reverse transcribed into double-stranded DNA and (d) integrated into the host cell's genome. The transcription of viral mRNA (multiply-spliced, singly-spliced and unspliced) occurs in the nucleus. Rev mediates transport of singly-spliced and unspliced mRNA to the cytoplasm, where translation occurs. Viral proteins and RNA are assembled into the virion at the cell membrane, followed by budding and maturation of HIV-1 (reproduced from Turner and Summers, 1999).

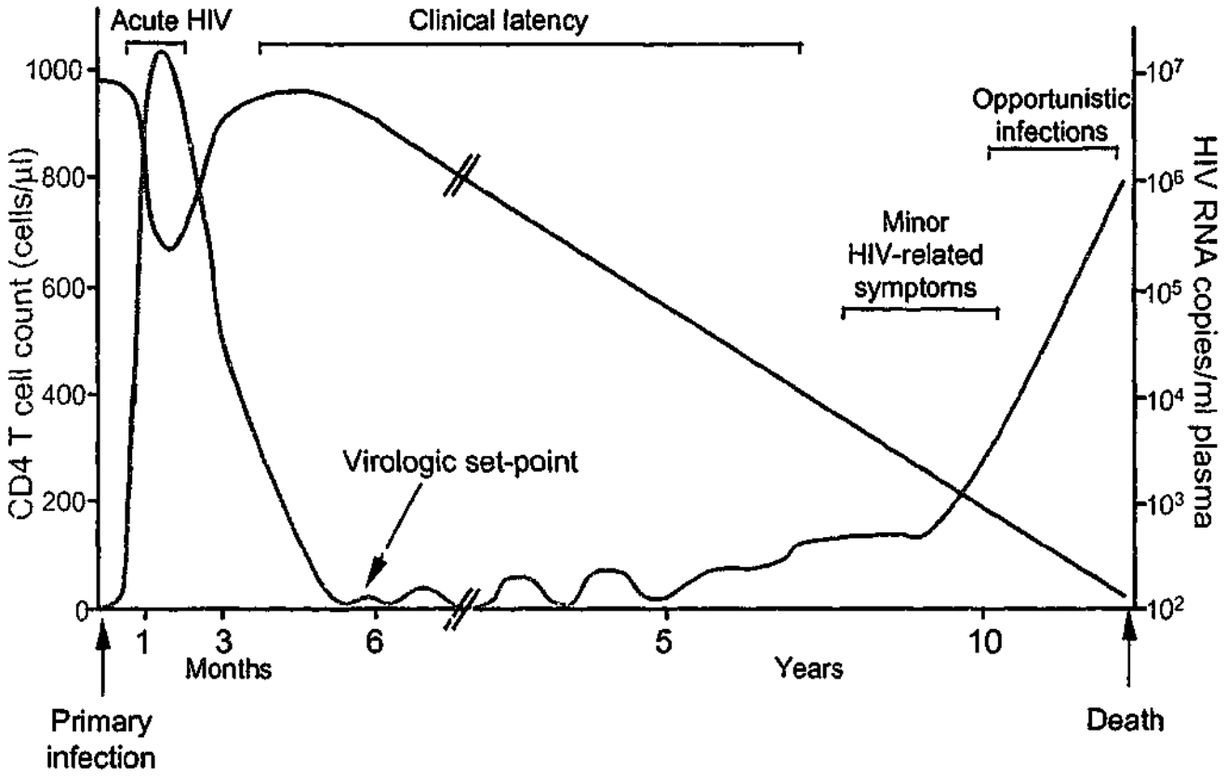

Figure 1.4 Typical course of HIV-1 infection in untreated patients.

Acute HIV-1 infection is characterised by high levels of plasma HIV-1 RNA (viral load, red line) and a transient decline in CD4 T cell count (blue line). This is usually followed by a period of clinical latency in which plasma viral load varies from patient to patient but is often undetectable or very low, and CD4 T cell counts are maintained in the normal range. The virologic set point which occurs approximately 6 months after infection (shown to be low in this figure) reflects the equilibrium between the host's immune responses and HIV-1, and predicts the long term clinical outcome of infection. Without any treatment, the CD4 T cell count steadily declines and HIV-1-related symptoms develop. The onset of AIDS-defining opportunistic infections generally occurs in individuals with CD4 T cell counts <200 cells/µl and high plasma viral load on average 9 to 10 years after the initial infection.

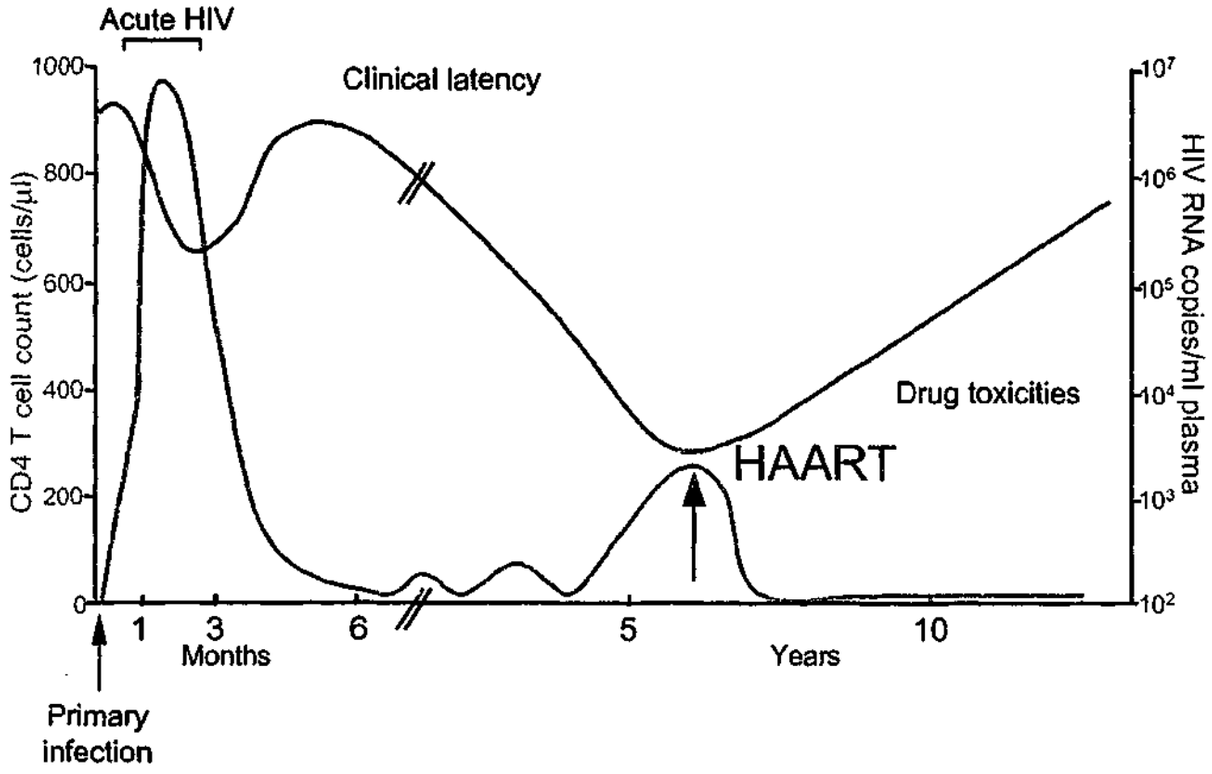

Figure 1.5 Typical course of HIV-1 infection in the era of HAART.

Highly active antiretroviral therapy (HAART) delays the progression to AIDS and results in dramatic reductions in plasma viral load (blue line), but does not completely eradicate the virus. HAART improves CD4 T cells count (red line), and decreases occurrence of opportunistic infections, but is associated with increasing risk of drug toxicities.

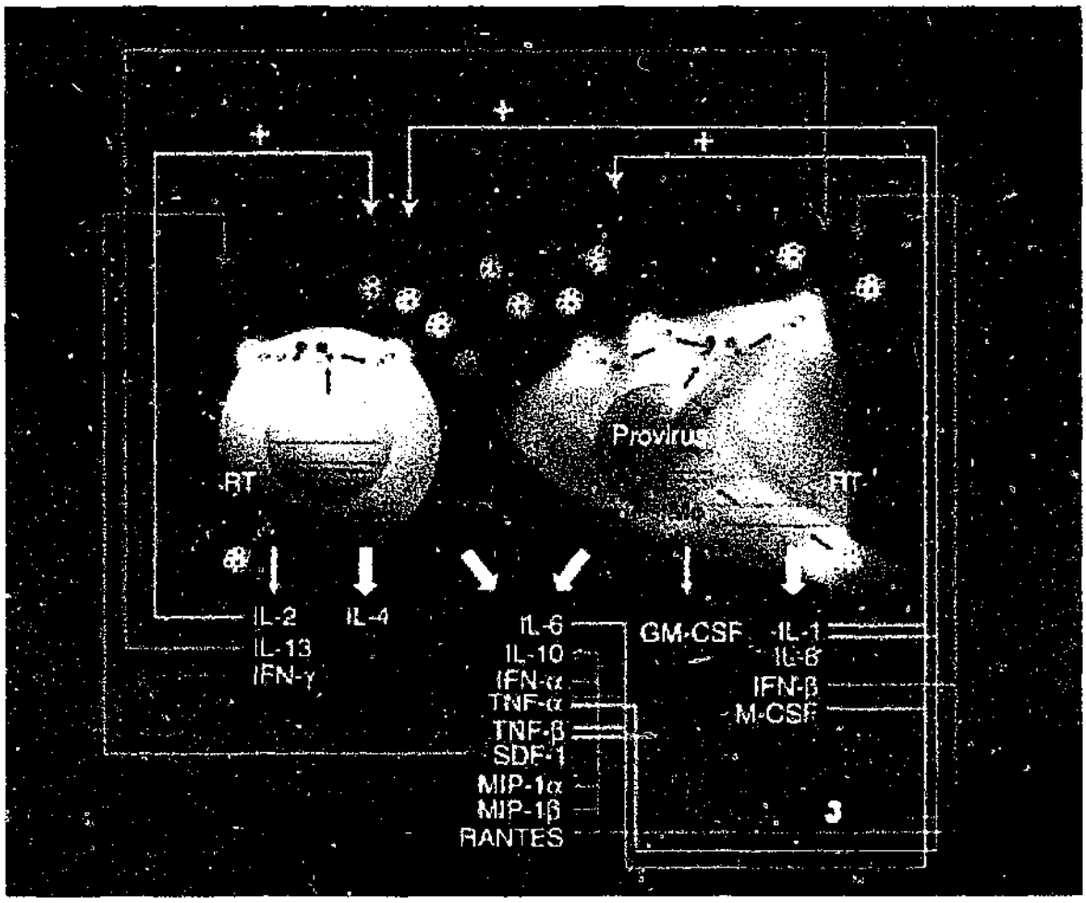

Figure 1.6 Interactions between HIV-1 and cytokines.

HIV-1 infection is associated with dysregulated cytokine production by T cells and cells of macrophage lineage. During the course of HIV-1 infection secretion of T helper (Th) type 1 cytokines (IL-2, IFN-γ) is generally decreased, whereas production of Th type 2 cytokines (IL-4, IL-10), pro-inflammatory cytokines (IL-1, IL-6, IL-8, TNF-α), α-chemokine (SDF-1) and β-chemokines (MIP-1α, MIP-1β, RANTES) is increased. These cytokines subsequently increase (orange line), decrease (green line) or have bi-functional effects (blue line) on HIV-1 infection and replication, indicating a complex and dysregulated interplay between cytokines and HIV-1 *in vivo* (reproduced from Kedzierska and Crowe, 2001).

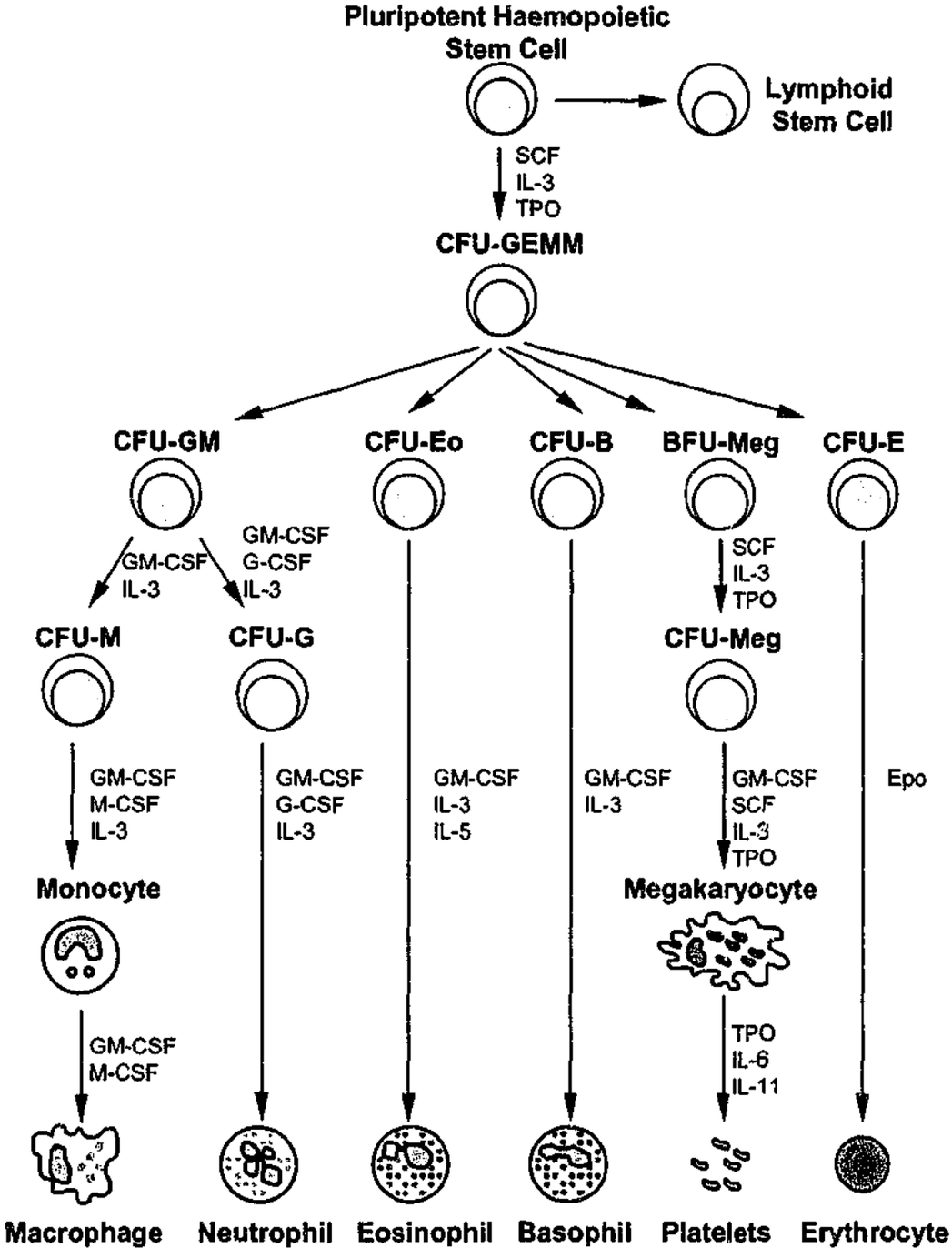

Figure 1.7 Development of granulocytes and monocytes.

Pluripotent haemopoietic stem cells generate the progenitor of myeloid lineage CFU-GEMM, which gives rise to granulocytes, erythrocytes, monocytes and megakaryocytes. Cytokines such as IL-3, GM-CSF and M-CSF promote proliferation and differentiation of mature monocytes, and subsequent differentiation into specialised tissue macrophages (modified from Kedzierska *et al.*, 1998).

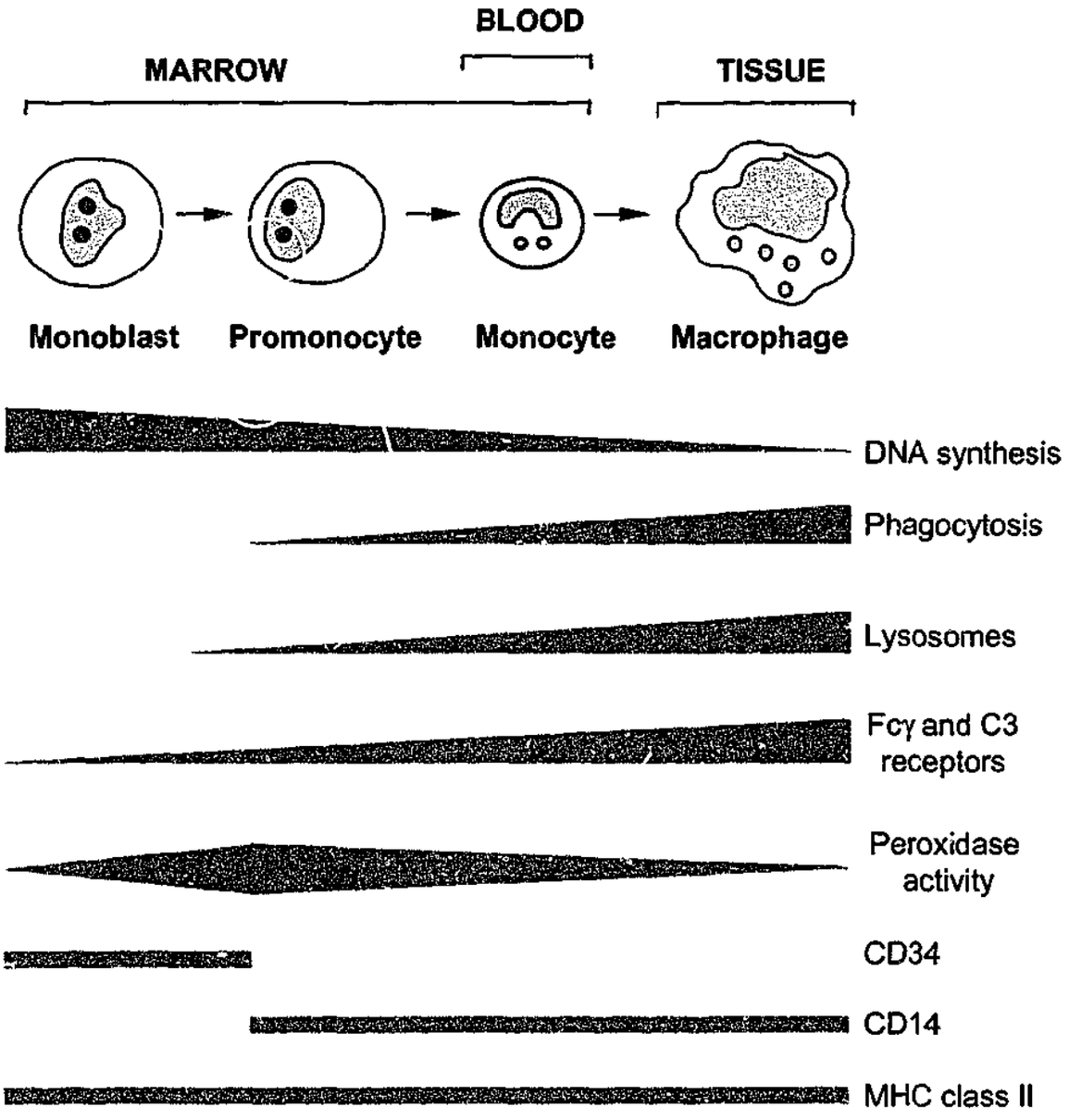

Figure 1.8 Characteristics of cells of macrophage lineage.

Phenotypical and functional changes during monocyte and macrophage differentiation. Mature cells of macrophage lineage lose CD34 surface expression, gain the CD14 marker and maintain MHC class II expression. They have increased surface expression of phagocytic receptors, increased phagocytic and peroxidase activity and intracellular levels of lysosomal enzymes, but decreased DNA synthesis (modified from Auger and Ross, 1992).

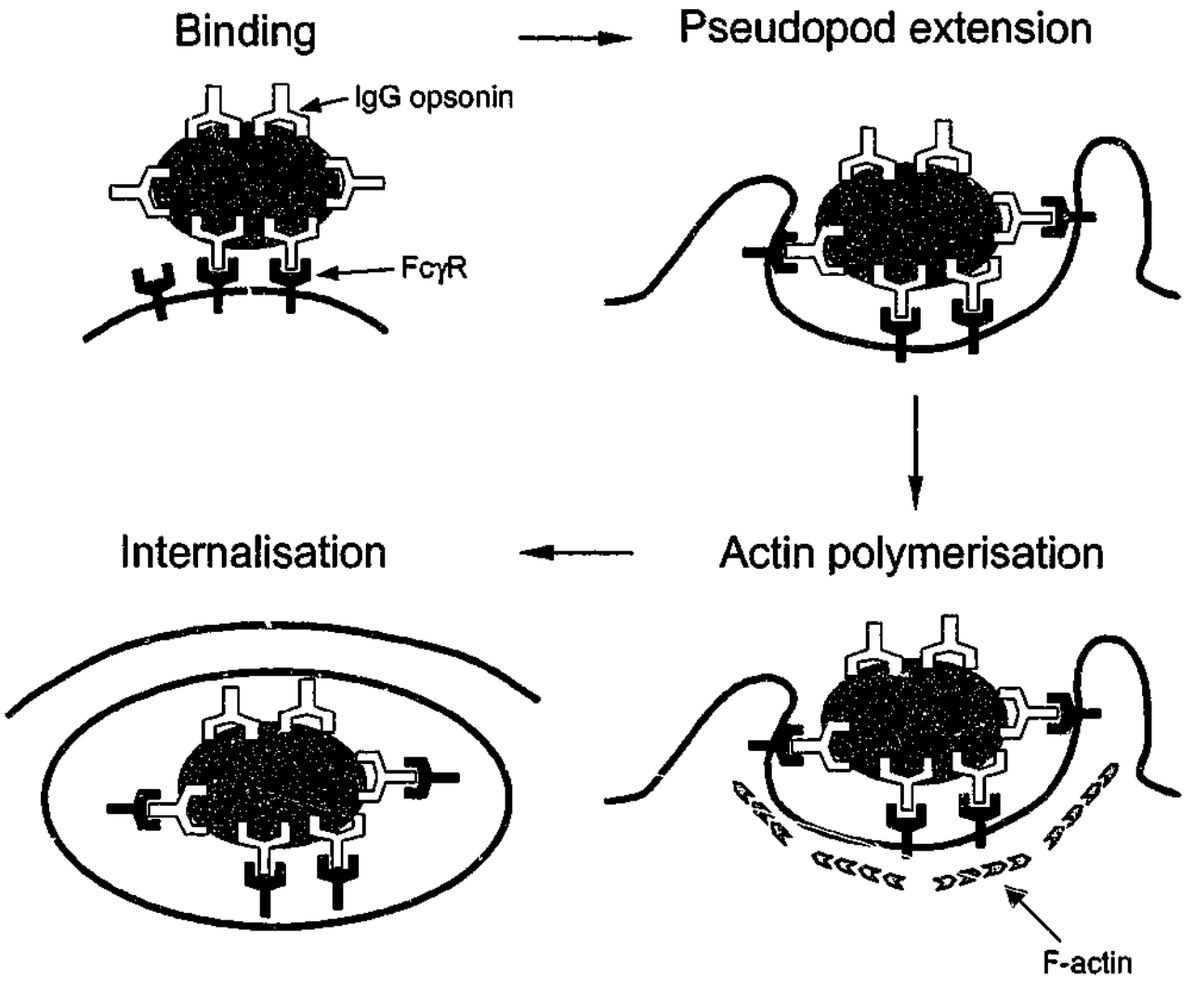

Figure 1.9 Mechanism of phagocytosis.

Schematic representation of FcγR-mediated phagocytosis. Phagocytosis is initiated by the interaction of phagocytic receptors with an IgG-opsonised particle. This is followed by a cascade of signalling events resulting in pseudopodia formation, actin polymerisation at the site of ingestion and internalisation of the phagocytosed particle.

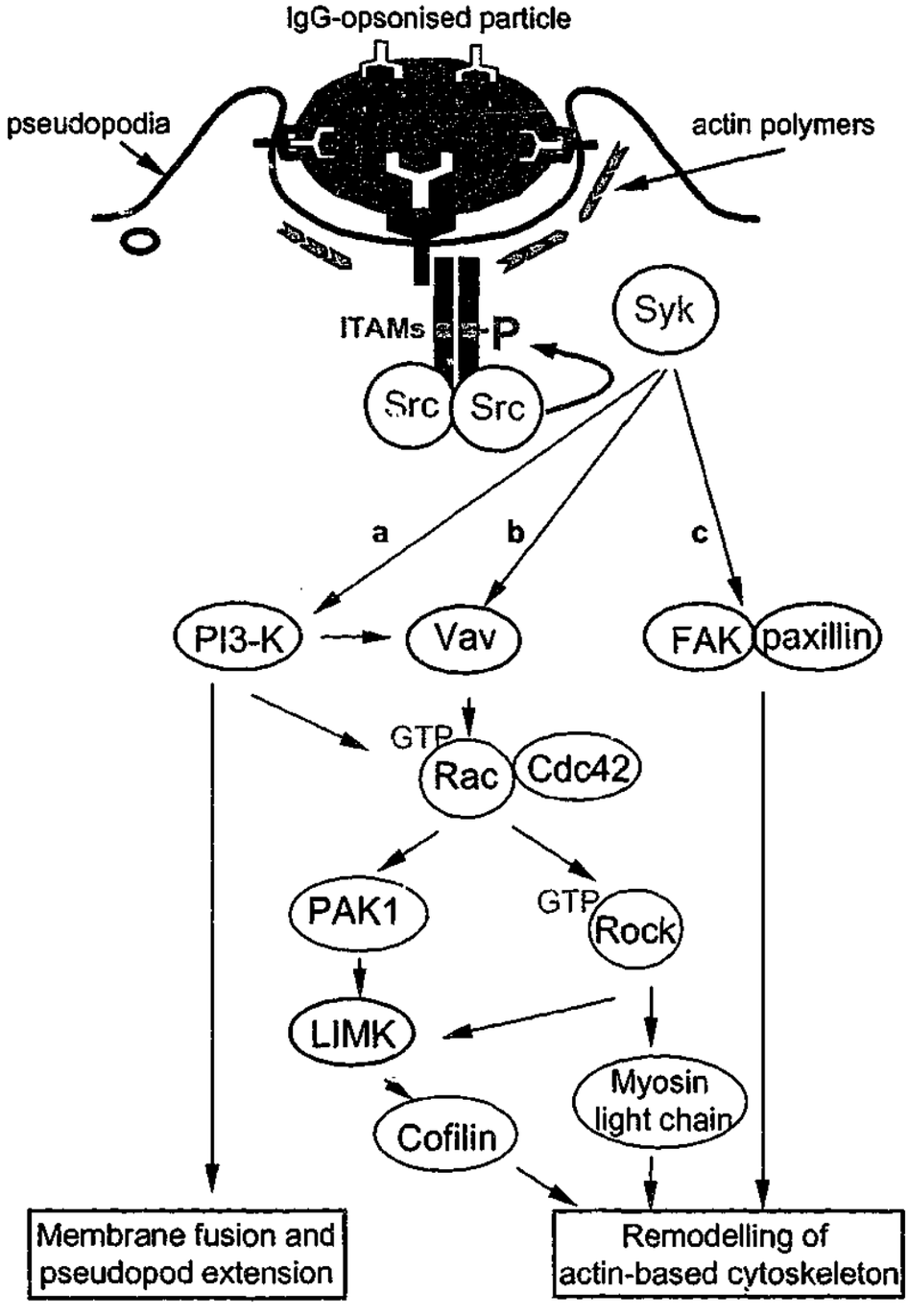

Figure 1.10 FcγR-mediated phagocytosis - signal transduction.

Schematic representation of signalling events occurring during FcγR-mediated phagocytosis. Following the binding of IgG-opsonised particles, clustering of FcγR leads to activation of tyrosine kinases from Src family associated with immunoreceptor tyrosine-based activation motif (ITAM)-containing γ-chain of FcγR I, III or cytoplasmic domain of FcγR II. Activation of Src kinases results in phosphorylation of tyrosine residues within ITAMs, which subsequently create docking sites for Syk. Activated Syk promotes phosphorylation of a number of downstream effectors leading to rearrangement of the actin-based cytoskeleton and engulfment of the phagocytosed particle.

Table 1.1 Accessory proteins of HIV-1.

Protein	Product	Function (s)
Tat	Transcriptional transactivator of LTR	Transcriptional activator, which up-regulates all viral proteins.
Rev	Regulator of virus protein expression	Nuclear RNA export factor, which facilitates the export of unspliced and singly spliced viral mRNAs to the cytoplasm.
Nef	Negative factor	Numerous effector functions: down-regulation of cell surface CD4 and MHC I; enhances virion infectivity; effects on cellular signal transduction and activation.
Vpr	Viral protein R	Weak transactivator; nuclear import; G2 arrest in cell cycle.
Vif	Virion infectivity factor	Enhances infectivity of viral particles.
Vpu	Viral protein U	Enhances virion release from the cell; selective degradation of CD4 in the ER.

Modified from Wang, 2000.

Table 1.2 Effects of HIV-1 infection on cytokine production *in vivo* and *in vitro*.

Cytokine	Secretion level	Source	HIV-1 infection	Reference
IL-1	↑	MDM	*in vitro*	Esser, 1991; 1996; 1998
	↑	monocytes	*in vivo*	Sadeghi, 1995; Baqui, 2000
IL-2	↓	CD4 T cells	*in vitro*	Fan, 1997
	↓	PBMC, CD4 T cells	*in vitro* – gp160	Hu, 1994
	↓	PBMC	in vivo	Barcellini, 1994; Meyaard, 1996
IL-4	↑	PBMC, CD4 T cells	*in vivo*	Barcellini, 1994; Maggi, 1994
	-	CD4 T cells	*in vivo*	Meyaard, 1996
	↓	CD4 T cells	*in vivo*	Re, 1992
IL-6	↑	MDM	*in vitro*	Esser, 1991; 1996, Foli, 1997;
	↑	THP-1 cell line, PBMC	*in vitro*:gp41, gp120	Takeshita, 1995; Capobianchi, 1996
	↑	Serum	*in vivo*	Birx, 1990
IL-8	↑	MDM	*in vitro*	Esser, 1991; 1996; 1998
	↑	Serum, BA fluid	*in vivo*	Denis, 1994
IL-10	↑	Monocytes, MDM	*in vitro*	Borghi, 1995
	↑	PBMC, Monocytes/MDM	*in vitro*:gp41, gp120	Borghi, 1995; Capobianchi, 1996
	↑	PBMC, BA fluid	*in vivo*	Barcellini, 1994; Denis, 1994
IL-12	↑	Monocytes, MDM	*in vitro* – gp120	Fantuzzi, 1996
	↓	PBMC	*in vivo*	Marshall, 1999
IL-13	↓	PBMC, CD4, CD8 T cells	*in vivo*	Bailer, 1999
IFN-α	↓	Monocytes, MDM	*in vitro*	Gendelman, 1990; Perno, 1994
	↑	CD4 T cells, Monocytes	*in vitro* – gp120	Capobianchi, 1996
	↑	Serum	*in vivo*	Ambrus, 1989
IFN-β	↑	Monocytes, MDM	*in vitro* - gp120	Gessani, 1994; 1997
IFN-γ	↓	CD4 T cells	*in vitro*	Fan, 1997
	↑	CD4 T cells	*in vitro*	Vyakarnam, 1990
	↓	PBMC	*in vivo*	Meyaard, 1996; Bailer, 1999
GM-CSF	↓	MDM	*in vitro*	Esser, 1996; Esser, 1998
	-	Monocytes	*in vivo*	Delemarre, 1998
M-CSF	↑	MDM	*in vitro*	Gruber, 1995; Kutza, 2000
	↑	Cerebrospinal fluid	*in vivo*	Gallo, 1994
TNF-α	↑	PBMC, CD4 T cells,MDM	*in vitro*	Vyakarnam, 1990; Esser, 1991, Esser, 1996; Foli, 1997
	↑	Monocytes, PBMC, Serum	*in vivo*	Lahdevirta, 1988; Baqui, 2000
TNF-β	↑	PBMC, CD4 T cells	*in vitro*	Vyakarnam, 1990
	↑	Cerebrospinal fluid	*in vivo*	Jassoy, 1993
SDF-1	↑	PBMC	*in vivo*	Ohashi, 1998
MIP-1α,β, RANTES	↑	CD4 T cells, MDM	*in vitro*	Greco, 1999
	↑	PBMC, Plasma	*in vivo*	Cocchi, 1995; Garzino-Demo, 1999

MDM: monocyte-derived macrophages; PBMC: peripheral blood mononuclear cells;

BA: bronchoalveolar (modified from Kedzierska and Crowe, 2001).

Table 1.3 Effects of cytokines on HIV-1 replication.

Cytokine	Replication level	Source of cells	HIV-1 infection	Reference
IL-1	↑	PBMC, MDM, U1	*in vitro*	Poli, 1994; Granowitz, 1995
IL-2	↑	PBMC, T cell line	*in vitro*	Foli, 1995; Weissman, 1996
	↑	PBMC	*in vivo*	Kinter, 1995
IL-4	↑	Monocytes, MDM, PBMC	*in vitro*	Kazazi, 1992; Naif, 1994; 1997
	↓	Monocytes, MDM	*in vitro*	Schuitemaker, 1992; Wang, 1998
IL-6	↑	PBMC, MDM, U1	*in vitro*	Poli, 1990; 1992; 1994
	-	Plasma	*in vivo*	Marfaing-Koka, 1996
IL-7	↑	PBMC	*in vitro*	Moran, 1993
	↑	PBMC	*in vivo*	Smithgall, 1996
IL-10	↓	MDM, U1	*in vitro*	Weissman, 1994; 1995
IL-12	↑	PBMC, T cell lines	*in vitro*	Foli, 1995; Al-Harthi, 1998
	↓	MDM	*in vitro*	Akridge, 1996
IL-13	↓	MDM	*in vitro*	Montaner, 1993
IL-15	↑	PBMC, T cell lines	*in vitro*	Lucey, 1997
	↑	PBMC	*in vivo*	Lucey, 1997
IL-16	↓	CD4 Tcells, PBMC, MDM	*in vitro*	Maciaszek, 1997; Amiel, 1999
IL-18	↑	U1	*in vitro*	Shapiro, 1998
IFN-α, IFN-β	↓	Monocytes, MDM, U937	*in vitro*	Kornbluth, 1989; Poli, 1989
	↓	Plasma	*in vivo*	Schnittman, 1994
IFN-γ	↓	Monocytes, MDM	*in vitro*	Fan, 1994
	↑	U1, U937, PBMC, CD4 T cells	*in vitro*	Vyakarnam, 1990; Biswas, 1992, Han, 1996
GM-CSF	↑	MDM, U937	*in vitro*	Koyanagi, 1988; Schuitemaker, 1990; Perno, 1992
	Variable	MDM	*in vitro*	Kornbluth, 1989; Hammer, 1990
	↓	MDM	*in vitro*	Matsuda, 1995; Di Marzio, 1998;
M-CSF	↑	MDM	*in vitro*	Kornbluth, 1989, Kalter, 1991; Perno, 1992
TNF-α, TNF-β	↑	T cells, MDM, U1	*in vitro*	Folks, 1989; Novak, 1990; Naif, 1994, Han, 1996
SDF-1	↓	PBMC, HeLa/CD4 cells	*in vitro*	Oberlin, 1998
MIP-1α, MIP-1β, RANTES	↓	Monocyte, MDM, PBMC, Migroglial cells	*in vitro*	Cocchi, 1995; 1996; Alhatib, 1996; Capobianchi, 1998

MDM: monocyte-derived macrophages; PBMC: peripheral blood mononuclear cells

(modified from Kedzierska and Crowe, 2001).

Table 1.4 Distribution of tissue macrophages.

Tissue	Tissue macrophages
Liver	Kupffer cells
Lung	Alveolar macrophages
Lymph node	Macrophages
Spleen	Splenic macrophages
Brain	Microglial cells
Thymus	Macrophages
Peritoneum	Peritoneal macrophages
Skin	Langerhans' cells
Bone	Osteoclasts
Connective tissue	Histiocytes
Gastrointestinal tract	Macrophages
Genitourinary tract	Macrophages
Placenta	Macrophages

Modified from Auger and Ross, 1992.

Table 1.5 Phagocytosis-promoting receptors.

Phagocytic receptors (Rs)	
Opsonin-dependent Rs	**Opsonin-independent Rs**
IgG R (FcγR I, II, IIIA)	Complement R 3 (CR3)
Complement R 1 and 3 (C'R1, C'R3)	Macrophage mannose R
IgA R (FcαR I)	β_1 integrins
High affinity IgE R (FcεR I)	β-Glucan R
Low affinity IgE R (CD23, FcεR II)	Macrophage scavenger R
Vitronectin R ($\alpha_V\beta_3$)	Phosphatidylserine R

Modified from Greenberg, 1995.

Table 1.6 Signalling proteins required for FcγR-mediated phagocytosis.

Abbreviation	Name	Description / Function (s)
Cdc42	Cdc42	-a member of Rho family of GTPases, -accumulates at the phagosome and promotes cytoskeletal rearrangements, -involved in the appearance of the membrane around the IgG-opsonised particles.
cofilin	cofilin	-an enzyme required for actin depolymerisation, -phosphorylated and inactivated by LIMK.
FAK	Focal Adhesion Kinase	-a 125kDa cytoplasmic tyrosine kinase, -binds to paxillin LD motifs, -localises with integrins at the focal adhesions.
Hck	Hematopoietic cell kinase	-a 58-60kDa cytoplasmic tyrosine kinase of Src family -phosphorylated following clustering of FcγRs, -leads to phosphorylation of ITAMs.
ITAMs	Immunoreceptor Tyrosine-based Activation Motifs	-motifs positioned in the γ chain of FcγR I and FcγR III or the cytoplasmic domain of FcγR II, -contain tyrosine residues, which are phosphorylated upon activation of Src kinases, -provide a docking site for Syk.
LIMK	LIM-Kinase	-a serine kinase containing two LIM domains, -phosphorylated by activated ROCK during FcγR-mediated phagocytosis, -leads to subsequent phosphorylation and inactivation of cofilin,
PAK-1	P21-Activated Kinase	-a 68kDa serine/threonine kinase activated by Rac1, -co-localises in pseudopodia, membrane ruffles and phagocytic cups -associated with remodelling of the actin-based cytoskeleton -called Nef-associated kinase (NAK), essential for Nef-induced cytoskeletal rearrangements and cellular signalling.
paxillin	paxillin	-a 68kDa cytoskeletal protein, -localises beneath phagocytosed particles, required for actin polymerisation and cytoskeletal rearrangement, -a potential downstream effector of Syk
PI3-kinase	Phosphatidyl Inositide 3-kinase	-an enzyme catalysing phosphorylation of phosphoinositides in D3 position of the inositol ring, -a downstream effector of Syk, -required for pseudopod extension, phagosome closure and engulfment of IgG-opsonised targets.
Rac1	Rac1	-a member of Rho family GTPases activated by PI3-kinase or Vav, -accumulates at the phagosome, promotes phagocytic signalling, the closure of the phagosome and particle internalisation.
ROCK	Rho-associated kinase	-a serine/threonine kinase stimulated by Rac1, -inactivates myosin phosphatase, results in increased actomyosin contractility, -phosphorylates its downstream substrate LIM-kinase.

Syk	Syk	-a 72kDa cytoplasmic tyrosine kinase, -binds to tyrosine phosphorylated ITAMs of FcγRs via its SH2 domains, -results in the activation of downstream substrates, PI3-kinase, GTP-binding proteins, actin-binding proteins.
Vav	Vav	-a 95kDa guanine nucleotide exchange factor, -stimulated during FcγR-mediated phagocytosis by either products of the PI3-kinase reaction or Syk, -promotes GDP/GTP exchange activity of Rac1.

CHAPTER 2

MATERIALS AND METHODS

Materials and methods applied throughout this thesis are detailed in this chapter. Materials and methods which apply to specific chapters only are described in the individual chapters.

2.1 CELL ISOLATION.

2.1.1 Isolation of peripheral blood mononuclear cells.

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of HIV-, HBV-, HCV-, HTLV- and syphilis-seronegative blood donors (supplied by the Red Cross Blood Bank, Melbourne) by density gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Bioteck, Uppsala, Sweden) as previously described (Crowe *et al.*, 1987). Whole blood was diluted 1:2 with calcium- and magnesium-free phosphate-buffered saline (PBS-CMF) (GibcoBRL, Life Technologies, NY, USA). Diluted blood (25ml) was layered over 15ml of Ficoll-Paque in 50-ml conical tubes (Cellstar, Greiner Labortechnik, Frickenhausen, Germany) and centrifuged at 914×*g* for 25 min at room temperature in a Beckman centrifuge (Fullerton, CA, USA), with no brake. PBMC were collected from the interface of the Ficoll and plasma using a plastic transfer pipette (Samco Scientific, CA, USA), washed twice in PBMC-CMF and pelleted by centrifugation at 500×*g* for 10 min, followed by two washes in PBMC-CMF and centrifugation at 200×*g* for 10 min at 4^{0}C to remove contaminating platelets. Purified PBMC were either used immediately for monocyte isolation (Section 2.1.2) or resuspended in RPMI-1640 medium (GibcoBRL, Life Technologies, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; PA Biologicals Co,

Sydney, Australia), 2mM L-glutamine (GibcoBR) and 24μg/ml gentamycin (Delta West, Bentley, Western Australia, Australia) (supplemented RPMI-1640 medium) and used for amplification of HIV-1 stocks (Section 2.3).

2.1.2 Isolation of monocytes.

Human peripheral blood monocytes were isolated from freshly purified PBMC by plastic adherence. PBMC were resuspended at approximately $2x10^7$ cells/ml in Iscove's modified Dulbecco medium (GibcoBRL) supplemented with 10% heat-inactivated human AB-positive serum (supplied by the Red Cross Blood Bank, Sydney), 2mM L-glutamine and 24μg/ml gentamycin (supplemented Iscove's medium), and plated onto 150-cm^2 plastic petri dishes (Nalge Nunc International, Naperville, IL, USA) at no more than $3x10^8$ cells/plate. Plates were incubated for 1 to 2 hr in a humidified CO_2 incubator at 37^0C. Non-adherent cells were removed from monocyte monolayers by repeated (six times) washing and aspiration using pre-warmed (37^0C) PBS (containing calcium and magnesium). Adherent monocytes were detached by incubating cells with 20ml of cold (4^0C) PBS-CMF for 15 min on ice, followed by gentle scraping with a cell lifter (Costar, Cambridge, MA, USA). Monocytes were collected into 50-ml conical tubes containing 2.5ml FCS by washing the plate with cold (4^0C) PBS-CMF. Cells were centrifuged at 500×*g* for 10 min at 4^0C and resuspended in supplemented Iscove's medium at 0.5 to $1x10^6$ cells/ml. Monocytes were cultured in suspension in polytetrafluorethylene (Teflon) jars (Savillex, Minnetonka, MO, USA) or adherent to plastic in 24-well plates (Costar, Cambridge, MA, USA) in a 5%CO_2 humidified incubator at 37^0C.

Immediately after isolation, cell viability was greater than 95% as assessed by trypan blue dye (Sigma, St Louis, MO, USA) exclusion using an inverted phase microscope (Olympus, Tokyo, Japan). The purity of monocytes was greater than 90% as determined by immunofluorescence staining with anti-CD14 monoclonal antibody

(Mab) conjugated to phycoerythrin (CD14-PE; Becton Dickinson, Mountain View, CA, USA) and flow cytometric analysis using FACStar^{Plus} (Becton Dickinson). Briefly, freshly isolated monocytes ($2x10^5$ cells) were placed into 4-ml round bottom polypropylene tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA), washed with cold (4^0C) PBS-CMF and pelleted at 330x*g* for 5 min at 4^0C. Cells were resuspended in 100µl of PBS-CMF, stained with 0.5µg/ml of CD14-PE for 30 min on at 4^0C, followed by a wash in PBS-CMF, fixation with 200µl of 1% ultrapure formaldehyde (Polysciences, Warrington, PA, USA) and flow cytometric analysis. Using the same method, cells were also stained with anti-CD3 Mab conjugated to PE (CD3-PE; Becton Dickinson) or directly conjugated isotype-matched control (IgG1-PE; Becton Dickinson) to assess the level of contaminating T cells or background fluorescence respectively.

2.2 CELL LINES.

The 293T cells, a primary human embryonal kidney cell line transformed with SV40 large T cell antigen (ATCC McMaster University, Hamilton, Ontario, USA), were maintained in Dulbecco's modified Eagle medium (GibcoBRL) supplemented with 10% heat-inactivated FCS, 2mM L-glutamine and 100U/ml penicillin (GibcoBRL) and 100µg/ml streptomycin (GibcoBRL) (supplemented Dulbecco's medium) at 37^0C in a 5% CO_2 humidified incubator. Every three to four days, the 293T cells were passaged by resuspending $1x10^6$ cells in 20ml of supplemented Dulbecco's medium and transferring them to a new 150-cm^2 plastic petri dish (Nalge Nunc), for a maximum of 50 passages. The 293T cells were used for transfection of proviral DNA and subsequent virus production as described in Section 2.3.2.

2.3 AMPLIFICATION OF VIRUS STOCKS.

2.3.1 HIV-1 $_{Ba-L}$

Viral stocks of HIV-1_{Ba-L}, a laboratory adapted M-tropic strain of HIV-1 (the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, NIH, Bethesda, MD, USA) were amplified in monocyte-derived macrophages (MDM) in small volumes (up to 50ml), followed by amplification in PBMC in volumes up to 200ml as previously described by our laboratory (Crowe *et al.*, 1994). Briefly, freshly isolated monocytes (approximately $5x10^7$ cells) were adhered to a 80-cm^2 flask (Nalge Nunc) in 50ml of supplemented Iscove's medium for 5 days. The medium was removed and replaced with 10ml of HIV-1_{Ba-L} stock at multiplicity of infection (MOI) of 0.1 to 1.0 infectious particles/cell. After 4 to 8 hr of infection 40ml of fresh supplemented RPMI-1640 medium was added to the culture. Virus culture supernatant was collected on days 7, 10 and 14 post-infection, centrifuged to remove cell debris and clarified by using 0.2µm filters (Schleicher and Schuller, Dassel, Germany). HIV-1_{Ba-L} stocks were stored at -70^0C in small volumes and thawed immediately before use.

HIV-1_{Ba-L} viral stocks passaged through MDM were subsequently amplified in PBMC a maximum of three times. PBMC at a concentration of $2x10^6$ cells/ml were adhered to 175-cm^2 flasks (Nalge Nunc) and stimulated with phytohemagglutinin (PHA, 10µg/ml, Murex Diagnostics, Dartford, UK) for 3 days in 100 to 200ml of supplemented RPMI-1640 medium. The non-adherent cells were removed from the flask, centrifuged at 500x*g* for 7 min, resuspended in 20ml of HIV-1_{Ba-L} stock at MOI of 0.1 to 1.0 and returned to the flask. PBMC were infected for 4 to 8 hr and subsequently cultured in 100 to 200ml of supplemented Iscove's medium containing interleukin-2 (IL-2, 10U/ml; Boehringer-Mannheim, Mannheim, Germany). The culture supernatants were collected

on days 7, 10 and 14 post-infection and clarified as described for HIV-1_{Ba-L} stocks cultured in MDM. All the HIV-1 stocks were tested by the reverse transcriptase (RT) micro assay (Section 2.6.1) and the 50% tissue culture infectivity dose ($TCID_{50}$) assay (Section 2.4).

2.3.2 HIV-1 $_{NL(AD8)}$

The DNA construct pNL(AD8) was prepared by substituting the envelope coding DNA sequence from NL4.3 with the M-tropic AD8 envelope coding sequence, converting a T-tropic virus to M-tropic (a kind gift from Dr. Damien Purcell, Department of Microbiology and Immunology, The University of Melbourne). Virus stocks were generated using a calcium phosphate transfection method as previously described by our laboratory (Shehu-Xhilaga *et al.*, 2001). Briefly, the 293T cells ($1.2x10^6$) were plated onto 20-cm^2 plastic petri dishes (Nalge Nunc) and cultured for 24 hr in supplemented Dulbecco's medium as described in Section 2.2. DNA precipitate for transfection was prepared by adding drop wise 500µl of "DNA mix" (10µg pNL(AD8) DNA and 0.2M $CaCl_2$) to 500µl of Hepes buffered saline (280mM NaCl, 50mM Hepes acid and 1.5mM Na_2HPO_4; pH of 7.05) in 15-ml conical tubes (Nalge Nunc), followed by adequate mixing approximately 10 times. $Ca_3(PO_4)_2$-DNA complex was added drop wise onto 293T cells, distributed evenly and cultured for 12 hr at 37^0C in a 5% CO_2 humidified incubator. Cells were washed twice with pre-warmed (37^0C) PBS, resuspended in fresh supplemented Dulbecco's medium and cultured for an additional 24 hr. The viral supernatants were collected into 50-ml conical tubes, centrifuged at 1600x*g* for 30 min to remove cell debris, clarified with 0.22µm filters and stored at -70^0C for later use.

2.3.3 Primary isolates of HIV-1.

Primary isolates of HIV-1 from D36 and C18 (two of the Sydney Blood Bank Cohort members from which virus could be successfully isolated) were kindly prepared and an aliquot donated by Ms. Antoniette Violo (AIDS Pathogenesis Research Unit, Macfarlane Burnet Centre for Medical Research) and Ms. Kathy Chatfield (AIDS Cellular Biology Unit, Macfarlane Burnet Centre for Medical Research) respectively. Expansion of these M-tropic primary isolates was subsequently performed in CD8-depleted PBMC from HIV-1 seronegative donors. Uninfected donor PBMC, at a concentration of $2x10^6$ cells/ml, were stimulated with PHA (10µg/ml) and M-CSF (750U/ml, Genzyme, Cambridge, MA, USA) and cultured in supplemented RPMI-1640 medium in suspension for 3 days prior to CD8-depletion and use in co-culture for virus amplification. Depletion of CD8-positive T cells from uninfected donors was performed using anti-CD8 magnetic beads (Dynal, Oslo, Norway) according to manufacturer's instructions. Briefly, the required number of beads conjugated to anti-CD8 Mab (100µl of beads/$1x10^7$cells) was transferred into a 14-ml round-bottom polylpropylene tube (Becton Dickinson Labware). Beads were washed twice by resuspending them in 1ml of supplemented RPMI-1640 medium, placing on a magnetic particle concentrator (Dynal) for 3 min and removing the medium. Washed beads were mixed with PBMC resuspended in 5ml of supplemented RPMI-1640 medium in a 14-ml round-bottom tube and incubated on a sampler mixer (Dynal) for 30 min at 4^0C. The tube was placed on a magnetic particle concentrator for 3 min and CD8-depleted cells were collected into a new tube. A second round of CD8-depletion was performed to remove any remaining CD8-positive T cells as described for the first round of depletion, this time using 10µl of beads/$1x10^7$ cells.

CD8-depleted PBMC ($2x10^7$) were transferred into 15-ml conical tube and centrifuged at 500xg for 7 min. Cells were resuspended in 2ml of M-tropic primary HIV-1 isolate at

MOI of 0.1 to 1.0, placed into a 25-cm^2 flask (Nalge Nunc) and infected for 4 hr. Supplemented RPMI-1640 medium containing IL-2 (10U/ml) and M-CSF (750U/ml) was added to a final volume of 10ml. The culture supernatant (5ml) was collected on days 3, 7, 10 and 14 post-infection by centrifugation at 700x*g* for 10 min, clarifying the supernatant through a 0.22µm filter and stored at -70^{0}C until used. Cell pellets were resuspended in 5ml of fresh supplemented RPMI-1640 medium containing IL-2 (10U/ml) and M-CSF (750U/ml) and returned to the cultures.

2.4 DETERMINATION OF INFECTIOUS VIRUS TITRES.

The 50% tissue culture infectivity dose expressed as $TCID_{50}$/ml of the virus stock was determined by setting up 10-fold dilutions of HIV-1 stock in PBMC. HIV-1 stock virus suspension was serially diluted (ranging from undiluted to 10^{-7}) in a total of 200µl of supplemented RPMI-1640 medium per well of a 96-well plate (Nalge Nunc). Dilutions were performed in triplicate. PBMC, pre-stimulated with PHA (10µg/ml) for 3 days, were resuspended at a concentration of $5x10^6$/ml in supplemented RPMI-1640 medium containing 10U/ml of IL-2, added to the plate at $1x10^5$ cells/well and cultured at 37^{0}C in a 5% CO_2 humidified incubator. On day 3 post-infection, 100µl of medium was removed and replenished with fresh supplemented RPMI-1640 medium containing IL-2. On day 7 post-infection, 100µl of culture supernatant was collected and stored at -70^{0}C until assessed for RT activity in a standard RT micro assay (Section 2.6.1).

The $TCID_{50}$ of viral stock was determined by the last dilution at which more than 50% of wells were positive for RT activity and calculated as the number of infectious particles/ml using the formula as originally described (Reed and Muench, 1938):

$$TCID_{50} = \text{log dilution} - \frac{(\%\text{ +ve for this dilution} - 50}{(\%\text{ +ve for this dilution} - \%\text{ +ve for next dilution})}$$

2.5 HIV-1 INFECTION OF MONOCYTE-DERIVED MACROPHAGES.

On day 5 after monocyte isolation, MDM cultured in suspension were counted and centrifuged at 500x*g* for 7 min at room temperature. After removing the supernatant, cells were infected with an M-tropic strain of HIV-1 at MOI of 0.01 to 1.0 unless otherwise indicated at 37^{0}C, 5% CO_2 for 2 to 4 hr. Control cells were mock-infected and cultured under identical conditions. The virus was removed by washing cells with PBS-CMF and centrifugation at 500x*g* for 7 min at room temperature. MDM were resuspended in pre-warmed (37^{0}C) supplemented Iscove's medium at a concentration of 0.5 to 1x10^6 and cultured in suspension in a 5% CO_2 humidified incubator at 37^{0}C until used for phagocytosis assays on day 7 post-infection.

In selected experiments monocytes were cultured after isolation adherent to plastic in 24-well plates (Costar) at a concentration of 0.5 to 1x10^6/well. Cultured MDM on day 5 post-isolation were infected with HIV-1_{Ba-L} at an MOI of 0.01 to 1.0 for 2 to 4 hr, washed twice with pre-warmed (37^{0}C) PBS-CMF and resuspended in fresh pre-warmed (37^{0}C) supplemented Iscove's medium. In experiments where HIV-1 PCR products were assessed, HIV-1_{Ba-L} was pre-treated with 10U/ml of RNase-free DNase (Boehringer Mannheim Australia, Castle Hill, New South Wales) for 20 min at room temperature in the presence of 10mM $MgCl_2$ to remove contaminating viral DNA.

2.6 HIV-1 QUANTITATION ASSAYS.

2.6.1 Reverse transcriptase activity assay.

HIV-1 replication in supernatant from MDM or PBMC was quantified by monitoring RT activity using a micro RT assay. Briefly, 10 µl of culture supernatant was added to 10µl of 0.3% Nonidet-40 (NP-40; Sigma, St Louis, MO, USA) in a 96-well round-bottom plate. Thereafter, 40µl of RT mixture (50mM Tris pH 7.8, 7.5mM KCl, 5mM $MgCl_2$, 2mM

dithiothreitol (Sigma), 5µg/ml of template-primer p.An.dT12-18 (Pharmacia-Biotec, Australia) and 3µCi ^{33}P-dTTP (Amersham, UK)) was added and the mixture was incubated for 2 hr at 37^{0}C. The reaction products (10µl) were spotted on to DE81 chromatography paper (Whatman International, Maidstone, UK) and air-dried overnight. Dry filters were washed 6 times with 2x SSC buffer (0.3M sodium chloride and 34mM sodium citrate) (Merck, Kilsyth, Victoria, Australia) to remove unincorporated radioactive dNTP, rinsed once in 95% ethanol and air-dried. Meltilex scintillant (Wallax, Turku, Finland) was melted on a heater and spotted onto the filters, followed by quantifying radioactivity incorporated into DNA using an LKB micro β-counter (Wallax).

2.6.2 HIV-1 p24 ELISA.

In selected experiments HIV-1 replication in MDM was quantified by measuring p24 antigen in culture supernatant (HIV-1 p24 antigen immunoassay, Abbott Laboratories, Abbott Park, IL, USA) according to manufacturer's instructions. Briefly, 200µl of culture supernatant treated with 0.5% Triton-X 100 (Merck) together with positive or negative controls and recombinant p24 quantitation standards of 0, 12.5, 25, 50 and 100pg/ml (HIV-1 p24 antigen quantitation panel; Abbott Laboratories) were dispensed into a supplied reaction plate. Beads coated with human anti-HIV antibody were dispensed into the wells (one bead/well) and incubated for 2 hr at 40^{0}C, followed by two washes in distilled water using a Qwik Wash plate washer (Abbott Laboratories). Beads were subsequently incubated with 200µl of rabbit anti-HIV antibody for 1 hr at 40^{0}C, washed twice as above and incubated with 200µl of goat anti-rabbit IgG for 1 hr at 40^{0}C. Beads were washed, transferred to assay tubes (Abbott Laboratories) and incubated with 300µl of OPD substrate (o-phenylene-diamine, 2 HCl in citrate-phosphate buffer) for 30 min at room temperature in the dark. The reaction was stopped with 1ml of 1M sulphuric acid. The absorbance was measured at 490nm using a Quantumatic reader

(Abbott Laboratories) and converted to pg/ml values using the standard curve generated from the quantitation standards.

2.6.3 Intracellular staining for p24 antigen.

HIV-1 replication in MDM cultured in suspension in teflon jars was quantified by measuring intracellular p24 antigen and flow cytometric analysis as previously described (Crowe *et al.*, 1987). HIV- and mock-infected MDM ($2x10^5$ cells) were dispensed in polypropylene tubes (Becton Dickinson), washed twice in PBS-CMF by centrifugation at 500x*g* for 7 min at 4^0C. Cells were fixed in 1ml of 3% ultrapure formaldehyde (Polysciences, Warrington, PA, USA) for 45 min at 4^0C, followed by two washes with 0.1M glycine in PBS-CMF and permeabilisation with 1ml of 0.1% Triton-X 100 (Merck) for exactly 1min at 4^0C. MDM were washed twice in PBS-CMF containing 1% FCS and stained for intracellular p24 antigen using a Mab directed against p24 (2µg/ml; IgG1, Olympus, Lake Success, NY) or isotype-matched control (MOPC 21; 2µg/ml; IgG1, Bionetics, Charlestone, SC) for 30 min at 4^0C. After a wash with PBS-CMF and centrifugation at 500x*g* for 7 min at 4^0C, cells were stained with goat anti-mouse IgG conjugated to FITC (FITC-GAM; Tago, Burlingame, CA, USA). The mean fluorescence for intracellular p24 antigen was quantified by flow cytometric analysis, converted to MESF (molecules of equivalent soluble fluorochrome) units and corrected for background fluorescence.

2.7 QUANTIFICATION OF ENDOTOXIN.

As bacterial endotoxin (lipopolysaccharide; LPS) has been shown to affect HIV-1 replication within MDM (Kornbluth *et al.*, 1989; Bernstein *et al.*, 1991), all Teflon jars and glassware used for macrophage culture were treated in a hot air-oven for 4 hr after autoclaving to prevent endotoxin contamination. Culture supernatants, reagents and

media were intermittently tested for the presence of endotoxin using the Limulus Amebocyte Lysate Assay (Biowhitaker, Walkersville, MD, USA) according to manufacturer's instructions. Briefly, 50µl of each sample to be tested, LPS standards (0.1, 0.25, 0.5 and 1.0 endotoxin units/ml from *Escherichia coli*) and LPS-free water were dispensed into pre-warmed (37^{0}C) 96-well plates (Nunc). All the samples were incubated with 50µl of limulus amebocyte lysate (LAL) for 10 min at 37^{0}C, followed by the addition of 100µl of chromogenic substrate solution and further incubated for 6 min at 37^{0}C. The reaction was stopped by addition of 50µl of 0.1M HCl and the absorbance was measured at 410nm using a plate reader (Labsystems, Multiskan, Helsinki, Finland). Samples of >0.5 U/ml were considered positive for the presence of endotoxin. All culture supernatants, viral stocks, reagents and media tested in this study were negative for LPS contamination.

2.8 PHAGOCYTOSIS ASSAYS.

2.8.1 Flow cytometric phagocytosis assay.

2.8.1.1 Preparation of target particles.

Opportunistic pathogens relevant to HIV-1 pathogenesis such as *Mycobacterium avium* complex (MAC) and *Toxoplasoma gondii* (*T.gondi*) were used as phagocytic targets. Both targets were fluoresceinated (Sections 2.8.1.1.1 and 2.8.1.1.2 respectively), adjusted to a concentration of 5x10^8 cells/ml and stored at -70^{0}C until used for phagocytosis.

2.8.1.1.1 Fluoresceination of *Mycobacterium avium* complex.

MAC cultures (*Mycobacterium avium* and *Mycobacterium intracellulare*, a mixture of 4 serovars) were inoculated into 40ml of Middlebrook 7H9 broth (Difco Labs, Detroit, MI,

USA) and incubated at 37^{0}C for 14 days. These cultures were kindly donated by Ms Aina Sievers (Victorian Infectious Disease Reference Laboratory, Melbourne). The organisms were transferred into a 50-ml conical tube (Cellstar) and heat-killed at 95^{0}C for 20 min. MAC was washed in PBS-CMF by centrifugation at 2500x*g* for 10 min and labelled with FITC as previously described by our laboratory (Hewish *et al.*, 1996). Briefly, the MAC pellet was resuspended in 5ml of 0.1mg/ml FITC (Sigma, St Louis, MO, USA), aliquoted into 1.5ml micro tubes (Sarstedt, Numbrecht, Germany), vortexed for 30 sec and sonicated for 15 sec (Bransonic, B1200, Branson Ultrasonic Corp., Danbury, CT, USA). MAC was incubated with FITC for 18 hr at room temperature in the dark, followed by two washes in PBS-CMF by centrifugation at 6500x*g* for 10 min at 4^{0}C. Fluoresceinated MAC (MAC-FITC) was pooled into a 15-ml conical tube, resuspended in 1ml of PBS-CMF, vortexed and sonicated. Bacterial concentration was determined by comparison with McFarland turbidity standards (bioMerieux, Marcy l'Etoile, France). After adjusting the concentration to 5x10^8 cells/ml, the intensity of MAC-FITC fluorescence was assessed by flow cytometry.

2.8.1.1.2 Fluoresceination of *Toxoplasma gondii.*

T.gondii tachyzoites maintained in supplemented RPMI-1640 medium through serial passages in human embryonic lung fibroblasts (HEL fibroblasts; ATCC) were kindly donated by Ms Kay Dixon (Victorian Infectious Disease Reference Laboratory, Melbourne). Tachyzoites were subsequently purified and fluoresceinated as previously described by our laboratory (Biggs *et al.*, 1995). Briefly, the monolayer of infected fibroblasts were detached with a cell lifter (Costar) and passaged together with culture supernatant through a blunt 25-gauge needle. Tachyzoites were subsequently purified by passage through a column containing glass wool fibre (Sigma, St. Louis, MO, USA), followed by filtration through a 3µm polycarbonate membrane (Poretics, Livermore, CA, USA). Filtrate was dispensed into 50-ml conical tubes (Cellstar), washed in PBS-CMF and centrifuged at 1600x*g* for 10 min at 4^{0}C. Purified *T.gondii* tachyzoites were heat-

killed at 60^{0}C for 30 min and labelled with FITC as described for MAC in Section 2.8.1.1.1.

2.8.1.2 Phagocytosis by monocytes within peripheral blood.

Phagocytosis of MAC-FITC or *T.gondii*-FITC by monocytes was performed using a whole blood assay as previously described by our laboratory (Hewish *et al.*, 1996). Briefly, peripheral blood from HIV-infected and -uninfected individuals was collected in lithium heparin anticoagulant, dispensed into 4-ml polypropylene tubes (100µl per tube) within 3 hr of collection and cooled on ice for 20 min. MAC-FITC or *T.gondii*-FITC was thawed immediately prior to phagocytosis, vortexed for 1 min and sonicated for 1 min (Bransonic B1200; full settings). Target particles at concentrations ranging from 5x10^6 to 1.5x10^7 in 30µl PBS-CMF were added to duplicate blood samples and incubated in a shaking water bath at 37^{0}C (or on ice as controls) for 10 min. After phagocytosis was arrested by placing tubes in ice, the fluorescence of MAC-FITC or T.gondii-FITC adherent to the cell surface was quenched by the addition of 100µl of Quenching agent (Orpegen, Heidelberg, Germany), followed by two washes with cold (4^{0}C) PBS-CMF at 500x*g* for 5 min at 4^{0}C. Monocytes within the sample were stained with anti-CD14-PE Mab (Becton Dickinson) for 30 min on ice. After washing with cold (4^{0}C) PBS-CMF, erythrocytes were lysed with 2ml of FACS lysing solution (Becton Dickinson) for 10 min at room temperature. White blood cells were washed twice with PBS-CMF and fixed with 1% ultrapure formaldehyde (Polysciences). The percentage of CD14-positive monocytes that phagocytosed MAC-FITC or *T.gondii*-FITC was calculated as the percentage of FITC-labelled cells in the PE-positive cell population.

2.8.1.3 Phagocytosis of MAC-FITC by monocyte-derived macrophages.

On day 7 following HIV-1 infection, MDM (2x10^5 cells) were dispensed in polypropylene tubes, washed twice in PBS-CMF by centrifuging at 500x*g* for 5 min at 4^{0}C. Cells were

resuspended in 100µl of PBS-CMF and cooled on ice for 20 min prior to phagocytosis. MDM were incubated for 2hr at 37°C (or on ice as controls) with MAC-FITC, at various MAC-FITC to MDM ratios (ranged from 1:10 to 1:75), in duplicate. Phagocytosis was arrested by plunging the tubes into ice. The fluorescence of adherent MAC-FITC was quenched using Quenching Agent (Orpegen). MDM were washed twice with cold (4°C) PBS-CMF by centrifugation at 500x*g* for 5 min at 4°C and fixed with 1% formaldehyde. The percentage of MDM that phagocytosed MAC-FITC was quantified by flow cytometry.

2.8.2 Colorimetric FcγR-mediated phagocytosis assay.

2.8.2.1 Preparation of IgG-opsonised sheep red blood cells.

Sheep red blood cells (E; ICN-Cappel, Aurora, Ohio, USA) were opsonised immediately prior to the phagocytosis assay. E ($2x10^8$ cells) were placed into a 15-ml conical tube and washed three times in PBS by centrifugation at 1430x*g* for 10 min at 4°C. Cells were opsonised with a sub-agglutinating titre (1:300) of rabbit anti-E antibody (ICN-Cappel) in 5ml of PBS. Opsonisation was performed on a sample mixer (Dynal) for 30 min at room temperature. Cells were washed five times in cold (4°C) PBS by centrifugation at 1430x*g* for 10 min at 4°C. Both IgG-opsonised E (E-IgG) and unopsonised E (treated under the same conditions) were resuspended at a concentration of $1x10^8$/ml in Iscove's medium.

2.8.2.2 Phagocytosis by monocyte-derived macrophages.

Cultured MDM were plated onto 96-well plates (Nalge Nunc) at $5x10^4$ cells/well in 100µl of supplemented Iscove's medium and allowed to adhere for 2 hr at 37°C in a 5%CO_2 humidified incubator. Phagocytic targets (E-IgG or unopsonised E) were added to adhered MDM at a MDM:E ratio of 1:10, in triplicate. The plate was centrifuged at

100x*g* for 5 min at 4^{0}C and then placed at 37^{0}C for phagocytosis to proceed. Phagocytosis was terminated after 10 min by placing the plates on ice and washing cells with cold (4^{0}C) PBS using a multichannel pipette (Labsystem, Helsinki, Finland). Phagocytosis of E was quantified using a colorimetric assay (Gebran *et al.*, 1992). Briefly, after unbound E were removed by washing with PBS, non-phagocytosed E attached to MDM were lysed with 0.2% NaCl for 3 min, followed by three washes with pre-warmed (37^{0}C) Iscove's medium. Phagocytosed E were measured by reaction of haemoglobin with 2,7-diaminoflurene (Sigma, St Louis, MO, USA) after lysis of MDM in 0.2M Tris-HCl buffer containing 6M urea for 10 min at room temperature. Absorbance at 620nm was determined using a plate reader (Labsystems, Multiskan, Helsinki, Finland), and compared to a standard curve generated using known numbers of E (ranging from 4x10^3 to 5x10^5). The estimated E per well were divided by the number of MDM (5x10^4) and multiplied by 100 to obtain the phagocytic index (the number of phagocytosed E per 100 MDM).

2.8.3 FcγR-mediated phagocytosis for biochemical analyses.

2.8.3.1 Preparation of IgG-opsonised latex beads.

Latex beads optimised previously for the bead size (Reece *et al.*, 2001) (3μm in diameter, Sigma) were prepared immediately prior to phagocytosis. Beads (4x10^9) were washed once in PBS by pelleting at 1400x*g* for 5 min and coated with bovine serum albumin (BSA) by incubation in 1ml of PBS containing 10mg BSA (Sigma) overnight at 4^{0}C. Beads were washed 5 times with PBS by centrifugation at 1400x*g* for 5 min and opsonised with 10% rabbit anti-BSA antiserum (ICN-Cappel) on a sample mixer for 30 min at room temperature. IgG-opsonised beads were washed 3 times in cold (4^{0}C) PBS and resuspended in PBS at a concentration of 4x10^8/ml.

2.8.3.2 Phagocytosis by monocyte-derived macrophages.

MDM ($2x10^6$ cells) were dispensed into 4-ml polypropylene tubes (Becton Dickinson), washed twice in PBS-CMF by centrifugation at 500x*g* for 5 min at 4^0C and cooled on ice for 20 min in 100µl of PBS-CMF prior to phagocytosis. MDM were incubated with or without IgG-opsonised beads at MDM to target ratio of 1:10 in a shaking water bath at 37°C. At specified time points phagocytosis was arrested by plunging the tubes into ice and washing MDM with cold (4^0C) PBS-CMF. Cells were pelleted at 20,000x*g* for 45 sec at 4^0C and used for preparation of cell lysates as described in Section 2.8.3.3.

2.8.3.3 Preparation of cell lysates.

For immunoblotting and immunoprecipitation experiments, washed MDM were lysed in 100µl of Triton-X 100 lysis buffer (TLB) containing 25mM Tris-HCl (pH 7.5), 0.14M NaCl, 1mM EDTA, 1% Triton-X 100, supplemented with the following phosphatase inhibitors: 50mM NaF, 1mM sodium orthovanadate (Sigma, St Louis, MO), 40mM β-glycerophosphate (Sigma, St Louis, MO), and the following protease inhibitors: 1mM pefabloc, 1µM pepstatin, 1µM leupeptin (Boehringer-Mannheim, Mannheim, Germany). After 30 min of lysis on ice, cell lysates were centrifuged at 20,000x*g* for 10 min at 4^0C, transferred to fresh 1.5-ml micro tubes (Sarstedt), frozen in liquid N_2 and stored at -70°C until used for biochemical analysis.

2.9 IMMUNOBLOTTING AND IMMUNOPRECIPITATION

For immunoblotting analysis the amount of proteins within MDM lysates were determined by DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. MDM extracts containing equal amounts of proteins (30 to 50µg) were boiled in 2x SDS sample buffer (10mM Tris pH 8.0, 2mM EDTA, 1% SDS, 5% β-mercaptoethanol, 5% glycerol) for 10 min at 95°C. Samples were resolved

by 10% SDS polyacrylamide gel electrophoresis (PAGE) as described in Section 2.12.2. Proteins were transferred to nitrocellulose (Amersham International, Buckinghamshire, UK) at 70V for 2.5 hr using a CBC Scientific (Del Mar, Ca, USA) electrophoretic transfer tank. The nitrocellulose filter was blocked with either 3% BSA (for anti-phosphotyrosine Mab directly conjugated to horseradish peroxidase; HRPO) or with 5% skim milk in Tris Buffered Saline, pH 7.5 containing 0.3% Tween-20 (TBST) (Astral, Gymea, Australia) for 2 hr at room temperature. The blots were probed with a primary antibody overnight at 4^0C, followed by five washes in TBST for 15 min at room temperature. Blots were subsequently probed with a secondary antibody conjugated with HRPO for 1hr at room temperature, washed three times in TBST for 10 min at room temperature and developed for enhanced chemiluminescence (ECL) according to manufacturer's instructions (Amersham Pharmacia).

Alternatively, to determine the phosphorylation of various proteins during FcγR-mediated phagocytosis, particular proteins were immunoprecipitated with relevant antibodies from MDM extracts containing equal amount of proteins (50 to 100µg) in 100µl of TLB overnight at 4^0C. Immunoprecipitates were collected with 15µl of protein G sepharose beads (Pharmacia Biotech, Uppsala, Sweden) by incubation on a sample mixer (Dynal) for 1 hr at 4^0C, followed by five washes in TLB by centrifugation at 420x*g* for 2 min at 4^0C The washed beads were mixed with 20µl of 2x SDS sample buffer, boiled for 10 min at 95^0C and centrifuged at 20,000x*g* for 10 min. Supernatant containing proteins of interest was resolved by 10% SDS-PAGE and transferred to nitrosellulose as described above. Blots were probed with anti-phosphotyrosine antibody directly conjugated to HRPO (RC20; Transduction Laboratories, Lexington, KY, USA) to determine the phosphorylation status of particular proteins. To verify the equal protein input blots were re-probed with relevant antibodies overnight at 4^0C, followed by a secondary antibody directly conjugated to HRPO as described in the immunoblotting Section 2.9.

2.10 EXTRACTION OF DNA

DNA was extracted as previously described (Lee *et al.*, 1991). Briefly, 1 x 10^6 MDM cultured in 24-well plates were washed with 1ml of cold (4^0C) PBS-CMF and lysed in 0.5ml lysis buffer containing 50mM KCl, 10mM Tris-HCl (pH 8.3), 2.5mM $MgCl_2$, 0.5% Tween 20 (BDH Chemicals, Kilsyth, Australia), 0.5% NP40 (Sigma, St Louis, MO, USA) and 0.1mg/ml BSA (Sigma). Following the addition of 50µl/ml proteinase K (Boehringer Mannheim), cell lysates were heated at 60° C for 1 hr and 95° C for 30 min. Sample lysates were stored at -20^0C until used in the PCR reaction.

2.11 DETECTION AND QUANTIFICATION OF RNA

2.11.1 mRNA extraction and cDNA generation

mRNA was extracted from MDM lysates using oligo $(dT)_{25}$ beads (Dynabeads, Dynal, Oslo, Norway) according to the manufacturer's protocol. Briefly, 1x10^6 cultured MDM were lysed in Lysis Binding Buffer consisting of 100mM Tris-HCl (pH 8.0), 500mM LiCl, 10mM EDTA (pH 8.0), 0.1% lithium dodecyl sulphate (LiDS), 5mM dithiothreitol (DTT). Cell lysates were stored at -70^0C until mRNA extractions were performed.

Prior to extractions, beads were pre-conditioned by two washes in Lysis Binding Buffer. MDM lysates were thawed on ice and hybridised with beads (1x10^6 MDM/20µl beads) for 10 min at room temperature to form a Dynabeads oligo$(dT)_{25}$/mRNA complex. The samples were placed on magnetic particle separator (Dynal) to collect the beads/mRNA complexes. Supernatants were discarded and beads were washed twice with 100µl of washing buffer (10mM Tris-HCl (pH 8.0), 0.15M LiCl, 1mM EDTA) (BDH Chemicals) with 0.1%LiDS (ICN Pharmaceuticals), and then three times with 100µl washing buffer (without LiDS). Beads were then washed three times with RT buffer consisting of 10mM Tris-HCl (pH 8.3) and 75mM KCl, changing tubes between each

wash.

To convert mRNA to cDNA, beads containing mRNA were resuspended in 25µl AMV-RT mix consisting of 1mM dNTPs (Promega, Madison, WI, USA), 4mM sodium pyrophosphate, 25U RNasin (Promega), 25U AMV-RT (Promega), 1X RT buffer (Promega) and incubated for 1hr at 42°C. The samples were placed on the magnetic particle separator at room temperature. RT mix was removed and the samples were resuspended in 100µl elution solution (0.5M ammonium acetate, 1mM EDTA, 0.1%SDS) and heated at 95°C for 5 min to remove mRNA, leaving cDNA attached to the beads. Beads/cDNA complexes were resuspended in 100µl TE buffer consisting of 10mM Tris-HCl (pH 8.0), 1mM EDTA, and stored at 4°C until used for PCR reactions. To check for DNA contamination samples prepared without AMV-RT were included within each experiment.

2.11.2 Standardisation of cDNA

cDNA from each sample was standardised according to β-actin mRNA levels as previously described (Grassi *et al.*, 1995). Beads/cDNA complexes were serially diluted in TE buffer (ranging from amounts equivalent to $1x10^5$ cells - 10^0 cell) and subsequently amplified using β-actin primers: BA1 5'CATGTGCAAGGCCGGCTTCG3' and BA4 5'TGAAGGTCGGAGTGAACGGATTTGGT3' (Bresatec, Adelaide, SA, Australia) as described in Section 2.11.3. PCR products were separated by agarose gel electrophoresis as described in Section 2.12.1. The intensity of bands was assessed by laser densitometry to standardise the amount of cDNA in subsequent PCR reactions.

2.11.3 PCR amplification

DNA or cDNA templates were amplified in a 50µl reaction containing appropriate primers (1µM final concentration), 1x PCR reaction buffer (Perkin Elmer, Norwalk, WI, USA), 1.5mM $MgCl_2$, 1mM dNTPs and 1U of AmpliTaq (Perkin Elmer). PCR amplification was performed in a GeneAmp PCR System 9600 (Perkin Elmer). Unless otherwise specified, initial denaturation occurred at 95°C for 2 min, followed by 35 cycles of denaturation step at 95°C for 1 min, annealing step at 56°C for 1 min and extension step at 72°C for 2 min, followed by one cycle at 72°C for 2 min.

2.12 GEL ELECTROPHORESIS

2.12.1 Agarose gel electrophoresis

DNA samples in1x loading buffer consisting of 8% glycerol, 0.1% bromophenol blue and 0.1% xylene cyanol were separated based on their size in 2% (w/v) agarose gels containing 0.05µg/ml of ethidium bromide (EthBr). Samples were electrophoresed at 100V for 30 min in 0.5x TBE electrophoresis buffer consisting of 50mM Tris-HCl, boric acid pH 8.3 and 1mM EDTA). The DNA markers used were 100 base pair (bp) ladder standards (Promega). DNA fragments were visualised on a short wave length UV Transilluminator and the image was taken with Polaroid 665 film.

2.12.2 Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Protein samples boiled in 2x SDS sample buffer (Section 2.9) were electrophoresed using a CBC Scientific Electrophoresis tank at 100V constant voltage. A 40% stock solution of acrylamide (Astral, Gymea, Australia) was used to make the 10% resolving

gel (together with 0.375M Tris at pH 8.8, 0.1%SDS, 0.07% ammonium persufate, 0.05% TEMED) and the 4% stacking gel (together with 0.125M Tris at pH 6.8, 0.1% SDS, 0.13% ammonium persufate, 0.05% TEMED). The SDS-PAGE electrode buffer consisted of 0.1% SDS, 25mM Tris, 192mM glycine, pH 8.3. SeeBlue Pre-Stained Standards (Novex, San Diego, CA, USA) were used as protein molecular mass markers. The electrophoresis was conducted at 100V for 2.5 hr.

2.13 RELEVANT PUBLICATIONS.

The following publication has arisen from the work presented in this chapter:

Kedzierska, K., and Crowe, S.M. (2001) Culture of HIV in monocytes and macrophages. *Current Protocols in Immunology.* John Wiley & Sons, Inc. 12.4.1-12.4.11.

CHAPTER 3

SIGNAL TRANSDUCTION PATHWAYS ACTIVATED DURING FCγR-MEDIATED PHAGOCYTOSIS BY HUMAN MACROPHAGES.

3.1 ABSTRACT.

Receptors for the constant region of immunoglobulin G (FcγR) are widely expressed on cells of haemopoietic lineage and play an important role in host defense. The signalling pathways activated during FcγR-mediated phagocytosis in human monocyte-derived macrophages (MDM) as well as the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) on these events were examined in this study. FcγR-mediated phagocytosis resulted in enhanced tyrosine phosphorylation of a wide range of cellular proteins, activation of the protein tyrosine kinases Hck, Syk and Pyk2, and phosphorylation of the multidomain adapter protein, paxillin. Stimulation of MDM with GM-CSF augmented FcγR-mediated phagocytosis and increased the levels of tyrosine phosphorylation in phagocytosing MDM cultures, suggesting activation of tyrosine kinases or an effect on protein tyrosine phosphatase pathways. GM-CSF treatment of MDM without a phagocytic stimulus did not activate Syk, suggesting that GM-CSF may act either distal to Syk in the FcγR-mediated signalling cascade or on a parallel pathway activated by the FcγR. This study shows that early signalling events during FcγR-mediated phagocytosis in human MDM involve activation of Syk, Hck and paxillin. It also provides the first evidence for Pyk2 activation during phagocytosis and a baseline for further studies on the effect of GM-CSF on FcγR-mediated phagocytosis.

3.2 INTRODUCTION.

The receptors for the constant region of IgG (FcγRI, FcγRII, FcγRIIIA) are the major means by which cells of the macrophage lineage recognise IgG opsonised particles and promote host defenses including phagocytosis, antibody-dependent cellular cytotoxicity as well as secretion of cytokines and chemokines. The major Fcγ receptors expressed on monocytes are the high affinity FcγRI (CD64) and a low affinity FcγRII (CD32), while macrophages also express FcγRIIIA (CD16) (reviewed in Daeron, 1997). Most studies delineating the specific signalling events during FcγR-mediated phagocytosis have been performed using murine macrophages (Greenberg *et al.*, 1993; Greenberg *et al.*, 1994; Aderem, 1995; Crowley *et al.*, 1997; Strzelecka *et al.*, 1997; Kiefer *et al.*, 1998), or cell lines transfected with FcγR (Indik *et al.*, 1994; Cox *et al.*, 1996; Greenberg *et al.*, 1996), with only two studies done in human macrophages (Kusner *et al.*, 1999) or monocytes (Zheleznyak and Brown, 1992). Several signal transduction pathways utilised by macrophages activated during FcγR-mediated phagocytosis have been described (reviewed in Aderem and Underhill, 1999), including requirements for isoforms of protein kinase C (Zheleznyak and Brown, 1992), phosphatidylinositol 3-kinase (Kanakaraj *et al.*, 1994; Ninomiya *et al.*, 1994) and the Rho family of GTPases (Hackam *et al.*, 1997; Cox *et al.*, 1997). These signalling events are initiated following clustering of FcγR via activation of tyrosine kinases of the Src family (Jouvin *et al.*, 1994; Sarmay *et al.*, 1994). Src kinase activation results in the rapid and transient phosphorylation of ITAM (immunoreceptor tyrosine-based activation motifs) on either the ligand binding subunit in the cytoplasmic domain of Fcγ RII or on the associated γ chain of Fcγ RI and IIIA (Park *et al.*, 1993; Indik *et al.*, 1994; Indik *et al.*, 1995a). Phosphorylated ITAMs may represent docking sites for Syk allowing its subsequent activation (Indik *et al.*, 1995c; Bonnerot *et al.*, 1998). A requirement for Syk in FcγR-mediated phagocytosis was initially demonstrated in human monocytes by using anti-sense oligodeoxynucleotides to eliminate Syk mRNA (Matsuda *et al.*, 1996).

Gene knockout studies subsequently confirmed an absolute and specific requirement for Syk FcγR-mediated phagocytosis by murine macrophages (Crowley *et al.*, 1997). Macrophages derived from the fetal livers of Syk-deficient mice had defective FcγR-mediated phagocytosis and actin assembly, but unimpaired phagocytosis of *E.coli*, yeast or latex particles (Crowley *et al.*, 1997), suggesting that Syk is specifically required for FcγR-mediated functions, but not phagocytosis per se.

Activated Syk is thought to promote phosphorylation and localised accumulation of a number of cytoskeletal substrates (reviewed in Greenberg, 1995), including the actin-binding proteins paxillin, vinculin, talin and alpha-actinin (Greenberg et al., 1990; Greenberg et al., 1994; Allen and Aderem, 1996). Paxillin, a multidomain adapter protein, is thought to interact with a variety of proteins, such as FAK (focal adhesion kinase), Pyk2 and Vav, and in this way it organises focal adhesion complexes and cytoskeletal rearrangement (reviewed in Turner, 1998). Human monocytes and macrophages do not express FAK (Li *et al.*, 1998), but they activate and phosphorylate another member of the FAK family, Pyk2. This kinase is 45% identical in amino acid sequence to FAK (Avraham *et al.*, 1995), and shown to be involved in cytoskeletal engagement upon adherence and subsequent calcium or PKC co-stimulatory activation in human monocytes (Li *et al.*, 1998). A role for Pyk2 in phagocytosis has not yet been reported.

The results presented in this Chapter demonstrate that FcγR-mediated internalisation of IgG-opsonised targets by human monocyte-derived macrophages (MDM) is dependent on protein tyrosine phosphorylation, with transient activation of the protein tyrosine kinases Hck, Syk and Pyk2, and redistribution of the multidomain adaptor protein paxillin between Triton-soluble and a Triton-insoluble cell fractions. In addition, acute stimulation of MDM with granulocyte-macrophage colony-stimulating factor (GM-CSF) augments phagocytosis of IgG-opsonised targets, and concomitantly tyrosine

phosphorylation of cellular proteins in an additive manner but does not activate Syk. These observations suggest that GM-CSF may either stimulate phagocytic pathways activated by the FcγR downstream of Syk kinase or independent of Syk activation.

3.3 METHODS.

3.3.1 Phagocytosis assays.

On day 5 to 7 after monocyte isolation (Section 2.1.2), MDM were used for phagocytosis assays. Cells ($5x10^4$) were plated onto 96 well plates, adhered for 2 hr and exposed to IgG-opsonised or unopsonised E (Section 2.8.2.1) at a E:MDM ratio of 10:1. Phagocytosis of erythrocytes was quantified by a colorimetric assay (Section 2.8.2.2). In selected experiments MDM were incubated with the protein tyrosine kinase inhibitor, genistein (Calbiochem, Croydon, Australia), at concentrations ranging from 0.1 to 30μM at 37°C for 30 min prior to phagocytosis assay.

Alternatively, MDM ($2x10^6$ cells) in 4ml polypropylene tubes were incubated with or without IgG-opsonised beads (Section 2.8.3.1) at 37°C with a target to MDM ratio of 10:1 (Section 2.8.3.2). At specified time points, phagocytosis was arrested by plunging the tubes into ice and washing the MDM in ice-cold PBS-CMF, followed by cell lysis in Triton-X 100 lysis buffer (Section 2.8.3.3) for immunoblotting and immunoprecipitation experiments.

3.3.2 GM-CSF stimulation.

MDM were stimulated with GM-CSF at 100ng/ml (Genzyme; kindly provided by Prof. A. Lopez, Hanson Centre, Adelaide, Australia), immediately prior to addition of phagocytic targets. Phagocytic assays commenced within 2 min of adding GM-CSF. Lysates were

either analysed for phagocytosis by a colorimetric assay, tyrosine phosphorylated proteins by immunoblotting or Syk phosphorylation by immunoprecipitation.

3.3.3 Immunoblotting and immunoprecipitation.

Cell extracts containing equal amounts of proteins were resolved by 10% SDS-PAGE (Section 2.12.2) and transferred to nitrocellulose (Section 2.9). The blots were probed with a recombinant antibody directed against phosphotyrosine (RC20; Transduction Laboratories, Lexington, KY) or paxillin (Transduction Laboratories) overnight at 4^0C, followed by secondary antibody conjugated with horseradish peroxidase (Amersham Pharmacia, Buckinghamshire, England), and developed for enhanced chemiluminescence (ECL) according to manufacturer's instructions (Amersham Pharmacia). Alternatively, to determine the involvement of various proteins in FcγR-mediated phagocytosis, cell lysates were immunoprecipitated with either a rabbit antibody against Hck (gift from Dr H.C. Cheng, School of Biochemistry, Melbourne University), anti-Syk Mab (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-paxillin Mab, and then probed with anti-phosphotyrosine antibody (PY20; Transduction Laboratories, Lexington, KY), or conversely, immunoprecipitated with anti-phosphotyrosine Mab and then probed with anti-Pyk2 (Transduction Laboratories) or anti-Syk Mab. Proteins were immunoprecipitated from extracts with antibodies overnight at 4^0C as detailed in Section 2.9. To verify equal protein input, blots were re-probed with relevant antibodies.

3.3.4 Immunofluorescence microscopy.

Cells fixed with 1ml of 3% ultrapure formaldehyde (Polysciences, Warrington, PA, USA) for 20 min were washed twice with cold 0.1M glycine in PBS-CMF and permeabilised with 0.1% Triton-X 100 (Merck, Kilsyth, Australia) for 1 min. After two

washes with 1% Fetal Bovine Serum in PBS-CMF, cells were stained for intracellular proteins with mouse anti-phosphotyrosine Mab (PY20) or isotype-matched control IgG1 (MOPC 21; Bionetics, Charlestone, SC) for 30 min on ice. After two washes with cold PBS-CMF, cells were incubated with biotin-conjugated anti-mouse Ig (Silenus, Melbourne, Australia) for 30 min on ice, followed by two further washes with cold PBS-CMF. Subsequently, MDM were incubated with Texas Red-conjugated streptavidin (Amersham Pharmacia, Buckinghamshire, England) for 30 min on ice, washed once with cold PBS-CMF, fixed with 200µl of 1% formaldehyde and cytocentrifuged onto glass slides. Protein tyrosine phosphorylation in both resting and phagocytosing MDM were analysed by confocal laser microscopy (Bio-Rad MRC500, Hercules, CA). For some samples, cells were also stained with phalloidin conjugated to Alexa 488 (Molecular Probes, Eugene, OR) to determine co-localisation of F-actin and phosphotyrosine.

3.4 RESULTS.

3.4.1 Enhanced protein tyrosine phosphorylation during FcγR-mediated phagocytosis by monocyte-derived macrophages.

Two minutes after the addition of IgG-opsonised latex beads, increased tyrosine phosphorylation of a number of human MDM proteins was evident by immunoblot analysis, with phosphorylation reaching a peak between 5 and 15 min (Figure 3.1a). Phagocytosis of IgG-opsonised E was inhibited by the protein tyrosine kinase inhibitor, genistein, in a dose-dependent manner (Figure 3.1b), suggesting that tyrosine kinase activity was required for FcγR-mediated phagocytosis. Phagocytosis of unopsonised E by MDM cultured in the presence or absence of genistein was below the detection level of the assay.

Confocal microscopy revealed that the majority of antiphosphotyrosine immunoreactivity in MDM was localised around the internalised IgG-opsonised latex beads (Figure 3.2b) co-localised with sites of actin polymerisation (Figure 3.2d). This finding is consistent with binding of tyrosine phosphorylated proteins to a cytoskeleton which has been rearranged around the phagosome. Within 2 min of initiation of phagocytosis, paxillin, a multidomain adapter protein involved in organising focal adhesion complexes, was also found to be preferentially localised around the phagosome (Figure 3.2c). Staining with isotype-matched control antibodies verified a lack of non-specific staining (Figure 3.2a).

3.4.2 Activation of Hck, Syk and Pyk2 kinases.

Having demonstrated a requirement for tyrosine kinase activity during FcγR-mediated phagocytosis by MDM, the proteins, which were tyrosine phosphorylated in the experiments depicted in Figure 3.1, were subsequently identified. Based on estimated size, several tyrosine kinases were investigated, which could potentially be activated during this process. Enhanced tyrosine phosphorylation of Hck (58-60kDa), Syk (72kDa) and Pyk2 (120kDa) was observed within 2 to 5 min of FcγR-mediated phagocytosis (Figure 3.3). The input of Syk, Hck and Pyk2 proteins was standardised according to protein estimations in each lysate. Protein levels are shown by re-probing the blots with relevant antibodies (Figure 3.3 a,b; lower panels).

3.4.3 Redistribution of paxillin.

Within 2 to 5 min of FcγR-mediated phagocytosis there was an increase in paxillin phosphorylation which had declined by 30 min (Figure 3.4a). Immunoblot analysis of Triton-soluble lysates probed with anti-paxillin antibody showed that the total level of extracted paxillin levels changed in a corresponding fashion (Figure 3.4b). Total

extracted paxillin levels did not, however, vary during phagocytosis when MDM were lysed in SDS sample buffer (total cell extract) (Figure 3.4c, lower panels). Taken together, these results suggest that in different phases of FcγR-mediated phagocytosis, paxillin is distributed predominantly in either the cytoskeletal (detergent-insoluble) fraction or cytoplasmic (detergent-soluble) fraction.

3.4.4 Stimulation of FcγR-mediated phagocytosis by GM-CSF.

Stimulation of MDM with GM-CSF (100ng/ml) augmented the level of FcγR-mediated phagocytosis by 20-30% (Figure 3.5a). GM-CSF stimulated tyrosine phosphorylation of MDM cellular proteins and this effect was further augmented during phagocytosis of IgG-opsonised latex beads in an additional manner (Figure 3.5b). The effect of GM-CSF on Syk phosphorylation was subsequently determined since Syk is activated in MDM during FcγR-mediated phagocytosis (Figure 3.3b) and it is a well-established critical intermediate in FcγR signalling in murine macrophages (Crowley *et al.*, 1997). Incubation of MDM with GM-CSF for 2 min did not alter the extent of tyrosine phosphorylation of Syk above the levels observed during FcγR-mediated phagocytosis (Figure 3.5c). In addition, control MDM which had not been exposed to target particles did not display detectable Syk phosphorylation in either GM-CSF-treated or untreated samples. The level of Syk protein was equal for all the samples (Figure 3.5c, lower panel). Syk co-immunoprecipitated from Triton-X 100 lysates with several other phosphoproteins in a reproducible pattern. This pattern may represent a stable complex of Syk with Fcγ receptors and/or other signalling molecules located around the phagosome since a similar pattern was observed in experiments when MDM lysates were immunoprecipitated with anti γ-chain antibody (data not shown). Taken together, these data suggest that GM-CSF augments the process of FcγR-mediated phagocytosis by triggering tyrosine phosphorylation of a number of cellular proteins,

and most probably by acting downstream of Syk (and, by extension, downstream of Src) in the FcγR-mediated cascade or via a pathway parallel to these events.

3.5 DISCUSSION.

This study shows that FcγR-mediated phagocytosis by human macrophages is mediated by tyrosine phosphorylation of a number of cellular proteins which localise around the phagocytic cup, and that phagocytosis is blocked by a protein tyrosine kinase inhibitor, genistein. Both Hck (a macrophage-specific Src family kinase) and Syk are phosphorylated following engagement of human MDM FcγRs. The results presented in this chapter also provide the first evidence for activation of Pyk2 and redistribution of the phosphorylated form of paxillin between the cytoplasm and cytoskeleton during FcγR-mediated phagocytosis. In addition, augmentation of FcγR-mediated phagocytosis by GM-CSF, concomitant with increased tyrosine phosphorylation of a number of cellular proteins which do not however include Syk, has also been demonstrated in this study.

A role for Hck in FcγR signalling has been suggested, based on data showing that macrophages prepared from mice deficient in three Src family tyrosine kinases, Hck, Fgr and Fyn exhibit poor signalling downstream of FcγR (i.e., Syk activation) as well as reduced levels of FcγR-induced functional responses such as phagocytosis, actin cup formation and respiratory burst (Crowley *et al.*, 1997; Fitzer-Attas *et al.*, 2000). A specific requirement for Hck in phagocytosis has also been demonstrated using Hck knock-out mice, which fail to internalise IgG-opsonised erythrocytes, while other haemopoietic functions remain intact (Lowell *et al.*, 1994). This study also suggested that some functions of Hck are subsumed through compensatory increases in the activity of another Src kinase, Lyn, although Lyn could not substitute for Hck in phagocytosis. Both Hck and Lyn have been also shown to co-immunoprecipitate with

FcγR following cross-linking of receptors on THP-1 monocytic cell lines (Ghazizadeh *et al.*, 1994) and human monocytes (Wang *et al.*, 1994). In the present work activation of Hck during FcγR-mediated phagocytosis in human macrophages is demonstrated.

Phosphorylation of ITAMs creates docking sites for Syk, a kinase shown by a number of studies to play a critical role in coupling phagocytosis-promoting FcγRs to the actin-based cytoskeleton (reviewed in Greenberg, 1995). A study employing stem-loop Syk antisense oligonucleotides in human monocytes (Matsuda *et al.*, 1996) as well as transfection studies using COS and DT40 cell lines and gene knockout studies have demonstrated that Syk activation is absolutely required for FcγR-mediated phagocytosis, actin assembly and FcγR-mediated transport to lysosomes (Cox *et al.*, 1996; Crowley *et al.*, 1997; Kiefer *et al.*, 1998; Bonnerot *et al.*, 1998). Furthermore, Syk has also been found to be a part of the activated FcγR complex following cross-linking of receptors in U937 and THP-1 cell lines (Darby *et al.*, 1994; Ghazizadeh *et al.*, 1995). This study confirms that Syk is phosphorylated in human MDM following FcγR-mediated phagocytosis.

Activation of Syk is thought to result in the phosphorylation of various substrates located in the submembranous region beneath phagocytosed particles, which are required for actin polymerisation and cytoskeletal rearrangement (Cox *et al.*, 1996). Paxillin is a potential downstream effector of Syk, as this protein has been shown to interact with a variety of proteins involved in growth control and cytoskeletal reorganisation (reviewed in Turner, 1998). The abundance of binding motifs in paxillin for structural and regulatory proteins has led to a suggestion that it is important in recruiting signalling molecules at sites of actin rearrangement and to facilitate their interaction during phagocytosis (Turner *et al.*, 1999). Paxillin has been previously shown to be tyrosine phosphorylated in murine peritoneal macrophages during FcγR-mediated phagocytosis (Greenberg *et al.*, 1994) and to co-localise with phagosomes

(Greenberg *et al.*, 1994; Allen and Aderem, 1996). This study confirms and extends those observations by showing that the phosphorylated form of paxillin is redistributed between cytoplasm and cytoskeleton during phagocytosis in human macrophages.

Paxillin phosphorylation following cell adhesion has been postulated to involve the kinase p125 FAK, shown previously to bind to paxillin LD motifs (Turner, 1998). The involvement of FAK in FcγR-mediated phagocytosis remains controversial, with one study showing no enhancement of tyrosine phosphorylation of FAK during phagocytosis in murine macrophages (Greenberg *et al.*, 1994), and another report demonstrating the expression and phosphorylation of FAK as a result of FcγR cross-linking in human monocytes (Pan *et al.*, 1999). However, it has been reported that human monocytes/macrophages isolated under stringent conditions and free of platelet contamination do not express FAK (Li *et al.*, 1998) (Jaworowski, unpublished). Therefore the activation of Pyk2, a kinase with 45% sequence identity to FAK (Sasaki *et al.*, 1995), known to be expressed in human monocytes/macrophages (Li *et al.*, 1998) and bind to paxillin (Hiregowdara *et al.*, 1997), during FcγR-mediated phagocytosis was determined. This study demonstrates that tyrosine phosphorylation of Pyk2 is increased during FcγR-mediated phagocytosis. This is the first report of stimulation of Pyk2 tyrosine phosphorylation in response to phagocytosis, although this kinase has been implicated in other processes involving reorganisation of the cytoskeleton, such as locomotion and adhesion (Astier *et al.*, 1997; Li *et al.*, 1998; Duong *et al.*, 1998).

FcγR-phagocytosis by both human (Capsoni *et al.*, 1992) and murine macrophages (Coleman *et al.*, 1988) has been found to be upregulated by GM-CSF, a cytokine known to augment a number of macrophage effector functions (reviewed in Metcalf, 1985; Armitage, 1998). Since GM-CSF receptor does not possess an intrinsic tyrosine kinase catalytic domain, the activation of protein tyrosine kinases associated with the β

subunit of the receptor, ie JAK (Janus Kinase), and members of the Src family as well as the transcription factor STAT5 (signal-transducing activator of transcription-5) mediate GM-CSF-stimulated proliferation, differentiation and gene expression (Corey and Anderson, 1999; Mui *et al.*, 1995). Stimulation of human monocytes with GM-CSF for 48 hr resulted in increased expression of FcγR II, and increased binding of IgG-opsonised particles (Rossman *et al.*, 1993). In experiments presented in this thesis short-term pretreatment of MDM with GM-CSF (2 to 5 min) was unlikely to increase FcγR expression and *de novo* receptor synthesis. The observations of this study and those of Rossman *et al* suggest that GM-CSF may augment FcγR-mediated phagocytosis by several mechanisms, i.e. by stimulating the level of FcγR expression as well as a direct effect on phagocytic signal transduction. Since GM-CSF treatment of MDM did not enhance phosphorylation of Syk, GM-CSF may exerts its stimulatory effect on FcγR-phagocytosis by acting either distal to Syk kinase in FcγR-mediated signalling cascade or in a parallel pathway mediated via Fcγ receptor.

This study shows that the early signalling events during FcγR-mediated phagocytosis in human MDM are similar to those previously characterised in murine macrophages. In both species, key protein tyrosine kinases are activated during this process. In addition, activation of the FAK-related kinase, Pyk2, during phagocytosis has been demonstrated for the first time. Furthermore, these studies have provided a baseline for further studies on the stimulatory role of GM-CSF on macrophage function. Since recent clinical studies have demonstrated the successful outcome of adjunctive GM-CSF treatment for opportunistic infections in immunosuppressed patients (Vazquez *et al.*, 1998; Brites *et al.*, 2000), an understanding of the mechanism underlying macrophage stimulation by GM-CSF is potentially of therapeutic benefit.

3.6 RELEVANT PUBLICATIONS.

The following publication has arisen from the work presented in this chapter:

Kedzierska, K., Vardaxis, N., Jaworowski, A. and Crowe, S.M. (2001) FcγR-mediated phagocytosis by human macrophages involves Hck, Syk and Pyk2, and is augmented by GM-CSF. *J Leukoc Biol*, **70**:322-328.

I have performed all the experiments presented in this manuscript, analysed results and written the manuscript with input from my supervisors Prof. Suzanne Crowe and Dr. Anthony Jaworowski. Confocal microscopy was performed by A/Prof. Nicholas Vardaxis.

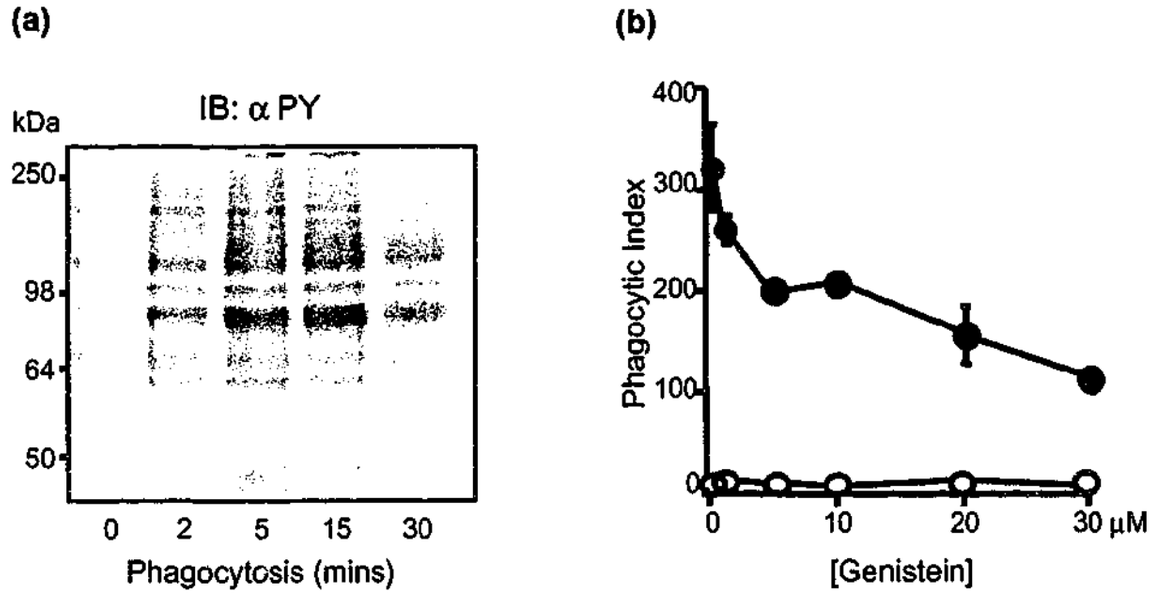

Figure 3.1 FcγR-mediated phagocytosis and protein tyrosine phosphorylation.

(a) MDM incubated with IgG-opsonised latex beads ($2x10^7$) for the indicated times were lysed in Triton-X 100 buffer and samples of lysate containing 50μg protein were resolved by SDS-polyacrylamide gel electrophoresis, then probed with anti-phosphotyrosine conjugated to horseradish peroxidise (RC20). Experiment is representative of 5 experiments using MDM prepared from different donors; (b) Inhibition of phagocytosis by Genistein (inhibitor of protein tyrosine kinases). Phagocytosis of E-IgG (●) or E (○) was measured in the presence of the indicated concentrations of Genistein via colorimetric assay as described in Materials and Methods. Data represent mean (±SD) of triplicate determinations. IB: immunoblotting; PY: phosphotyrosine.

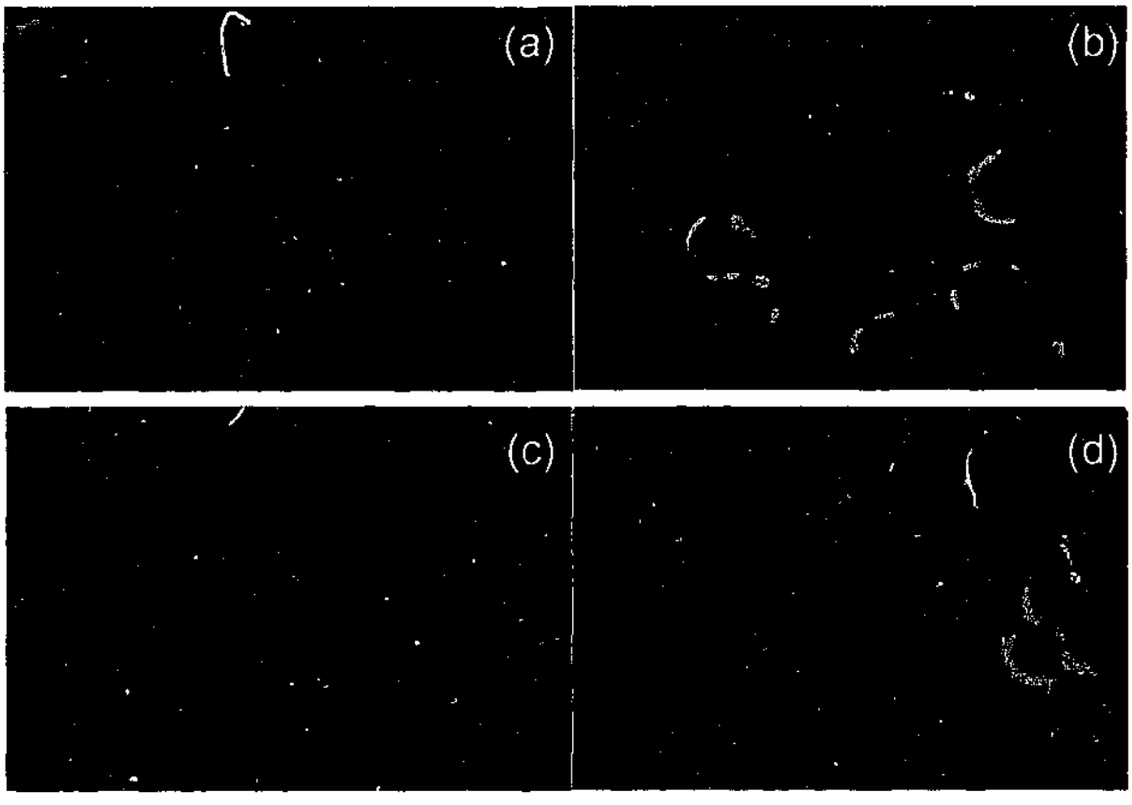

Figure 3.2 Co-localisation of tyrosine-phosphorylated proteins and polymerised actin around phagocytic cups in MDM ingesting IgG-opsonised latex beads.

MDM were incubated with IgG-opsonised latex beads (3μm) for 2 min, fixed with 3% formaldehyde, permeabilised with 0.1% Triton-X 100 and stained with (a) an isotype-matched control (MOPC 21); (b,d) mouse anti-phosphotyrosine monoclonal antibody; (c) mouse anti-paxillin monoclonal antibody, followed by biotinylated anti-mouse Ig and Texas Red streptavidin conjugate; (d) MDM were double stained for phosphotyrosine as above and with Alexa 488-labelled phalloidin. Areas of the cell staining with both antibodies are depicted yellow. MDM were examined by confocal laser microscopy. Bar = 3μm

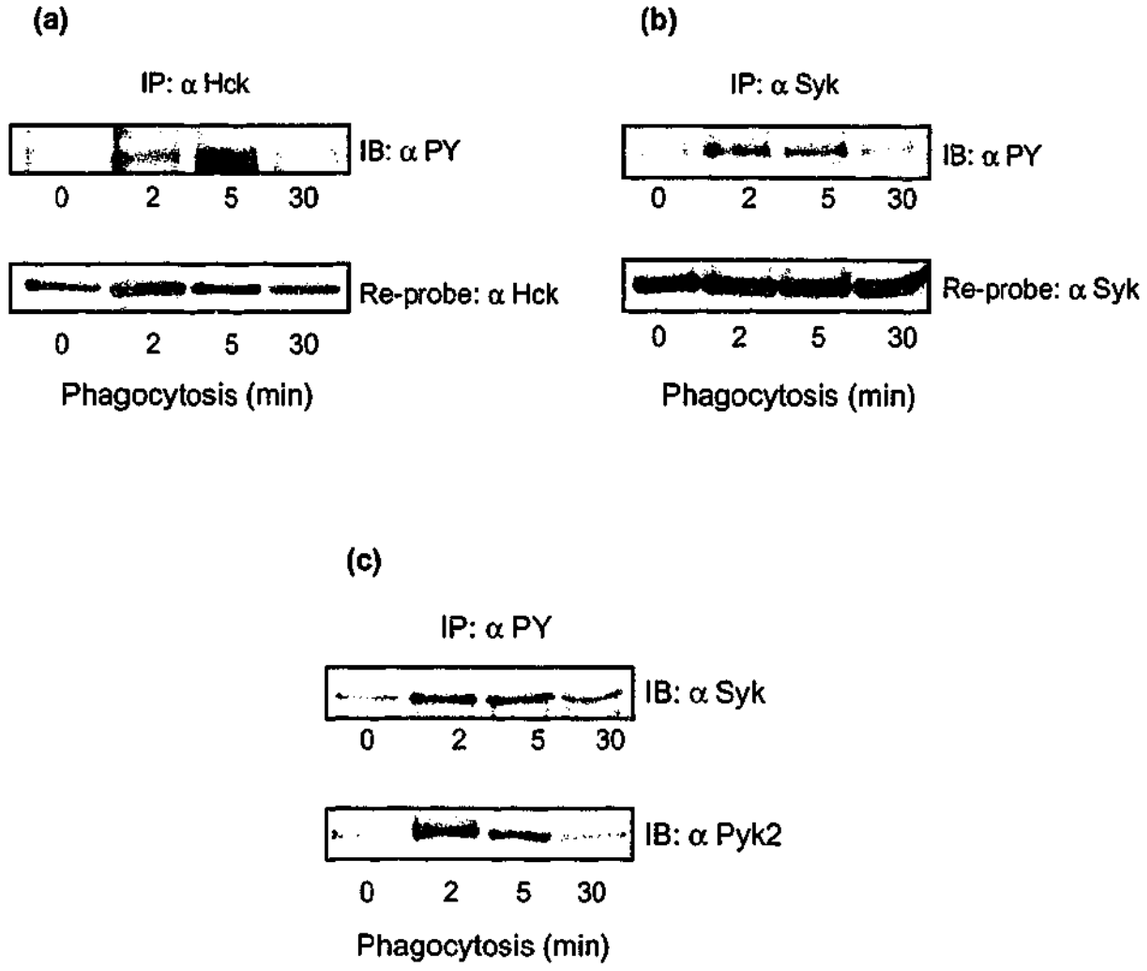

Figure 3.3 Activation of Hck, Syk and Pyk2 tyrosine kinases during FcγR-mediated phagocytosis in human MDM.

Cells ($2x10^6$) were incubated with IgG-opsonised latex beads ($2x10^7$) in polypropylene tubes in a shaking waterbath for the indicated times (0 to 30 min) at 37°C, then lysed in Triton-X buffer. Samples of lysate containing 100µg protein were immunoprecipitated with (a) anti-Hck or (b) anti-Syk monoclonal antibodies; resolved by SDS-polyacrylamide gel electrophoresis, and immunoblotted with anti-PY conjugated to horseradish peroxidase; or immunoprecipitated with (c) anti-PY monoclonal antibody, resolved by SDS- polyacrylamide gel electrophoresis and immunoblotted with anti-Syk or anti-Pyk2 Mab. The data are representative of 3 experiments using MDM from different donors. Protein levels are shown by re-probing the blots with (a) anti-Hck monoclonal antibody (lower panel) or (b) anti-Syk monoclonal antibody (lower panel). IB: immunoblotting; IP: immunoprecipitation; PY: phosphotyrosine.

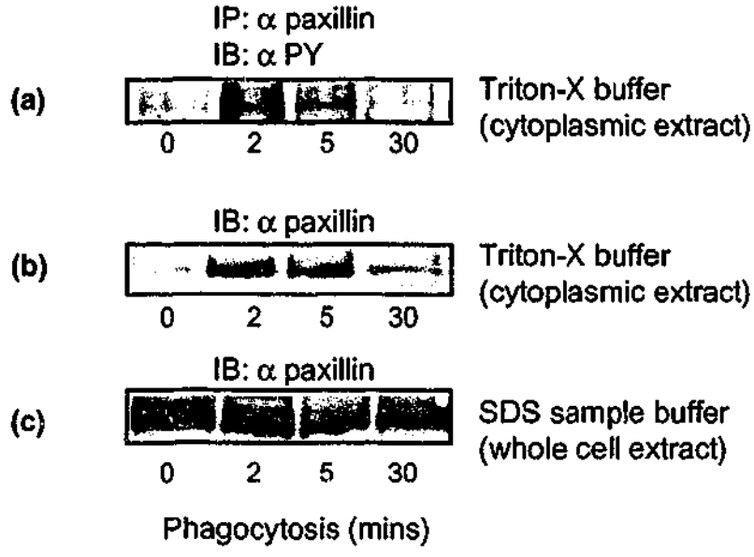

Figure 3.4 Redistribution of paxillin between cytoplasm and cytoskeleton during FcγR-mediated phagocytosis in MDM.

Cells were incubated with IgG-opsonised latex beads ($2x10^7$) in a shaking waterbath for the indicated times (0 to 30 min) at 37°C and lysed in either Triton-X or SDS sample buffer. (a) Triton-X 100 lysates containing 100μg protein were immunoprecipitated with anti-paxillin monoclonal antibody, resolved by SDS-polyacrylamide gel electrophoresis and probed with anti-PY monoclonal antibody conjugated to horseradish peroxidase. (b) Triton-X 100 lysates (cytoplasmic fraction) or (c) SDS lysates (whole cell extracts) containing 50μg protein were resolved by SDS- polyacrylamide gel electrophoresis and probed with anti-paxillin monoclonal antibody. The data shown are representative of three experiments. IB: immunoblotting; IP: immunoprecipitation; PY: phosphotyrosine.

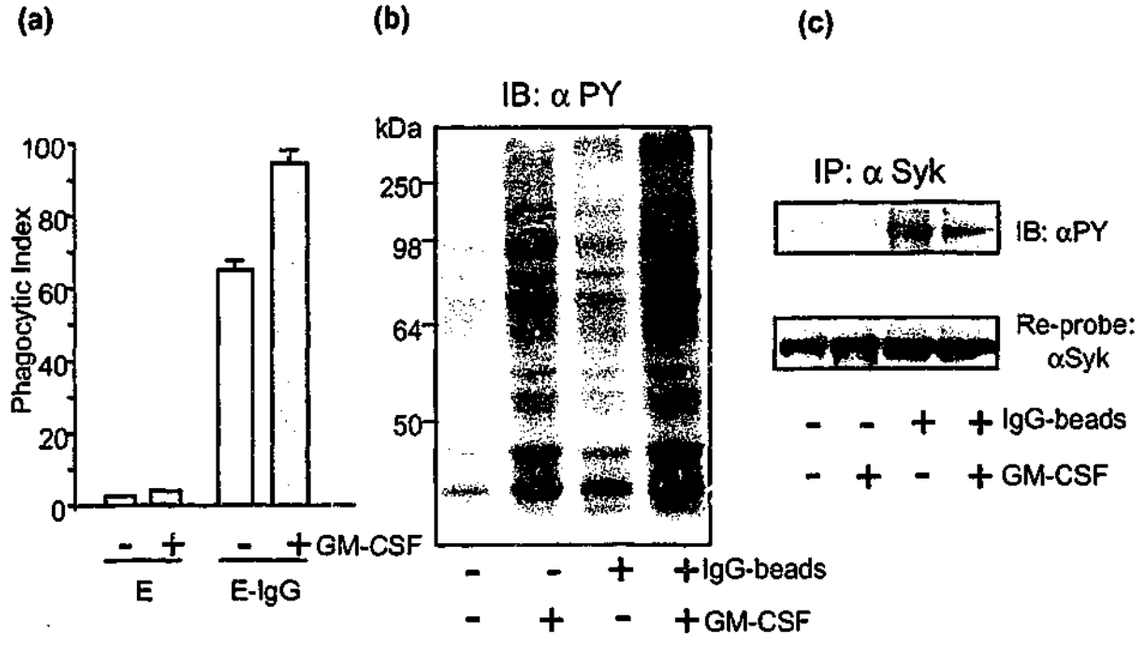

Figure 3.5 Effect of GM-CSF on FcγR-mediated phagocytosis, tyrosine phosphorylation and Syk activation.

MDM were treated with GM-CSF (100ng/ml), where indicated, immediately prior to addition of target particles. (a) MDM ($5x10^4$ per well) plated onto 96 well plates were incubated with E-IgG or E for 20 min and phagocytosis determined by colorimetric assay. Data are representative of 3 donors. Shown are mean±SD; (b) MDM ($2x10^6$) were incubated with IgG-opsonised latex beads ($2x10^7$) for 2 min and Triton-lysates analysed for tyrosine phosphorylated proteins by probing with HRPO-conjugated anti-PY antibody (representative of 5 experiments using MDM prepared from different donors); or (c) for Syk tyrosine phosphorylation by immunoprecipitation of Syk and immunoblotting with horseradish conjugated anti-PY antibody (representative of 5 MDM donors). Input level of Syk was determined by re-probing the blots with Syk monoclonal antibody. IB: immunoblotting; IP: immunoprecipitation; PY: phosphotyrosine.

CHAPTER 4

THE MECHANISM OF HIV-1 INHIBITION OF FCγR-MEDIATED PHAGOCYTOSIS BY HUMAN MACROPHAGES.

4.1 ABSTRACT.

HIV-1 infection impairs a number of macrophage effector functions, thereby contributing to development of opportunistic infections and the pathogenesis of AIDS. Fcγ receptor (FcγR)-mediated phagocytosis by human monocyte-derived macrophages (MDM) is inhibited by HIV-1 infection *in vitro*, and the underlying mechanism was investigated in this study. Inhibition of phagocytosis directly correlated with the multiplicity of HIV-1 infection. Expression of surface FcγRs was unaffected by HIV-1 infection, suggesting that inhibition of phagocytosis occurred during or after receptor binding. HIV-1 infection of MDM markedly inhibited tyrosine phosphorylation of the cellular proteins, which occurs following engagement of FcγRs, suggesting a defect downstream of initial receptor activation. FcγR-mediated phagocytosis in HIV-infected MDM was associated with inhibition of phosphorylation of tyrosine kinases from two different families, Hck and Syk, defective formation of Syk complexes with other tyrosine-phosphorylated proteins and inhibition of paxillin activation. Down-modulation of protein expression but not mRNA of the γ signalling subunit of FcγR (a docking site for Syk) was observed in HIV-infected MDM. Infection of MDM with a construct of HIV-1 in which *nef* was replaced with the gene for the γ signalling subunit augmented FcγR-mediated phagocytosis, suggesting that downmodulation of γ-chain protein expression in HIV-infected MDM caused the defective FcγR-mediated signalling and impairment of phagocytosis. This study provides the first possible mechanism by which HIV-1 inhibits macrophage effector function via interference with specific signalling events within the cell, and contributes to the understanding of how HIV-1 impairs cell-mediated immunity

leading to HIV-1 disease progression.

4.2 INTRODUCTION.

Cells of macrophage lineage including peripheral blood monocytes and tissue macrophages provide critical functions in the cell-mediated response to a variety of opportunistic pathogens such as *Mycobacterium avium* complex, *Toxoplasma gondii* and *Candida albicans*. A number of monocyte/macrophage functions are impaired following HIV-1 infection *in vivo* and *in vitro* including chemotaxis (Tas *et al.*, 1988; Wahl *et al.*, 1989), phagocytosis (Crowe *et al.*, 1994; Biggs *et al.*, 1995), intracellular killing (Biggs *et al.*, 1995) and cytokine production (reviewed in Kedzierska and Crowe, 2001). These defects contribute to the pathogenesis of AIDS by allowing reactivation and development of opportunistic infections (reviewed in Crowe, 1995). The mechanism by which HIV-1 impairs effector functions of cells of macrophage lineage is unknown.

The HIV-1 encoded proteins, Nef, Vif, Vpr and Rev have been shown to modulate a number of signalling pathways via interactions with cytoskeletal (Macreadie *et al.*, 1995; Karczewski and Strebel, 1996; Fackler *et al.*, 1997; Watts *et al.*, 2000) and cytoplasmic proteins (Saksela *et al.*, 1995; Greenway *et al.*, 1996; Nunn and Marsh, 1996; Fackler *et al.*, 2000; Hassaine *et al.*, 2001). These interactions include cellular proteins and kinases which are also involved in Fcγ receptor (FcγR)-mediated phagocytosis, eg. the Src kinases, Hck and Lyn (Lee *et al.*, 1991; Saksela *et al.*, 1995; Lee *et al.*, 1996; Greenway *et al.*, 1996), p21-activated kinase (Nunn and Marsh, 1996; Fackler et al., 2000), the guanine nucleotide-exchange factor, Vav (Fackler *et al.*, 1999) and actin (Fackler *et al.*, 1997). HIV-1 impairs FcγR-mediated phagocytosis and the mechanism is unknown, although studies using the promonocytic U937 cell line

suggest that inhibition occurs via a cylic AMP (cAMP)-dependent mechanism (Thomas *et al.*, 1997).

The receptors for the constant region of immunoglobulin (Ig) G (FcγRI, FcγRII, FcγRIII) are the major means by which cells of macrophage lineage recognise IgG-opsonised pathogens, thereby triggering phagocytosis and antibody-dependent cellular cytotoxicity. Peripheral blood monocytes express mainly the high-affinity FcγRI (CD64) and a low-affinity FcγRII, whereas macrophages also express FcγRIIIA (CD16A) (reviewed in Daeron, 1997). FcγR-mediated internalisation of IgG-opsonised particles requires tyrosine phosphorylation of proteins and involves activation of several kinases and their substrates. Most studies to date have examined these pathways in murine macrophages or cell lines transfected with FcγR (Greenberg *et al.*, 1993; Greenberg *et al.*, 1994; Indik *et al.*, 1995b; Park and Schreiber, 1995; Allen and Aderem, 1996; Greenberg *et al.*, 1996). Following clustering of FcγRs, tyrosine kinases from the Src family associated with γ chain of FcγR (including Hck and Lyn) are activated (Jouvin *et al.*, 1994; Fitzer-Attas *et al.*, 2000) leading to a rapid and transient phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) present on the γ signalling subunits associated with FcγR I and FcγR III or on the cytoplasmic domain of FcγR II (Park *et al.*, 1993). Phosphorylation of ITAMs create docking sites for the tyrosine kinase Syk, which is subsequently activated by phosphorylation (Indik *et al.*, 1995c; Greenberg *et al.*, 1996). An absolute and specific requirement for Syk in FcγR-mediated phagocytosis has been shown by gene knockout studies in mice (Crowley *et al.*, 1997). Activation of Syk results in phosphorylation of phosphatidylinositol 3-kinase (PI3-kinase) (Ninomiya *et al.*, 1994) and localised accumulation of kinases such as focal adhesion kinase (FAK) and cytoskeletal substrates including actin-binding proteins paxillin, vinculin, talin and α-actinin (Greenberg *et al.*, 1990; Greenberg *et al.*, 1994; Allen and Aderem, 1996) leading to cytoskeletal rearrangement and

phagocytosis of the opsonised particles. In my doctoral studies (Chapter 3) it was found that the early signalling events during FcγR-mediated phagocytosis by human monocyte-derived macrophages (MDM) also involve tyrosine phosphorylation of cellular proteins including Hck, Syk, Pyk-2 (a member of FAK family) and paxillin (Kedzierska *et al.*, 2001b).

This study examines the mechanism by which HIV-1 inhibits FcγR-mediated phagocytosis in human MDM. The results presented in this Chapter show that defective phagocytosis by HIV-infected MDM is due, at least in part, to decreased expression of the γ signalling subunit of the FcγR, which leads to specific signalling defects downstream of FcγRs.

4.3 METHODS.

4.3.1 Flow cytometric analysis of FcγR I, II and III expression.

MDM were infected with the M-tropic strain of HIV-$1_{Ba\text{-}L}$ on day 5 post-isolation (Section 2.5). On day 7 post-infection MDM were analysed for surface expression of FcγRs I (CD64), FcγR II (CD32) and FcγR III (CD16). Cells were stained with monoclonal antibody (Mab) directed against CD64 conjugated to PE (1μg/ml; Serotec, Raleigh, NC), CD32 conjugated to FITC (1μg/ml; Serotec) and CD16 conjugated to Cy-Chrome (1μg/ml; BD PharMingen, San Diego, CA) on ice for 30 min, followed by a wash in PBS-CMF. The mean fluorescence of monocytes expressing FcγRs was quantified by flow cytometric analysis. Cells were also stained with isotype-matched controls conjugated to the appropriate fluorochrome.

4.3.2 Intracellular staining of γ chain of FcγRs.

The intracellular γ chain staining was determined in uninfected and HIV-infected MDM on day 7 post-infection. Cells were fixed in 3% ultrapure formaldehyde for 45 min, followed by two washes in 0.1M glycine in PBS-CMF and permeabilisation in 0.1% Triton-X for 1 min. MDM were washed twice in PBS-CMF containing 1% FCS and stained for intracellular γ chain using rabbit anti-γ subunit (TCR, FcR) polyclonal antibody (1μg/ml; Upstate Biotechnology, New York, USA) or rabbit IgG control (1μg/ml); Upstate Biotechnology) followed by two washes in cold (4^0C) PBS-CMF and incubation with sheep anti-rabbit IgG conjugated to FITC (Sileneus, Mebourne, Australia). All staining procedures were performed in the presence of 50% FCS to reduce the level of non-specific staining. The fluorescence for intracellular γ chain was quantified by flow cytometric analysis, converted to MESF (molecules of equivalent soluble fluorochrome) units and corrected for background fluorescence.

4.3.3 Phagocytosis assays.

To determine the effect of HIV-1 on FcγR-mediated phagocytosis, MDM were exposed to IgG-opsonised or unopsonised E on day 7 post-infection, as described in Section 2.8.2.2. In selected experiments MDM were incubated with 8'bromo-cAMP (Sigma, St Louis, MO. USA) at concentrations ranging from 0.1μM to 1mM at 37^0C for 30 min or 48 hr prior to phagocytosis assay. Alternatively, MDM were incubated with or without IgG-opsonised beads (Section 2.8.3.2) and lysed in Triton-X 100 lysis buffer (Section 2.8.3.3) for immunoblotting and immunoprecipitation analysis (Section 2.9).

4.3.4 Immunoblotting and Immunoprecipitation.

MDM extracts containing equal amounts of proteins were resolved by 10% SDS-PAGE (Section 2.12.2), transferred to nitrocellulose (Section 2.9), and probed with antibodies directed against phosphotyrosine (RC20; Transduction Laboratories, Lexington, KY), Syk (Santa Cruz Biotechnology, Santa Cruz, CA), paxillin (Transduction Laboratories), Hck (gift from Dr H.C. Cheng, Department of Biochemistry, The University of Melbourne), γ chain of FcγR (Upstate Biotechnology), followed by secondary antibody conjugated with horseradish peroxidase (Section 2.9). To determine the phosphorylation of Hck, Syk and paxillin during FcγR-mediated phagocytosis, cell lysates were immunoprecipitated with the appropriate antibody and probed with anti-phosphotyrosine antibody (RC20) conjugated to HRPO (Section 2.9).

4.3.5 mRNA extraction and amplification of γ signalling subunit.

Using oligo $(dT)_{25}$ beads (Dynabeads, Dynal, Australia) mRNA was extracted from MDM as described in Section 2.11.1. Beads/cDNA complexes were resuspended in 100μl TE buffer (pH 8.0) and stored at 4°C. For PCR, 2-fold dilutions were made using equal amounts of cDNA based on GAPDH levels determined by real-time PCR as previously described (Lewin *et al.*, 1999). PCR for β-actin was performed to confirm equal template levels (Section 2.11.2). Reactions were performed in a total of 50μL comprising 0.2mM of each dNTP, 1.5mM $MgCl_2$, 0.4μM of each primer, 1.1U of Taq polymerase (Perkin Elmer, Norwalk, WI, USA) and 1× reaction buffer (Perkin Elmer). The γ chain primer set amplified 172 bp of the human γ chain cDNA sequence (GenBank accession NM004106) with the sequences 5'-GAGCCTCAGCTCTGCTATATCC-3' and 5'-TCTCGTAAGTCTCCTGGTTCC-3'. Samples were first denatured at 95°C for 2 min and then amplified for 25 cycles of 95°C for 30 sec, 55 °C for 30 sec, and 72°C for 1 min, with a final extension step at

72°C for 7 min. PCR products were analysed by 2% agarose gel electrophoresis (Section 2.12.1)

4.3.6 Construction of [NL(AD8)Δnef-γ(+)].

The DNA constructs [pNL(AD8)] and [pNL(AD8)Δnef] were prepared by substituting the respective envelope coding DNA sequences from NL4.3 and NL4.3Δnef with monocytotropic AD8 envelope coding sequences, converting a T-tropic virus to M-tropic as described in Section 2.3.2. The [NL(AD8)Δnef-γ(+)] plasmid was constructed using stitch PCR mutagenesis. HIV-1 sense f1 primer Rev 8392S 5'GGGGACCCGACAGGCCCG3' and HIV-1 antisense f1 primer 5'-GAGCAAGACCACTGCTGGAATCATCTTATAGCAAAATCCTTTCCAAGC-3' were used to amplify a 390-bp HIV-1 f1 fragment upstream of Nef. γ chain sense (5'-GCTTGGAAAGGATTTTGCTATAAGATGATTCCAGCAGTGGTCTTGCTC-3') and antisense (5'-CCCCTCGAGACGCGTCTACTGTGGTGGTTTCTCATGCTTCAG-3') f2 primers were used to amplify a 300-bp of γ chain coding f2 fragment. HIV-1 f1 and γ chain f2 fragments were joined by extension reaction under the following conditions - one cycle of 94^{0}C for 2 min, 35 cycles (each) of 94^{0}C for 30 sec. 60^{0}C for 45 sec, 72^{0}C for 2 min and 1 cycle of 72^{0}C for 7 min. The resulting fragment was amplified by PCR (conditions as above) and cloned into the *BamHI* and *XhoI* restriction sites of the [NL(AD8)Δnef] proviral DNA as previously described (Shehu-Xhilaga *et al.*, 2001). To ensure the presence of γ chain in this mutant proviral DNA and the absence of spontaneous mutations, the construct was sequenced using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Sequencing reaction products were separated on a model 373 automated DNA sequencer at the Department of Microbiology, Monash University, Clayton, Australia. The production of [NL(AD8)],

[NL(AD8)Δnef] viruses and [NL(AD8)Δnef-γ(+)] particles was achieved by introducing 10μg of DNA into 293T cells by calcium-phosphate transfection method (Section 2.3.2).

4.3.7 Statistical analysis.

The significance of the effects of HIV-1 and cAMP on FcγR-mediated phagocytosis, downmodulation of γ chain in HIV-infected MDM and the augmentation of phagocytosis by [NL(AD8)Δnef- γ(+)] construct were assessed using the Student's paired *t* test. A probability of 0.05 was used to reject null hypothesis.

4.4 RESULTS.

4.4.1 HIV-1 INHIBITS FCγR-MEDIATED PHAGOCYTOSIS VIA A POST-RECEPTOR MEDIATED MECHANISM.

In vitro infection of MDM with HIV-1 inhibited phagocytosis of IgG-opsonised E (mean inhibition of 50.3 ±. 4.3%, n=33, p<0.001) as assessed on day 7 post-infection (Figure 4.1a). Phagocytosis was inhibited progressively with increased multiplicity of infection in MDM cultures, indicating a direct effect of HIV-1 replication on the phagocytic capacity of macrophages (Figure 4.1b). HIV-1 infection of MDM did not alter the surface expression of FcγRs: FcγRI (CD64), FcγRII (CD32) or FcγRIII (CD16) (p=0.22, 0.84 and 0.09 respectively, n=5) at the time of phagocytosis, suggesting that inhibition of phagocytosis occurred during or after receptor binding (Figure 4.2).

4.4.2 HIV-1 inhibition of FcγR-mediated phagocytosis is not mediated via a cAMP-dependent mechanism.

As a cAMP-dependent mechanism has been proposed to be the cause of HIV-1 inhibition of FcγR-mediated phagocytosis in the promonocytic U937 cell line (Thomas *et al.*, 1997), the possibility of a cAMP-dependent mechanism being responsible for the inhibition of phagocytosis in MDM infected with HIV-1 was initially investigated. However, incubation of MDM with a non-metabolised cAMP analogue, 8'bromo-cAMP for either 30 min ($p>0.05$, $n=5$) or 48 hr ($p>0.05$, $n=4$) did not inhibit FcγR-mediated phagocytosis by human MDM (Table 4.1).

4.4.3 Tyrosine phosphorylation of cellular proteins is inhibited during FcγR-mediated phagocytosis by HIV-infected MDM.

Since FcγR-mediated internalisation of IgG-opsonised beads by human MDM requires rapid phosphorylation of tyrosine residues in a range of proteins (shown in Chapter 3), the effects of HIV-1 on tyrosine phosphorylation triggered by binding of IgG-opsonised targets was assessed. A range of cellular proteins was rapidly phosphorylated (by 2 min exposure to IgG-particles) in MDM exposed to IgG-opsonised beads, and phosphorylation reached a peak between 2 and 5 min (Figure 4.3a). HIV-1 infection of MDM inhibited FcγR-triggered tyrosine phosphorylation of cellular proteins, suggesting a defect downstream to initial receptor activation. Inhibition of tyrosine phosphorylation in HIV-infected MDM correlated with markedly impaired phagocytosis (Figure 4.3b). In some cultures, HIV-infected MDM displayed a relatively high basal level of tyrosine phosphorylation, but an increase in tyrosine phosphorylation was never observed during FcγR-mediated phagocytosis by those cells.

4.4.4 FcγR-mediated phagocytosis is impaired upstream of Hck, Syk and paxillin.

The effect of HIV-1 on specific tyrosine kinases necessary for FcγR-mediated phagocytosis and their substrates was subsequently assessed. HIV-1 infection of MDM inhibited phosphorylation of tyrosine kinases from two different families, Hck (a Src family member; 58-60 kDa) (Figure 4.4a) and Syk (72 kDa) (Figure 4.4b). HIV-1 infection also impaired formation of FcγR-stimulated Syk complexes with other tyrosine phosphorylated proteins (MW of 38, 42, 48, 52, 58, 64 and 95kDa) (Figure 4.4b). HIV-infected MDM also displayed reduced phosphorylation of paxillin (an adapter protein which localises to focal adhesions; 68 kDa) during phagocytosis (Figure 4.4c). The input of Hck, Syk and paxillin proteins was standardised according to protein estimations in each lysate. Protein levels are shown by re-probing with relevant antibodies where feasible (Figure 4, lower panels).

4.4.5 FcγR-mediated phagocytosis by HIV-infected MDM is associated with downmodulation of Fcγ chain protein expression.

Since the surface expression of FcγRs was not altered by HIV-1, the protein tyrosine phosphorylation data (Figure 4.4) suggest a defect upstream of Hck and Syk, at the level of the γ signalling subunit of FcγR (see Figure 1.10 for a schematic diagram of FcγR-mediated signalling). HIV-1 infection reduced protein levels of the ITAM-containing γ chain of FcγR as demonstrated by immunoblot analysis of Triton-X 100 soluble lysates from HIV-1 infected MDM when compared to lysates obtained from uninfected MDM and standardised according to total protein levels (Figure 4.5a). Intracellular staining using anti-γ chain antibody confirmed these results and demonstrated a significant decrease in intracellular levels of the FcγR γ signalling

subunit in HIV-infected MDM (mean inhibition of 57.3 ± 12.3%; p=0.009, n=4) compared to uninfected MDM (Figure 4.5 b,c).

Analysis of cell lysates from three donors showed no detectable change in γ chain mRNA levels in MDM infected with HIV-1 when compared to uninfected controls (Figure 4.6), showing that HIV-1 does not down-modulate γ chain at the mRNA level. Input of cDNA was standardised according to GADPH levels as assessed by real-time PCR and confirmed by conventional PCR using β-actin specific primers (Figure 4.6).

In vitro infection of MDM by either wild type [NL(AD8)] or *nef*-deleted [NL(AD8)Δ*nef*] HIV-1 inhibited FcγR-mediated phagocytosis (p=0.003 and p=0.01 respectively; n=5). In marked contrast, infection of MDM with a *nef*-deleted HIV-1 with the gene for the γ chain of FcγR [NL(AD8)Δnef-γ(+)] inserted in the *nef* site augmented FcγR-mediated phagocytosis (p=0.045, n=5) and partially restored the phagocytic capacity of HIV-infected MDM (mean restoration of 35.2 ± 15.8%) (Figure 4.7a). The observed increase in phagocytic capacity of MDM infected with [NL(AD8)Δnef-γ(+)] virus was associated with increased protein levels of the γ chain as assessed by immunobloting (n=2; data not shown). Replication of both of these *nef*-deleted strains of HIV-1 was equivalent to that of wild type virus, [NL(AD8)Δnef] (p=0.48, n=5) or [NL(AD8)Δnef-γ(+)] (p=0.89; n=5) (Figure 4.7b).

4.5 DISCUSSION.

This study shows that HIV-1 infection of MDM *in vitro* leads to a significant impairment of FcγR-mediated phagocytosis, resulting from inhibition of tyrosine phosphorylation of the signalling proteins required for phagocytosis. Phosphorylation of Hck, Syk and paxillin was specifically inhibited in HIV-infected MDM. Upstream of Hck and Syk decreased protein expression of the γ signalling subunit of FcγR was found. This

suggests that HIV-1 inhibits phagocytosis via interference with γ chain-specific signalling events within human MDM. Thus the finding of reduced protein levels of the ITAM-containing γ signalling subunit in HIV-infected MDM is likely to be responsible for the defective phagocytosis by these cells. To my knowledge, this study is the first to report inhibition of tyrosine phosphorylation and signalling events underlying defective effector function in HIV-infected macrophages.

The results presented in this study, in agreement with other reports, show impaired FcγR-mediated phagocytosis by human monocytes and macrophages following HIV-1 infection (Bender *et al.*, 1988; Capsoni *et al.*, 1992; Capsoni *et al.*, 1994), although the mechanism of inhibition was unknown. The majority of previous reports (Petit *et al.*, 1988; Capsoni *et al.*, 1992; Capsoni *et al.*, 1994; Dunne *et al.*, 1996), confirmed by observations presented in this study, showed that HIV-1 infection did not change surface expression of FcγRs, indicating that the inhibition of phagocytosis results from a signalling defect downstream of FcγRs. In the only study published to date on the mechanism of inhibition of phagocytosis by HIV-1, Thomas and colleagues reported that inhibition of FcγR-mediated phagocytosis is mediated via a cAMP-dependent mechanism in the promonocytic U937 cell line latently infected with HIV-1. This was established by showing that pre-treatment of these cells with 8'bromo-cAMP for 48 hr prior to phagocytosis significantly decreased their functional capacity (Thomas *et al.*, 1997). However, results obtained from primary human MDM presented in this chapter showed that FcγR-mediated phagocytosis is not inhibited by the presence of 8'bromo-cAMP. Similarly, others have shown that dibutryl cAMP inhibited complement-mediated phagocytosis but not FcγR-mediated phagocytosis in human MDM (Newman *et al.*, 1991), suggesting that elevated cAMP levels resulting from HIV-1 infection (Nokta and Pollard, 1991; Thomas *et al.*, 1997) are not responsible for impaired FcγR-mediated phagocytosis. However, it is possible that cAMP inhibits FcγR-mediated phagocytosis

in the promonocytic U937 cell line via affecting the differentiation of U937 cells, rather than inhibiting phagocytosis per se.

Data presented in this Chapter showing inhibition of tyrosine phosphorylation of any cellular proteins following stimulation with IgG-opsonised targets in HIV-infected MDM suggested dysfunction at an early stage in FcγR-mediated signalling. This was confirmed by demonstration of impaired phosphorylation of the tyrosine kinases Hck and Syk during phagocytosis by HIV-infected MDM. Specific requirements for both Hck (associated with γ chain) and Syk in FcγR-mediated phagocytosis have been demonstrated in numerous studies using knockout mice, monocytic cell lines and human MDM (Lowell *et al.*, 1994; Ghazizadeh *et al.*, 1994; Ghazizadeh *et al.*, 1995; Crowley *et al.*, 1997; Kedzierska *et al.*, 2001b). Although defective activation of either Syk or Hck in HIV-infected macrophages has not been previously reported, structural and functional defects of Lck and Fyn (members of Src kinases) as well as ZAP-70 (analogue of Syk) have been observed in T cells from HIV-infected individuals (Stefanova *et al.*, 1996).

Syk activation is absolutely essential for phagocytosis, as it couples phagocytosis-promoting FcγRs to rearrangements in the actin-based cytoskeleton (Crowley *et al.*, 1997). Therefore, impaired Syk phosphorylation triggered by FcγR engagement would inhibit Syk-mediated activation of substrates required for actin polymerisation and cytoskeletal rearrangement, thereby inhibiting phagocytosis (Cox *et al.*, 1996). Paxillin, a potential downstream effector of Syk, has been shown to interact with proteins involved in actin reorganisation including vinculin, talin and α-actinin (Turner, 1998). Consistent with data showing inhibition of Syk phosphorylation, HIV-infected MDM displayed reduced phosphorylation of paxillin during phagocytosis, compared to uninfected MDM.

Upstream of Hck, Syk and paxillin reduced levels of ITAM-containing γ signalling subunit of FcγR were found, not associated with an effect on surface expression of either FcγRI, FcγRII or FcγRIIIA. The γ chain of FcγR is not a prerequisite for transient expression of surface FcγRs, but it is important for their stable expression (Takai *et al.*, 1994; van Vugt *et al.*, 1996). The cytoplasmic γ chain of FcγRs, however, is critical for Syk activation and subsequent signalling events resulting in FcγR-mediated phagocytosis (Park *et al.*, 1993; Indik *et al.*, 1995b). Deletion of the intracellular γ chain of FcγR markedly impairs phagocytosis despite unchanged surface receptor level (Edberg *et al.*, 1999). Thus the finding of reduced protein levels of the ITAM-containing γ signalling subunit in HIV-infected MDM is likely to be responsible for the defective phagocytosis by these cells. The HIV-induced downregulation of γ chain expression was specific for this subunit as levels of other signalling molecules downstream FcγRs viz. Syk and paxillin were not affected. As γ chain mRNA levels were unaltered, this indicated that HIV-1 inhibited γ chain expression at a post-transcriptional point. This contrasts with T cells where HIV-1 infection reduces the levels of mRNA for the homologous CD3-ζ chain (Geertsma *et al.*, 1999) and CD3-γ chain (Segura *et al.*, 1999).

Data presented in this study confirmed my results (described in Chapter 5) that a functional *nef* gene was not essential for inhibition of FcγR-mediated phagocytosis. Infection of MDM with a *nef*-deleted HIV-1 expressing the γ chain of FcγR augmented FcγR-mediated phagocytosis, supporting the claim that reduced expression of the γ signalling subunit of FcγR may be responsible for impaired FcγR-mediated phagocytosis in HIV-infected MDM. As it was impossible to control the level of γ chain protein input in this model, it was not possible to rigorously correlate phagocytic efficiency of macrophages and γ chain levels. If HIV-1 infection impairs FcγR-mediated phagocytosis at more than one level along the signalling pathway, any HIV-induced

phagocytic defect would not be completely restored by overexpression of γ chain protein.

Taken together, these data suggest that the mechanism of inhibition of FcγR-mediated phagocytosis in HIV-infected MDM occurs upstream of Hck, Syk, paxillin activation and is associated with decreased protein expression of the γ chain signalling subunit of FcγR. It provides the first possible mechanism of defective cellular activation in HIV-infected macrophages not only during phagocytosis but potentially also underlying other functions mediated via FcγR. Impaired FcγR-mediated signalling may explain why HIV-infected macrophages fail to control opportunistic pathogens such as *Toxoplasma gondii* (Joiner *et al.*, 1990) and provides potential therapeutic targets for immunomodulatory therapies for the treatment of AIDS via restoration of host defense.

4.6 RELEVANT PUBLICATIONS.

The following publication has arisen from the work presented in this chapter:

Kedzierska K, Ellery P, Mak J, Lewin S, Crowe SM and Jaworowski A. (2001) HIV-1 downmodulates γ signalling chain of Fcγ receptor in human macrophages: a possible mechanism for inhibition of phagocytosis (*J Immunol;* responses to the reviewers' comments/questions submitted).

I have performed the experiments presented in this Chapter, analysed results and written the manuscript with input from my supervisors Prof. Suzanne Crowe and Dr. Anthony Jaworowski. Philip Ellery has provided his expertise with mRNA experiments. Dr. Johnson Mak designed the [NL(AD8)Δnef-γ(+)] clone. The real-time PCR data used for standardisation of cDNA levels were provided by Dr. Sharon Lewin.

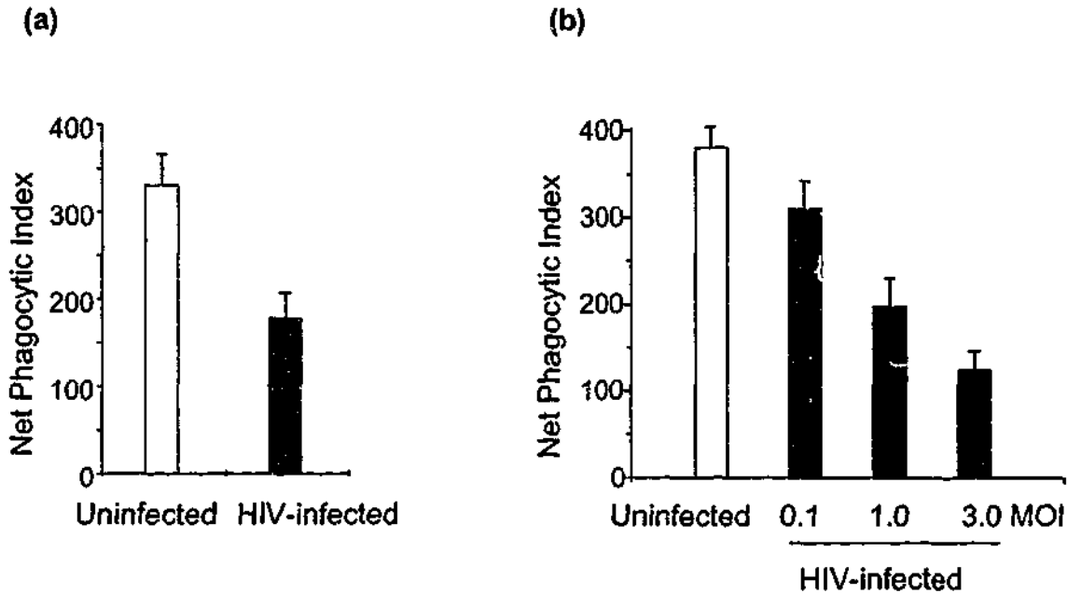

Figure 4.1 HIV-1 inhibits FcγR-mediated phagocytosis by MDM.

Phagocytosis of specific IgG-opsonised sheep red blood cells (IgG-E) was assessed by colorimetric assay. Monocyte-derived macrophages (MDM) on day 5 post-isolation were either mock-infected (white bars) or infected with HIV-1_{Ba-L} (black bars). Phagocytosis assays were performed on day 7 post-infection using IgG-E at target to MDM ratio of 10:1. These data represent means ± SEM of the phagocytic index (number of phagocytosed particles per 100 MDM) from (a) 33 donors used in this study; (b) representative donor infected with HIV-1 at varying multiplicity of infection (MOI).

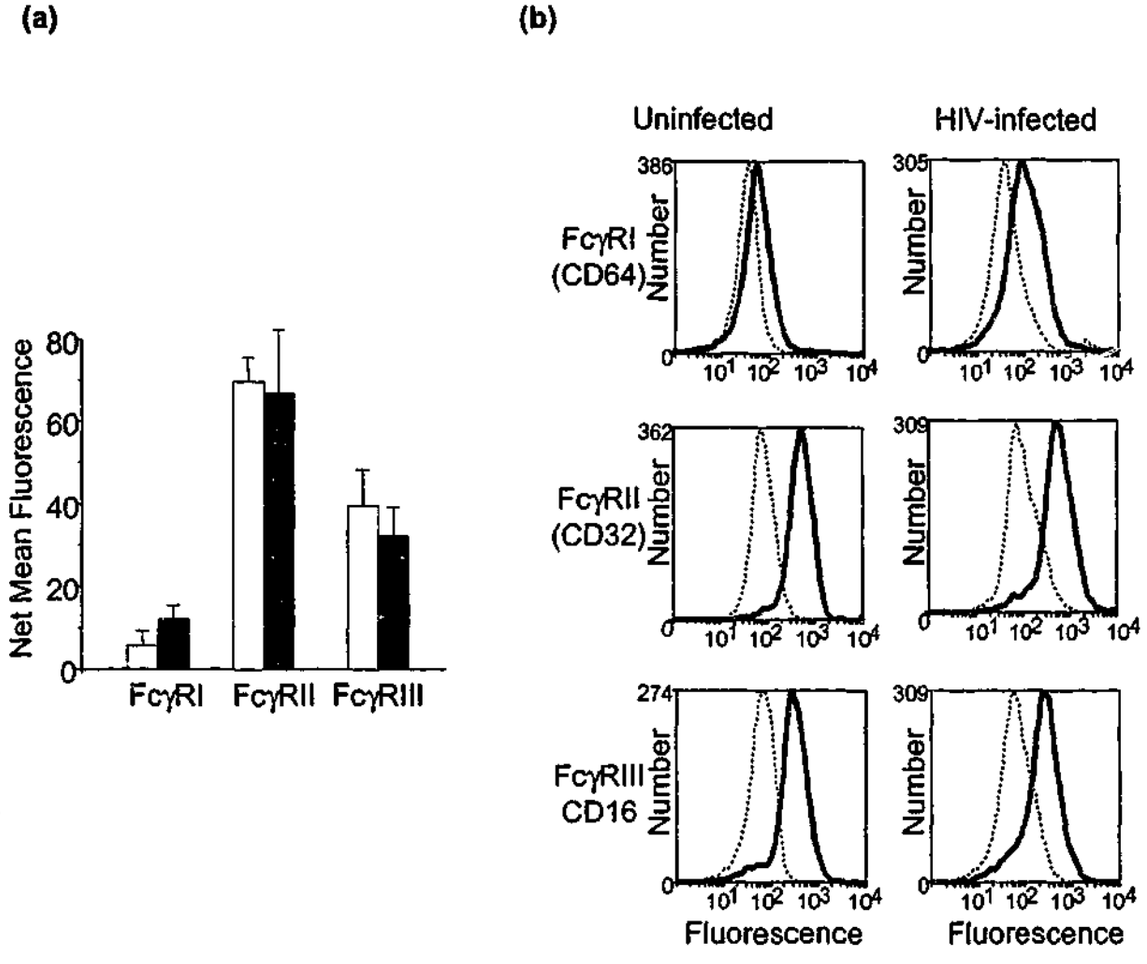

Figure 4.2 Flow cytometric analysis of surface expression of FcγR I (CD64), FcγR II (CD32) and FcγR III (CD16) on HIV-infected and uninfected MDM.

Expression of FcγRs on MDM on day 7 after HIV-1 infection was assessed by flow cytometric analysis using anti-CD64 monoclonal conjugated to PE, anti-CD32 monoclonal antibodies conjugated to FITC and anti-CD16 monoclonal antibody conjugated to Cy-Chrome. (a) Results are expressed as net mean fluorescence (corrected for background fluorescence) ± SEM and represent data from five experiments using uninfected (white bars) or HIV-infected (black bars) MDM. (b) Results from a representative single donor are provided. Histograms are unimodal and demonstrate the staining with monoclonal antibodies directed against FcγRs (solid lines) or isotype-matched controls (dotted lines) conjugated to appropriate fluorochrome.

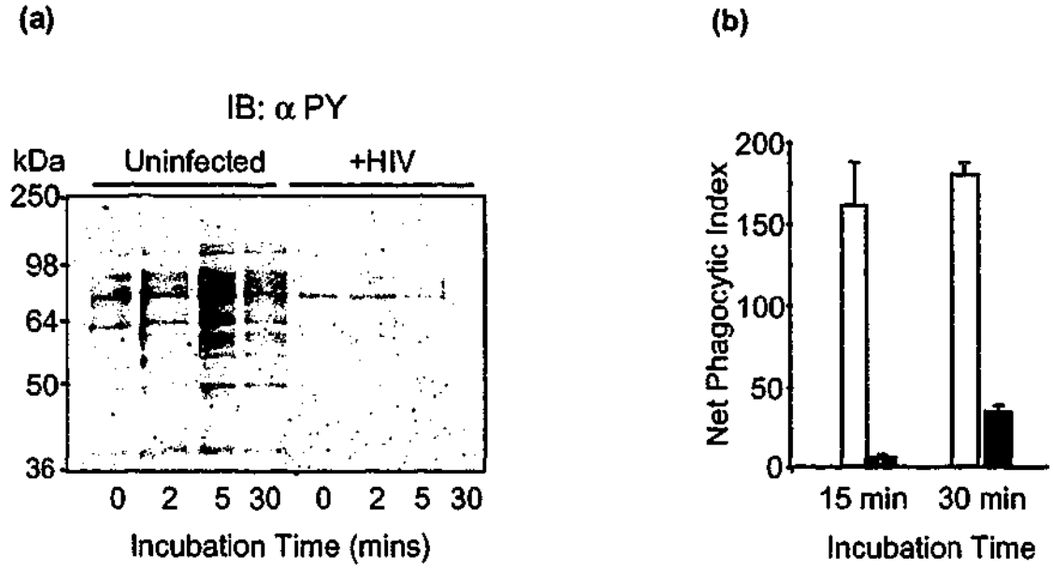

Figure 4.3 Inhibition of protein tyrosine phosphorylation during FcγR-mediated phagocytosis in HIV-infected macrophages.

(a) MDM incubated with IgG-opsonised latex beads (target to MDM ratio of 10:1) for the indicated times were lysed in Triton X-100 buffer, and samples of lysate containing of equal amount of protein (30μg) were resolved by SDS-polyacrylamide gel electrophoresis, then probed with anti-phosphotyrosine conjugated to horseradish peroxidase (RC20). Results shown are representative of three experiments using MDM prepared from different donors. (b) Inhibition of tyrosine phosphorylation was associated with decreased FcγR-mediated phagocytosis by HIV-infected MDM from the same donor. Phagocytosis of IgG-opsonised E by uninfected (white bars) and HIV-infected (black bars) MDM was measured using cells from the same cultures analysed in (a), after 15 or 30 min via a colorimetric assay as described in the Methods section. Data represent means ± SEM of triplicate determinations. IB: immunoblotting; PY: phosphotyrosine.

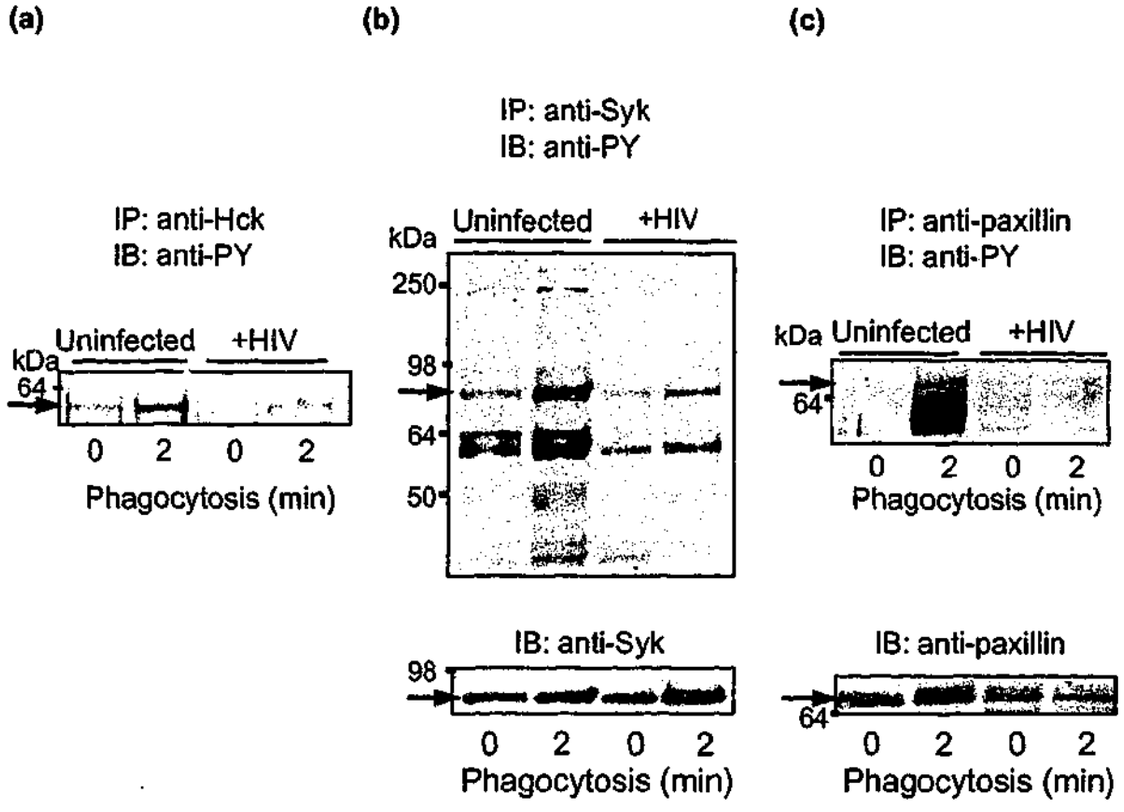

Figure 4.4 HIV-1 infection of MDM inhibits Hck (p58), Syk (p72) and paxillin (p68) activation during FcγR-mediated phagocytosis.

On day 7 post-infection, MDM ($2x10^6$) were incubated with IgG-opsonised latex beads ($2x10^7$) in 200µl of PBS-CMF for the indicated times at 37^0C, followed by lysis in Triton X-100 buffer. Sample of lysates containing equal amounts of protein (50µg) were immunoprecipitated with (a) anti-Hck; (b) anti-Syk and (c) anti-paxillin; the immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-PY-horseradish peroxidase. The data shown are representative of three experiments using MDM from different donors. Equivalent protein levels are shown by re-probing the blots with the relevant antibodies where feasible (lower panel). IB: immunoblotting; IP: immunoprecipitation; PY: phosphotyrosine.

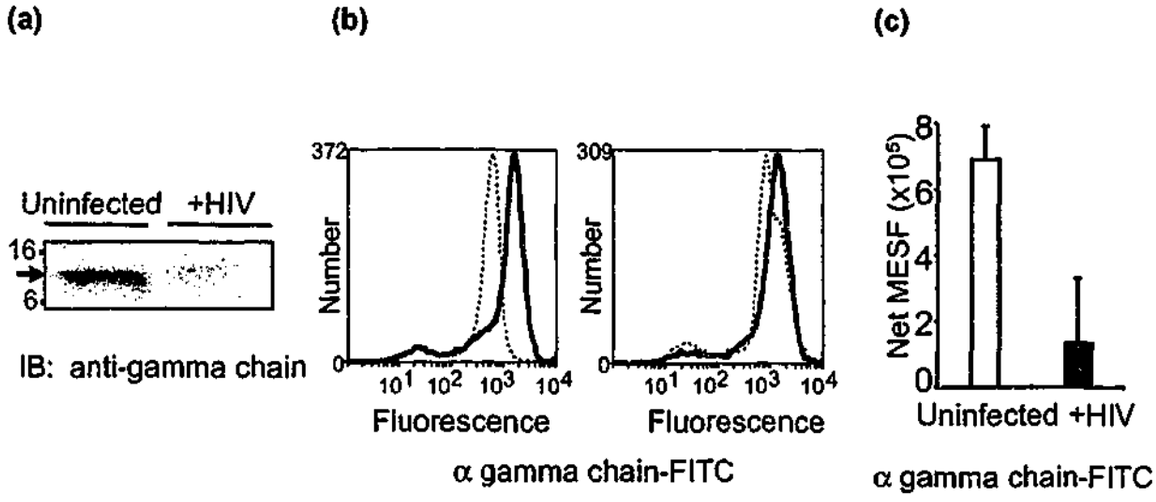

Figure 4.5 HIV-1 infection of MDM downmodulates protein levels of the γ signalling subunit of FcγR.

(a) MDM (1x10^6) infected with HIV-1 for 7 days were washed twice in PBS-CMF, lysed in Triton X-100 buffer and samples of lysate containing equal amount of protein (30μg) were resolved by SDS-polyacrylamide gel electrophoresis, probed with rabbit anti-γ chain antibody overnight followed by anti-rabbit antibody conjugated to horseradish peroxidase. The immunoblot shown is representative of six experiments using MDM from different donors. (b) The intracellular staining of γ chain of FcγRs on MDM on day 7 post-infection was assessed by flow cytometry using anti-rabbit γ chain antibody (solid lines) or isotype-matched control (dotted lines), followed by a secondary anti-rabbit antibody conjugated to FITC. Results are representative of four experiments using MDM prepared from different donors. (c) Net mean fluorescence values are shown for Fc γ-chain expression that has been converted to MESF (molecules of equivalent soluble fluorochrome) units using QuickCal program and corrected for background fluorescence. Results are representative of four experiments using uninfected (white bars) or HIV-infected (black bars) MDM. IB:immunoblotting.

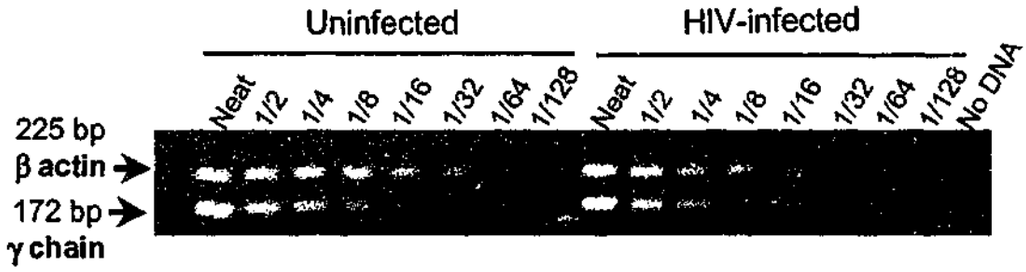

Figure 4.6 HIV-1 does not modulate γ chain mRNA expression.

MDM were infected with HIV-1 on day 5 post-isolation and mRNA extractions were performed 7 days after HIV-1 infection. γ chain mRNA was reverse transcribed to cDNA by PCR using γ chain-specific primers as described in Methods. Levels of cDNA were standardised according to GADPH by real-time PCR and confirmed by β-actin levels. PCR on samples prepared without reverse transcriptase were negative.

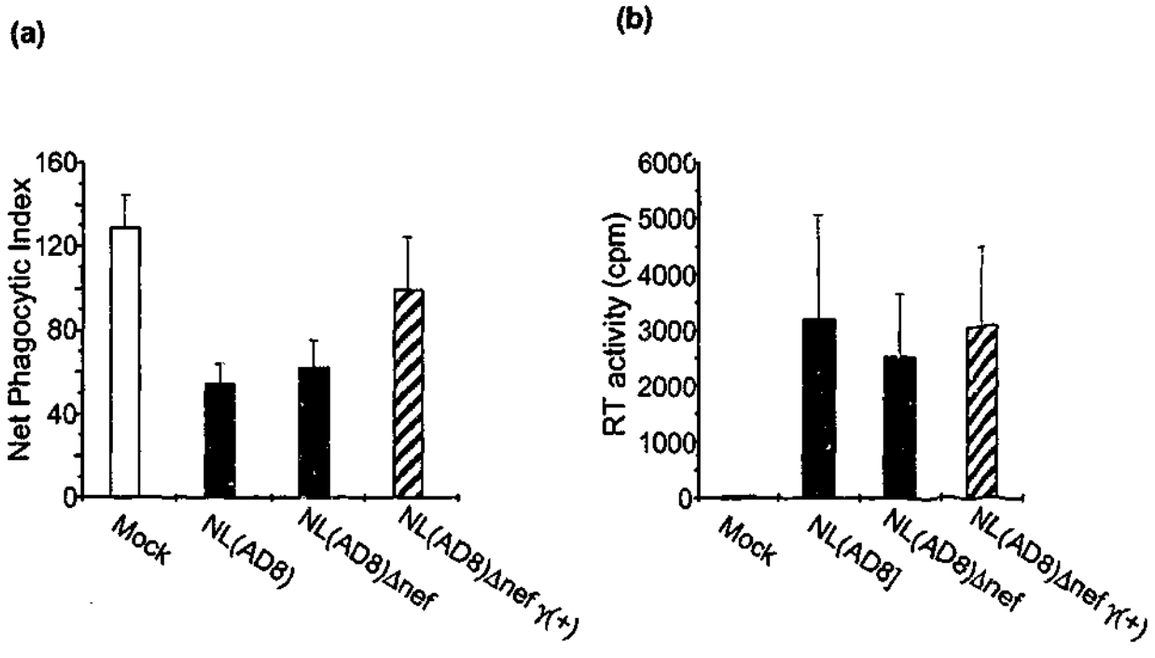

Figure 4.7 Expression of γ chain during HIV-1 infection augments FcγR-mediated phagocytosis.

MDM on day 5 post-isolation were mock-infected (white bars), infected with WT [NL(AD8)] and *nef*-deleted [NL(AD8)Δnef] (black bars) or [NL(AD8)Δnef γ(+)] (striped bars). (a) Phagocytosis was performed on day 7 post-infection and assessed via a colorimetric assay as described in the Methods section. These data represent means ±SEM of experiments using cells from five different donors; (b) Reverse transcriptase activity was quantified in culture supernatant on day 7 post-infection. Results represent means ±SEM of experiments using cells from five different donors.

Table 4.1 8-bromo-cAMP does not inhibit FcγR-mediated phagocytosis by human MDM.

	Phagocytic Index (% control)	
8-Bromo-cAMP (mM)	30 mins pre-incubation (n=5)	48 hrs pre-incubation (n=4)
10^{0}	111.3 ± 19.9	*61.5 ± 15.6
10^{-1}	109.2 ± 10.5	124.3 ± 11.1
10^{-2}	122.2 ± 9.4	116.0 ± 4.5
10^{-3}	125.0 ± 6.9	112.8 ± 10.6
10^{-4}	111.8 ± 10.6	111.5 ± 6.0

Phagocytosis using MDM from five different donors pre-incubated with 8-bromo-cAMP at the indicated comcentrations was performed in triplicate at an MDM to IgG-sRBC ratio of 1:10. Results are expressed as percentage of control (MDM cultured in the absence of cAMP) as mean ± SEM; p values <0.05 for all concentrations tested; *was toxic to the cells as assessed by viable count measurements using Trypan Blue exclusion (viability was <75%; under all other conditions viability was >95%).

CHAPTER 5

nef-DELETED HIV-1 INHIBITS PHAGOCYTOSIS BY MONOCYTE-DERIVED MACROPHAGES *IN VITRO* BUT NOT BY PERIPHERAL BLOOD MONOCYTES *IN VIVO*.

5.1 ABSTRACT.

HIV-1 infection impairs a number of monocyte and macrophage effector functions. The role of HIV-1 Nef in modulating phagocytosis by human monocytes and monocyte-derived macrophages (MDM) was investigated in this study. Using a flow cytometric assay, phagocytosis of *Mycobacterium avium* complex (MAC) by monocytes in peripheral blood of Sydney Blood Bank Cohort (SBBC) members infected with a *nef*-deleted (Δ*nef*) strain of HIV-1 was compared with that of monocytes from uninfected or wild-type (WT) HIV-infected subjects. The specific impact of Nef on phagocytosis by MDM was determined by either infecting cells *in vitro* with Δ*nef* strains of HIV-1 or electroporating Nef into uninfected MDM. MAC phagocytic capacity of monocytes from SBBC members was equivalent to that of cells from uninfected individuals ($p=0.81$); it was greater than that of cells from individuals infected with WT HIV-1 ($p<0.0001$), irrespective of CD4 counts and HIV viral load. In contrast, *in vitro* infection of MDM with either Δ*nef* or WT strains of HIV-1 resulted in similar levels of HIV-1 replication and equivalent impairment of phagocytosis via Fcγ and complement receptors. Electroporation of Nef into MDM did not alter phagocytic capacity. This study provides evidence demonstrating the complex indirect effect of Nef on phagocytosis by peripheral blood monocytes (infrequently infected with HIV-1) *in vivo*. Conversely, the fact that MDM infected with either Δ*nef* or WT HIV-1 *in vitro* (high multiplicity of infection) show comparably impaired phagocytosis, indicates that HIV-1 infection of macrophages can directly impair function, independent of Nef.

5.2 INTRODUCTION.

Cells of macrophage lineage including peripheral blood monocytes and tissue macrophages are important effector cells against a number of intracellular pathogens including *Mycobacterium avium* complex (MAC), *Toxoplasma gondii, Candida albicans* and *Pneumocystis carinii.* These cells provide critical functions in the cell-mediated response to these opportunistic pathogens such as chemotaxis, phagocytosis and intracellular killing. We and others have reported that a number of these functions are impaired following HIV-1 infection (Crowe *et al.*, 1994; Biggs *et al.*, 1995). These defects contribute to the pathogenesis of AIDS by allowing reactivation or infection with otherwise uncommon opportunistic pathogens (reviewed in Crowe, 1995) resulting in significant morbidity and mortality.

The exact mechanism by which HIV-1 impairs monocyte/macrophage function and promotes disease progression remains unclear. However, the importance of Nef (a 25- to 30-kDa myristylated accessory protein) for HIV-1 pathogenesis has been demonstrated in prospective studies of the Sydney Blood Bank Cohort (SBBC), a blood donor and eight transfusion recipients who were all infected with a strain of HIV-1 containing deletions within the *nef* gene and deletions and duplications within the overlapping long terminal repeat overlap (Learmont *et al.*, 1992; Deacon *et al.*, 1995; Learmont *et al.*, 1999). Whilst there is evidence of recent clinical progression in two of the cohort members after 16 years of infection, the virus is clearly attenuated in comparison to WT HIV-1 strains, supporting the role for a functional Nef in HIV-1 pathogenesis in humans (Learmont *et al.*, 1992; Deacon *et al.*, 1995; Learmont *et al.*, 1999). An intact *nef* gene has also been shown to be important for disease progression in simian immunodeficiency virus (SIV)-infected adult rhesus macaques (Kestler *et al.*, 1991) as well as in HIV-infected SCID-hu mice (Jamieson *et al.*, 1994). However, viral load may play an important role, as infant macaques infected with *nef*-deleted SIV at high multiplicity of infection developed disease (Ruprecht *et al.*, 1996).

Nef is thought to contribute to HIV-1 pathogenesis through a variety of mechanisms such as down-regulating CD4 and MHC class I expression (Garcia *et al.*, 1993; Anderson *et al.*, 1994; Rhee and Marsh, 1994; Schwartz *et al.*, 1996; Le Gall *et al.*, 1997), enhancing virion infectivity (Aiken and Trono, 1995; Chowers *et al.*, 1995; Schwartz *et al.*, 1995) and modulating signalling pathways via interactions with host cell proteins (reviewed in Herna Remkema and Saksela, 2000). Since these latter include interactions of Nef with cellular proteins and kinases which are also involved in the process of phagocytosis (such as Src kinases, Hck and Lyn (Saksela *et al.*, 1995; Lee *et al.*, 1996; Greenway *et al.*, 1996; Greenway *et al.*, 1999), p21-activated kinase (Nunn and Marsh, 1996; Fackler *et al.*, 2000), guanine-nucleotide exchange factor Vav (Fackler et al., 1999) and small GTPases Rac1 and Cdc42 (Lu *et al.*, 1996; Crespo *et al.*, 1997)), it is possible that Nef modulation of signalling pathways involving these proteins inhibits phagocytosis. The impact of Nef on phagocytosis by human monocytes and macrophages following HIV-1 infection has not yet been elucidated.

In this study the effects of Nef on phagocytosis of MAC, by comparing monocytes from members of SBBC to monocytes from subjects who are either uninfected or infected with WT HIV-1 were investigated. The effects of HIV-1 infection on phagocytosis by monocytes in whole blood from HIV-infected individuals including SBBC members and patients with WT HIV-1 infection were examined. Since monocytes are infrequently infected with HIV-1, these experiments measure the indirect effect of HIV-1 on monocyte function. To investigate the direct effects of HIV-1 replication within monocyte-derived macrophages (MDM), cells were infected with either *nef*-deleted (Δ*nef*) or WT strains of HIV-1 *in vitro*, at relatively high multiplicity of infection, and phagocytosis of both IgG- and complement (C')-opsonised targets was assessed. To examine the effects of Nef on phagocytosis directly, Nef protein has been electroporated into uninfected MDM. The results of this study demonstrate that the impact of HIV-1 Nef protein on phagocytosis differs markedly in MDM infected *in vitro*

and in monocytes studied *ex vivo* reflecting the potentially different mechanisms underlying inhibition of phagocytosis in these two situations.

5.3 METHODS.

5.3.1 Sources of monocytes.

Blood (2 ml) was collected from six members of the Sydney Blood Bank Cohort in lithium heparin anticoagulant, with their informed consent, and dispensed into polypropylene tubes. Similarly, blood was collected from HIV-1 infected patients and from uninfected controls. Peripheral blood was assessed for plasma HIV-1 RNA by bDNA assay (Chiron Corporation, Emeryville, USA) or RT-PCR (Roche Diagnostics, Nutley, New Jersey, USA) according to manufacturers' instructions by the Clinical Research Laboratory (Macfarlane Burnet Centre for Medical Research, Melbourne).

5.3.2 Whole blood phagocytosis assay.

Fluorescein isothiocyanate (FITC)-labelled MAC or *T. gondii* ($5x10^6$ and $1.5x10^7$) were added to 100µl of blood dispensed in polypropylene tubes and placed into a shaking waterbath at 37^0C for phagocytosis to proceed as described in Section 2.8.1.2. The proportion of monocytes that had ingested MAC-FITC was plotted against the ratio of MAC-FITC: monocyte as calculated from the total monocyte count.

5.3.3 F-actin content of peripheral blood monocytes during phagocytosis.

Blood (100µL) was dispensed in polypropylene tubes and cooled on ice for 20 min. To perform phagocytosis, blood samples were incubated with or without IgG-opsonised latex beads (3µm in diameter, Sigma, St Louis, MO) at a concentration of $5x10^7$

beads/ml, at 37°C. Phagocytosis was terminated at various times (0 to 10 min) by plunging tubes into ice and fixing the cells with 1ml of 3% formaldehyde (20 min, 4°C). Following two washes with cold 0.1M glycine in PBS-Calcium Magnesium free (CMF), monocytes within blood were stained with anti-CD14 Mab conjugated to PE for 30 min at 4°C. After a wash in cold (4°C) PBS-CMF, erythrocytes were lysed with FACS lysing solution at 4°C. The remaining white blood cells were permeabilised with 0.1% Triton-X 100 (Merck, Kilsyth, Australia) for 1 min, washed twice with 1% FCS/PBS-CMF and stained for F-actin levels with phalloidin-Alexa 488 (Molecular Probes, Eugene, OR) for 30 min at 4°C. Cells were washed with cold PBS-CMF, fixed with 1% formaldehyde and analysed by flow cytometry.

5.3.4 HIV-1 infection of MDM in vitro.

A subproviral Δnef construct, obtained from NIH AIDS Reagent Program (contributed by Ronald Desrosiers), was used to generate the full length NL4.3Δnef proviral DNA. The DNA constructs pNL(AD8) and pNL(AD8Δnef) were prepared by substituting the respective envelope coding DNA sequences from NL4.3 and NL4.3Δnef with monocytotropic AD8 envelope coding sequences, converting a T-tropic virus to M-tropic. A laboratory adapted M-tropic strain (HIV-$1_{Ba\text{-}L}$) of HIV-1 was also used for MDM infections as described in Section 2.3.1. The *nef*-deleted primary isolates from D36 and C18 (two of the SBBC members from which virus could be successfully isolated) were prepared by co-culturing PBMC from D36 or C18 with HIV-1 seronegative CD8-depleted PBMC (Section 2.3.3). Purified MDM from HIV-1 seronegative buffy coats were infected with HIV-$1_{NL(AD8)}$ chimera, HIV-$1_{NL(AD8\Delta nef)}$ chimera, HIV-$1_{Ba\text{-}L}$, and primary isolates HIV-1_{D36} or HIV-1_{C18} from SBBC members at the same multiplicity of infection. Control cells were mock-infected and cultured under identical conditions.

5.3.5 *Phagocytosis by MDM infected with HIV-1 in vitro.*

The phagocytic capacity of MDM for MAC-FITC was assessed 7 days after HIV-1 infection by flow cytometric analysis (Section 2.8.1.3). Phagocytosis of specific IgG-opsonised targets and complement (C')-opsonised targets was assessed by colorimetric assay (Section 2.8.2.2) on day 7 to 10 following HIV-1 infection. Sheep erythrocytes (E) used as targets were prepared immediately prior to the phagocytosis assay. IgG-E were opsonised with a rabbit anti-sheep red blood cell antibody (Section 2.8.2.1). C'-opsonised E were prepared by opsonisation of $2x10^8$ cells with pre-warmed 2% AB-negative human serum as a source of human complement components for 30 min at room temperature (Chan *et al.*, 2001). As controls E were opsonised AB-negative serum from the same source that has been heat-inactivated at 56^{0}C for 45 min (HI-E). Prior to the phagocytosis assay, MDM adhered to 96-well plates were activated by treatment with phorbol-12-myristate-13-acetate (PMA) at a final concentration of 200nM per well for 10 min at 37^{0}C. Subsequently those cells were exposed to C'-E or HI-E at E:MDM ratio of 20:1. C'R-mediated phagocytosis was performed at 37^{0}C for 60 min and assessed by colorimetric assay.

5.3.6 Phagocytosis by Nef-electroporated MDM.

To assess the direct effect of Nef on phagocytosis *in vitro*, purified recombinant Nef protein (Greenway *et al.*, 1994) was introduced by electroporation into MDM on day 5 post-isolation. Cells that were electroporated with purified recombinant GST or mock-electroporated (no protein) were used as controls. Proteins (300nM per $1x10^6$ cells) were introduced into MDM using a square wave electroporator (Bio-Rad, Richmond, CA; amplitude, 5kV; pulse frequency, 2^6; burst time, 0.8 sec; cycle number, 10). Electroporation of Nef into MDM was confirmed by immunofluorescence staining using specific anti-Nef Mab (AE6; AIDS Research and Reference Reagent Program, NIAID, NIH: HIV-1 Nef monoclonal antibody from Dr James Hoxie) immediately after

electroporation and at later times as previously described (Greenway *et al.*, 1994). Electroporated MDM were returned to cultures and allowed to recover for 24 hr at 37°C in a humidified incubator and subsequently used for phagocytosis assays. The impact of Nef on macrophage function was investigated by assessing its role on MAC-FITC phagocytosis (Section 2.8.1.3) as well as on the level of tyrosine phosphorylation during FcγR-mediated phagocytosis. The latter was performed by incubating MDM with IgG-opsonised latex beads at a ratio of 1:10 respectively at 37°C. At the indicated time points phagocytosis was arrested by plunging the tubes into ice and washing MDM in ice-cold PBS-CMF, followed by lysis in Triton-X 100 lysis buffer (Section 2.8.3.3) and immunoblot analysis (Section 2.9).

5.3.7 Statistical analyses.

Multiple regression was used to model the association between the percentage of phagocytosis and MAC-FITC:monocyte ratio, adjusting for HIV-1 infection status (HIV-1 positive, HIV-1 negative, and *nef*-deleted SSBC HIV-1 positive). Robust standard errors were used to account for repeat tests on individuals. To account for the highly skewed data, the MAC-FITC:monocyte ratio was transformed using natural logarithm. Interactions between MAC-FITC:monocyte ratio and HIV infection status were investigated by fitting interaction terms in the model. The analyses were performed using the Stata statistical software analysis package (Stata, Collage Station, TX).

The significance of F-actin contents of monocytes from HIV-positive and -negative individuals, *in vitro* infections with WT HIV-1(HIV-$1_{Ba\text{-}L}$ or HIV-$1_{NL(AD8)}$) and *nef*-deleted strains of HIV-1 (primary isolates HIV-1_{D36} and HIV-1_{C18} or HIV-$1_{NL(AD8)\Delta nef}$ chimera) as well as the impact of Nef electroporation on phagocytosis was assessed using the Student's t test (paired, 2-tailed).

5.4 RESULTS.

5.4.1 Subjects.

Blood was collected from a total of 6 Sydney Blood Bank Cohort members (median CD4: 805 cells/μl; range: 306 to 2331 cells/μl; median plasma HIV RNA <400 copies/ml; range <400 to 4000 copies/ml), 16 persons with WT HIV-1 infection (median CD4: 138 cells/μl; range: 20 to 713 cells/μl for 11 subjects tested at the time of assay; median plasma HIV RNA 900 copies/ml; range <500 to 385 500 copies/ml for 13 subjects tested) and 3 uninfected persons. Blood samples from the same HIV-uninfected donors were used as a source of control monocytes in every experiment (Table 5.1).

5.4.2 Phagocytosis by monocytes in whole blood from individuals infected with WT and nef-deleted HIV-1.

As SBBC members have been infected with an attenuated strain of HIV-1 for periods in excess of 16 years prior to the onset of any HIV-related symptoms or infections (Learmont *et al.*, 1999), it was of interest to determine whether they had unimpaired phagocytic function, consistent with slow or absent disease progression. Monocytes present in whole blood from members of SBBC phagocytosed MAC-FITC (ingested predominantly via C'R (Schlesinger and Horwitz, 1991)) with an efficiency similar to that of monocytes from HIV-1 uninfected individuals ($p=0.808$; n=16 tests in total), but significantly better than monocytes from individuals infected with WT HIV-1 ($p<0.0001$) (Table 5.1, Figure 5.1). In addition, monocytes from members of the SBBC appeared to phagocytose *Toxoplasma gondii*-FITC (mediated predominantly via FcγRII (Joiner *et al.*, 1990)) substantially better than monocytes from WT HIV-infected individuals (n=4; data not shown). These data suggest that phagocytosis by monocytes from WT HIV-

infected individuals is impaired, while those from subjects infected with the attenuated strain if HIV-1 containing deletions in *nef* gene are not. No correlation was found between the level of inhibition of phagocytosis by WT HIV-1 and either viral load (R=-0.27; p=0.38) or CD4 counts (R=0.35; p=0.29) (Data from Table 5.1).

5.4.3 Mechanism underlying inhibition of phagocytosis.

The surface expression of the complement receptor (CD11c) and Fcγ receptors (CD16, CD32) was not modulated in monocytes from individuals infected with WT or attenuated HIV-1 when compared to uninfected subjects (data not shown), suggesting another mechanism accounts for the inhibition of phagocytosis in individuals infected with WT HIV-1. As reorganisation of the actin-based cytoskeleton is essential for the formation of the phagocytic cup and the engulfment of phagocytosed particles, the mechanism of defective phagocytosis in HIV-infected individuals was further investigated at the actin polymerisation level. A flow cytometric assay was developed to measure the level of polymerised actin (filamentous actin; F-actin) during FcγR-mediated phagocytosis in blood monocytes (Kedzierska *et al*, unpublished). Monocytes in blood of HIV-infected individuals showed significantly increased basal levels of polymerised actin in comparison to F-actin levels in monocytes from uninfected controls (Figure 5.2a; $p<0.05$; n=5). During phagocytosis there was a significant net increase of F-actin from basal levels in monocytes from uninfected controls between 2 and 10 min of phagocytosis, but not in monocytes from HIV-infected individuals (Figure 5.2b; n=3). These data suggest defective actin rearrangement (and thus abnormal phagocytic cup formation) in blood monocytes from HIV-infected subjects. F-actin content in peripheral blood monocytes from SBBC members remains to be elucidated.

5.4.4 *Phagocytosis by purified MDM infected with HIV-1 in vitro.*

As phagocytosis by monocytes from SBBC members was not impaired, the direct effect of Nef on phagocytosis was assessed, initially by using Δ*nef* strains to infect MDM *in vitro* in comparison with WT HIV-1. *In vitro* infection of MDM by Δ*nef* strains HIV-1_{D36} (a primary isolate from SBBC member D36) or the HIV-$1_{NL(AD8)\Delta nef}$ chimera impaired phagocytosis of MAC-FITC to the same degree as infection with WT HIV-1 (Ba-L or NL(AD8)). All infected MDM cultures showed impaired phagocytosis compared with uninfected MDM ($p < 0.001$; Figure 5.3).

MDM infected with either WT HIV-1 (Ba-L or NL(AD8)), Δ*nef* HIV-1_{C18} (a primary isolate from SBBC cohort member C18) or with HIV-$1_{NL(AD8)\Delta nef}$ chimera had impaired phagocytosis mediated via either Fcγ or C' receptors using specifically opsonised targets (IgG- and C'-opsonised E) as compared to uninfected MDM ($p < 0.001$; Figure 5.4). Infection of MDM with *nef*-deleted strains of HIV-1 resulted in similar levels of inhibition of phagocytosis as MDM infected with WT HIV-1 ($p=0.08$ and $p=0.1$, for FcγR- and C'R-mediated phagocytosis respectively). The presence of a *nef*-deletion had no significant effect on HIV-1 replication *in vitro* as measured by RT activity in the culture supernatant (mean RT values of 2045 cpm and 1250 cpm for HIV-$1_{NL(AD8)}$ and HIV-$1_{NL(AD8)\Delta nef}$ chimera respectively; $n=5$; $p=0.48$) or by flow cytometric analysis of intracellular p24 antigen (mean of 59% and 74% p24-positive MDM for HIV-$1_{NL(AD8)}$ and HIV-$1_{NL(AD8)\Delta nef}$ chimera respectively; $n=2$).

5.4.5 Phagocytosis by MDM electroporated with Nef protein.

To address directly the effects of Nef on phagocytosis by MDM, cells were electroporated with Nef. Electroporation of Nef protein into MDM did not affect phagocytosis of MAC-FITC compared to mock- or GST-electroporated controls ($n=4$;

p=0.16 and 0.54 respectively) (Figure 5.5a). Since Nef interacts with cellular proteins and kinases, which are also phosphorylated during FcγR-mediated phagocytosis (eg Hck), we assessed the impact of Nef on the level of tyrosine phosphorylation during phagocytosis of IgG-opsonised targets. Stimulation of mock-electroporated MDM with IgG-opsonised beads triggered an increase in tyrosine phosphorylation of a wide range of cellular proteins after 2 min of phagocytosis. Nef- and GST-electroporated MDM displayed similar levels and patterns of phosphorylation during FcγR-mediated phagocytosis to that induced by mock electroporation (Figure 5.5b). These data suggest that HIV-1 Nef does not inhibit phagocytosis via FcγR or C'R in MDM infected *in vitro*.

5.5 DISCUSSION.

The data presented in this Chapter show that phagocytosis of MAC by blood monocytes from individuals infected with the attenuated strains of HIV-1 containing deletions in the *nef* gene was normal, whilst in agreement with data shown in Chapter 6, phagocytosis by blood monocytes from WT HIV-1 infected subjects was significantly impaired, compared to monocytes from uninfected controls. However, when MDM from HIV-1 seronegative individuals were infected *in vitro* at the same multiplicity of infection with WT or Δ*nef* HIV-1 strains, the phagocytic capacity of these MDM were equally reduced. These results suggest that if HIV-1 Nef protein has an effect on monocyte and macrophage phagocytic function, it is mediated through a complex indirect effect only evident *in vivo*.

A number of investigators have reported impairment of phagocytosis following WT HIV-1 infection, by monocytes and alveolar macrophages *in vivo* as well as by macrophages infected with WT HIV-1 *in vitro* (Musher *et al.*, 1990; Bravo-Cuellar et al., 1992; Capsoni *et al.*, 1992; Roilides *et al.*, 1993; Wehle *et al.*, 1993; Capsoni *et al.*,

1994; Dobmeyer *et al.*, 1995; Baqui *et al.*, 1999). However, others have shown normal monocyte and macrophage function following HIV-1 infection (Nottet *et al.*, 1993; Eversole *et al.*, 1994). These discordant results may be at least partially explained by the differing methods used by investigators, in particular whether cells have been isolated from HIV-infected individuals (where the proportion of infected cells is very low, in the range of 0.001% to 1%) or from seronegative donors whose purified MDM were then infected *in vitro* (with infection rates often in the range of 30 to 70%). As previously demonstrated by our group, monocytes and macrophages are distinct cell populations that differ in their susceptibility to HIV-1 infection (monocytes being highly refractory, whereas macrophages are fully permissive to HIV-1) (Sonza *et al.*, 1996), the expression level of surface receptors (including CD4, CCR5 and phagocytic receptors) (Sonza *et al.*, 1995; Kedzierska *et al.*, 2000a) as well as in their cytokine/chemokine production profile (reviewed in Fantuzzi *et al.*, 2000). This study, however, demonstrates defective phagocytosis by either monocytes or macrophages following HIV-1 infection *in vivo* and *in vitro.*

Phagocytosis of opportunistic pathogens such as MAC (utilising predominantly C'R for phagocytosis; (Schlesinger and Horwitz, 1991)) and *Toxoplasma gondii* (utilising FcγRII; (Joiner *et al.*, 1990)) was impaired by peripheral blood monocytes from WT HIV-infected individuals but not from SBBC members infected with a Δ*nef* strain of HIV-1. Impaired phagocytosis following HIV-1 infection is likely to directly contribute to HIV pathogenesis, by allowing reactivation of opportunistic pathogens normally controlled by macrophages. Data demonstrating normal phagocytic efficiency of monocytes in the blood of SBBC members is therefore consistent with their long term non/slow progression and the absence of opportunistic infections (Learmont *et al.*, 1999). Since individuals infected with *nef*-deleted HIV-1 displayed normal phagocytic efficiency, it might be considered that Nef might be responsible for the observed impairment of phagocytosis following HIV-1 infection. However, as the attenuated virus from this

group also has deletions and rearrangements within the LTR (Deacon *et al.*, 1995), a tight correlation between phagocytic efficiency and *nef*-deletion is not possible.

Since only a small proportion of blood monocytes (<1%) is infected with HIV-1, impaired phagocytosis may be predominantly an indirect consequence of HIV-1 infection and might reflect dysregulation of cytokine/chemokine production by monocytes and/or other cells present in blood. In support of this proposal, defective phagocytosis by neutrophils (not targets for HIV-1 infection) from HIV-positive individuals has also been reported (Dobmeyer *et al.*, 1995). Nef may alter phagocytosis through its influence on the cellular environment and subsequently on cytokine and chemokine production. Nef induces the production of two CC-chemokines, MIP1-α and MIP1-β by macrophages (Swingler *et al.*, 1999), induces synthesis of IL-15 by MDM (Quaranta *et al.*, 1999), IL-10 (Brigino *et al.*, 1997), TNFα, IL-6 and IL-1β (Greenway, unpublished) by PBMC or alters IL-2 production by T lymphocytes (Collette *et al.*, 1996a; Schibeci *et al.*, 2000). Since monocyte activation and their response to phagocytic stimuli are dependent on cytokine concentration (including IL-2 (Ennen *et al.*, 1989)) Nef may impair phagocytosis via dysregulated cytokine/chemokine production.

The *nef* gene of primate lentiviruses is necessary for high level virus replication *in vivo* (Kestler *et al.*, 1991). It has been also demonstrated by the work of Ruprecht *et al* that SIV with *nef* deletion is generally attenuated in adult macaques, but causes disease in infant macaques, likely to result from a "threshold" for pathogenicity relating to levels of viral replication and viral load (Ruprecht *et al.*, 1996; Montefiori *et al.*, 1996). Although it is possible that Nef may influence phagocytic capacity of monocytes *in vivo* by affecting viral load and CD4 counts, data presented in this thesis suggest that the reduction of phagocytosis was not due to viral load or CD4 counts. SBBC members who contributed to this study had low viral load and normal CD4 counts at the time of phagocytosis

measurements, whereas individuals infected with WT HIV-1 had a wide range of viral loads and CD4 counts. However, this study found no correlation between the observed inhibition of phagocytosis and either viral load (undetectable or low in approximately 50% of subjects) or CD4 counts in individuals infected with WT HIV-1, suggesting that the difference in phagocytosis between *nef*-deleted and WT HIV-infected individuals is not due to either of those factors. Those results are in the agreement with studies showing decreased phagocytosis in patients with both undetectable (<400 copies/ml) and high viral loads (>10,000 copies/ml) (Baqui *et al.*, 1999) as well as over a wide range of CD4 counts (Roilides *et al.*, 1993).

The mechanism of defective phagocytosis by peripheral blood monocytes from WT HIV-infected individuals remains unclear. Previous reports (Capsoni *et al.*, 1992; Capsoni *et al.*, 1994; Dunne *et al.*, 1996), confirmed by observations in this study, suggest that HIV-1 infection *in vivo* results in either elevated or unchanged expression of complement and Fcγ receptors on monocytes, indicating that inhibition of phagocytosis occurs at a post-receptor level. Recently, Elbim *et al* (Elbim *et al.*, 1999) showed increased basal levels of F-actin in monocytes from HIV-infected individuals, at different stages of disease, when compared to uninfected subjects. These findings have been confirmed and extended in this thesis. Data presented in this Chapter demonstrated that monocytes from HIV-infected individuals display either minimal or no elevation of F-actin above their basal level during phagocytosis. This is in contrast to significant net increases from the basal F-actin level in uninfected controls. As actin polymerisation plays a critical role in the formation of phagocytic cup and ingestion of phagocytosed particles, defective actin rearrangement is a potential mechanism underlying impaired phagocytic function in HIV-infected individuals. F-actin content in monocytes from individuals infected with *nef*-deleted HIV-1 remains to be elucidated.

There are other potential mechanisms by which Nef could directly influence phagocytosis. Nef associates with several cellular kinases, including Src family tyrosine kinases, Lck (resulting in impairment of Lck-mediated signalling events) (Collette *et al.*, 1996b) and Hck (resulting in its activation) (Greenway *et al.*, 1999). Since the same signalling pathways are involved in FcγR-mediated phagocytosis by MDM, (reviewed in Greenberg, 1995), Nef may influence phagocytosis by binding to Hck or other relevant kinases or cytoskeletal proteins. To assess this hypothesis the direct effects of infection and replication of HIV-1 on macrophage function as well as the effect of Nef in the absence of other HIV-1 proteins were investigated. Phagocytosis of MAC was defective in MDM infected *in vitro* with either WT or *nef*-deleted strain of HIV-1. Experiments to specifically address both FcγR- and C'R-mediated phagocytosis (occurring via different signalling mechanisms (Allen and Aderem, 1996)) by HIV-infected MDM showed similar levels of impairment. In results presented in this thesis, deletions within *nef* did not significantly alter the replicative capacity of HIV-1 in MDM (in agreement with previous reports (Hattori *et al.*, 1990; Meylan *et al.*, 1998), but in contrast to other studies (Miller *et al.*, 1994)). Therefore, results presented in this Chapter suggest that decreased phagocytosis *in vitro* is predominantly a direct consequence of HIV-1 replication in MDM, and that Nef does not contribute to this impairment of MDM function. Unaltered levels and pattern of tyrosine phosphorylation of key proteins during FcγR-mediated phagocytosis by Nef-electroporated MDM confirmed that Nef on its own is not sufficient to inhibit the process of phagocytosis.

In conclusion, there is no impairment in phagocytosis by monocytes in whole blood from individuals infected with Δ*nef* strains of HIV-1 in contrast to subjects infected with WT HIV-1, possibly reflecting an indirect effect of infection with HIV-1 resulting in altered actin polymerisation in these cells. However, *in vitro* infection of MDM with *nef*-deleted strains and WT HIV-1 at relatively high multiplicity of infection both equally impair phagocytosis, indicating a direct effect of HIV-1 infection and replication on

macrophage function, and suggesting that Nef does not directly impact on macrophage phagocytic activity.

5.6 RELEVANT PUBLICATIONS.

The following publications have arisen from the work presented in this chapter:

1. **Kedzierska, K.,** Mak, J., Jaworowski, A., Greenway, A., Violo A, Chan, H., Hocking, J., Purcell, D., Sullivan, J., Mills, J. and Crowe, S.M. (2001) *nef*-deleted HIV-1 inhibits phagocytosis by monocyte-derived macrophages *in vitro* but not by peripheral blood monocytes *in vivo*. *AIDS*, **15**:945-955.

I have performed the majority of the experiments presented in this manuscript, with some technical assistance from Antoniette Violo who did the pilot experiments for the SBBC/MAC phagocytosis part of the study. I have analysed results and written the manuscript with the intellectual input from Prof. Suzanne Crowe, Dr. Anthony Jaworowski and Prof. John Mills. Dr. Johnson Mak has supervised Antoniette Violo. Dr. Alison Greenway has electroporated Nef into macrophages used for some experiments. Hiu-Tat Chan performed the complement-mediated phagocytosis assays under my co-supervision, and Jane Hocking performed the statistical analysis presented in Table 5.1. Dr. Damien Purcell provided the *nef*-deleted constructs used in this study, and Dr. John Sullivan organised blood samples from SBBC members and provided the CD4 count and viral load details for each of the donors.

2. Chan, H., **Kedzierska, K.,** O'Mullane, J., Crowe, S.M. and Jaworowski, A. (2001) Quantifying complement-mediated phagocytosis by human monocyte-derived macrophages. *Immunol Cell Biol.*79:429-435.

During my doctoral studies I co-supervised Hiu-Tat Chan, a B Med Sci student, who worked on a parallel project to investigate defective complement-mediated phagocytosis by HIV-infected MDM. I performed experiments presented in Figure 4d and Table 2. I have contributed to the analysis of the results and writing and editing of the manuscript.

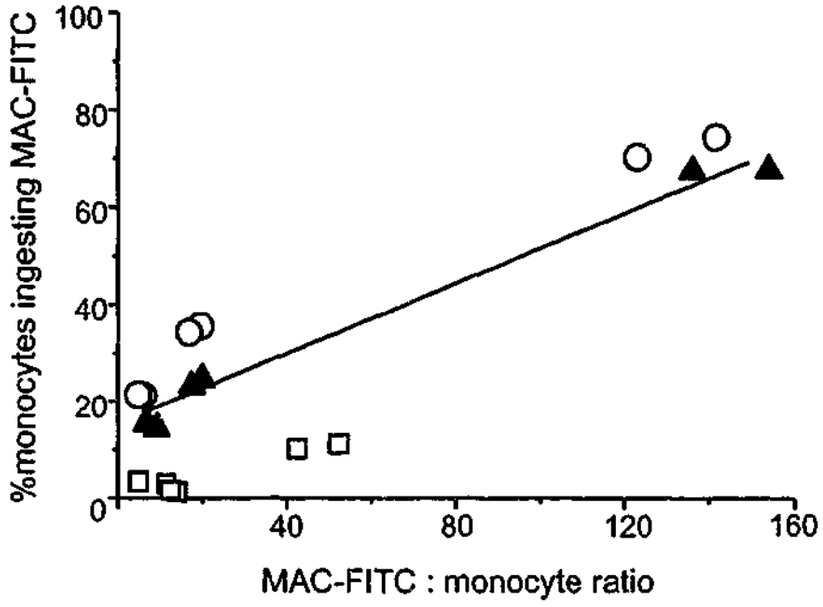

Figure 5.1 Quantitation of MAC-FITC phagocytosis by peripheral blood monocytes assayed *ex vivo*.

A representative phagocytic analysis for one SBBC member, C54 (○), WT HIV-infected individual (□) and HIV-seronegative controls (▲). Phagocytosis of MAC-FITC by monocytes obtained from C54 and WT HIV-infected individuals was compared with regression line fitted to the data points obtained from HIV-uninfected controls. Results are corrected for background fluorescence.

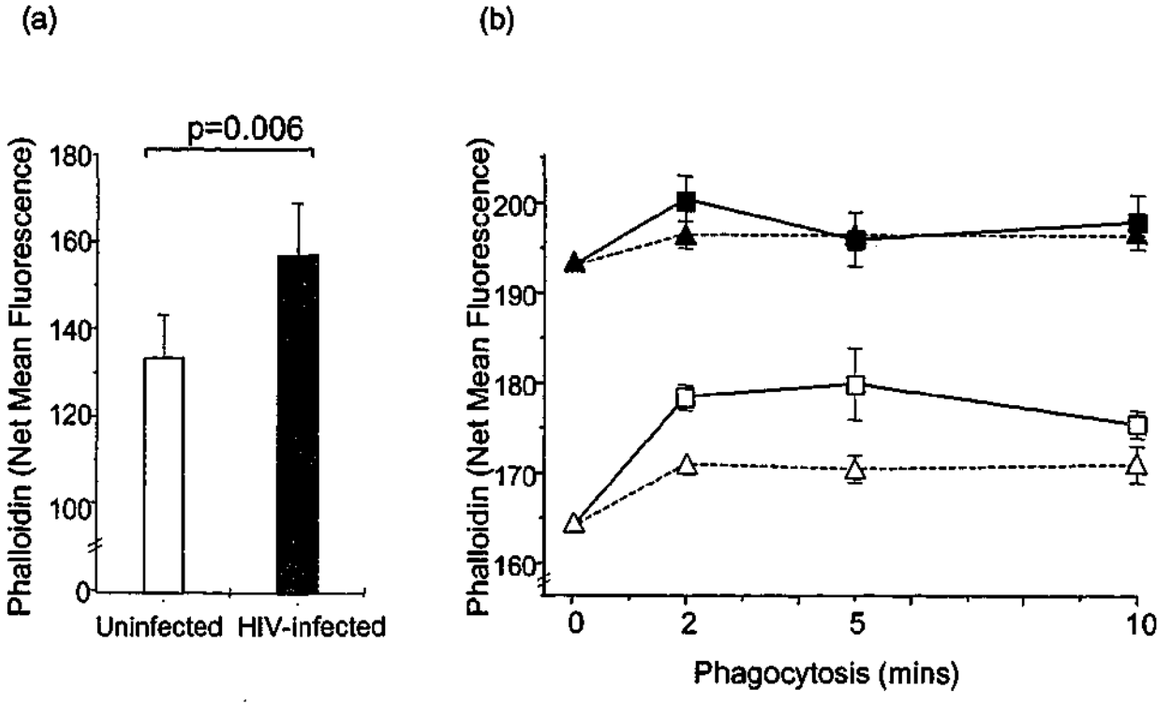

Figure 5.2 F-actin content in peripheral blood monocytes during phagocytosis.

Blood samples from HIV-positive individuals (solid symbols) or uninfected controls (open symbols) were stained for F-actin levels. Cells were (a) unstimulated; or (b) incubated with IgG-opsonised latex beads (squares, solid lines) or without beads (triangles, dotted lines) at 37°C for indicated times. Following fixation with 3% formaldehyde, monocytes were stained with CD14-PE Mab. Cells were permeabilised with 0.1% Triton-X, stained for F-actin levels with phalloidin-Alexa488 as described in Methods and analysed by flow cytometry. Data shown represent (a) means±SEM from 5 different experiments (b) representative of 3 experiments using blood samples from HIV-negative (n=4) and HIV-positive (n=3) donors.

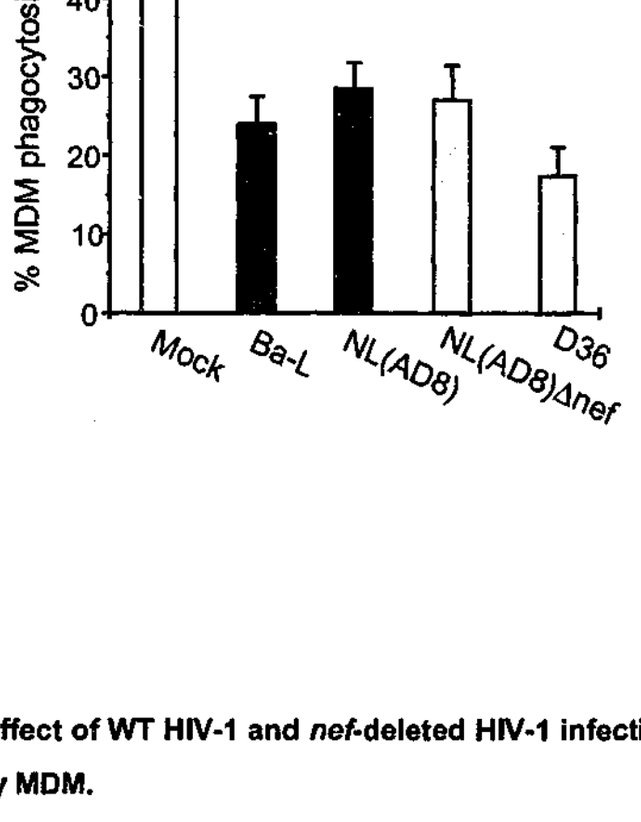

Figure 5.3 Effect of WT HIV-1 and *nef*-deleted HIV-1 infection on phagocytosis of MAC-FITC by MDM.

Phagocytosis of MAC-FITC by MDM infected *in vitro* with mock (open bar), WT HIV-1 (Ba-L or NL(AD8); black bars) or Δ*nef* HIV-1 (NL(AD8)Δnef chimera or primary isolate from SBBC member D36; grey bars) was assessed by a flow cytometric assay on day 7 to 10 post-infection. The proportion of MDM phagocytosing MAC labelled with FITC (mean±SEM) is shown using MDM to MAC-FITC ratio of 1:75. These data are the means compiled from results from 10 different MDM donors.

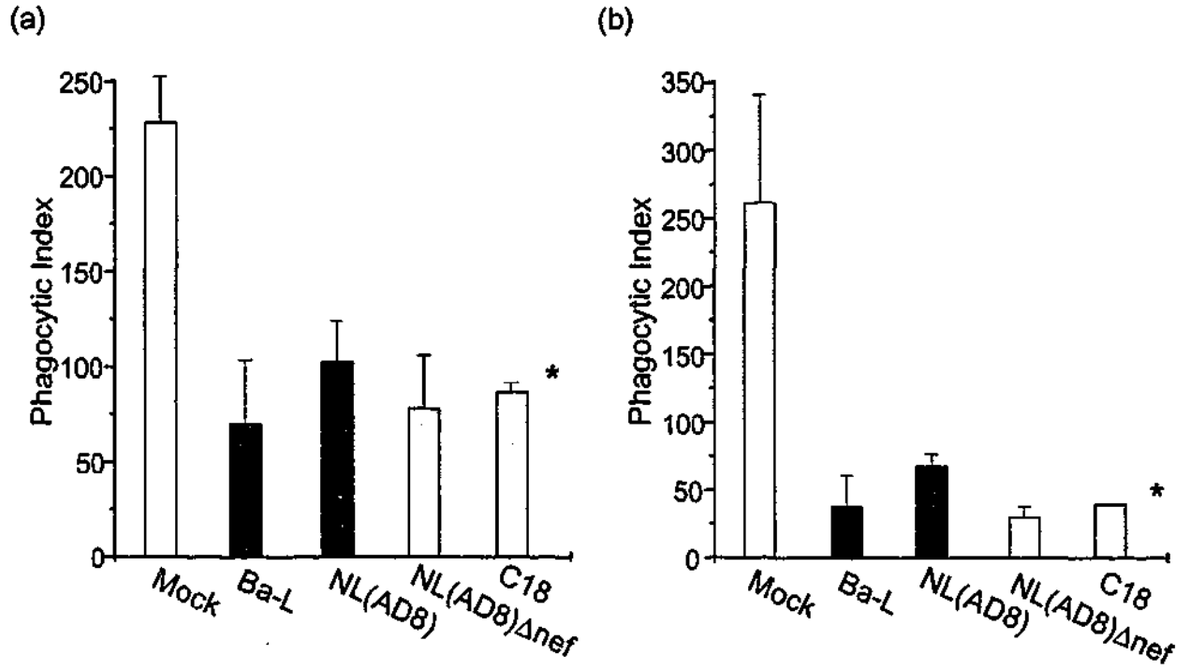

Figure 5.4 Effect of *nef*-deleted HIV-1 on FcγR- and C'R-mediated phagocytosis.

Phagocytosis of specific targets (a) Fc-opsonised and (b) C'-opsonised sheep erythrocytes (E) was assessed by colorimetric assay. MDM on day 5 post-isolation were infected with either WT HIV-$1_{Ba\text{-}L}$ and HIV-$1_{NL(AD8)}$ (black bars) or Δ*nef* strains (HIV-$1_{NL(AD8)\Delta nef}$ or HIV-1_{C18}, *nef*-deleted primary isolate; grey bars). Phagocytosis was performed on day 7 to 10 post-infection. These data represent means±SEM from (a) 5 different MDM donors; (b) 3 different donors. *Shown results from a single experiment.

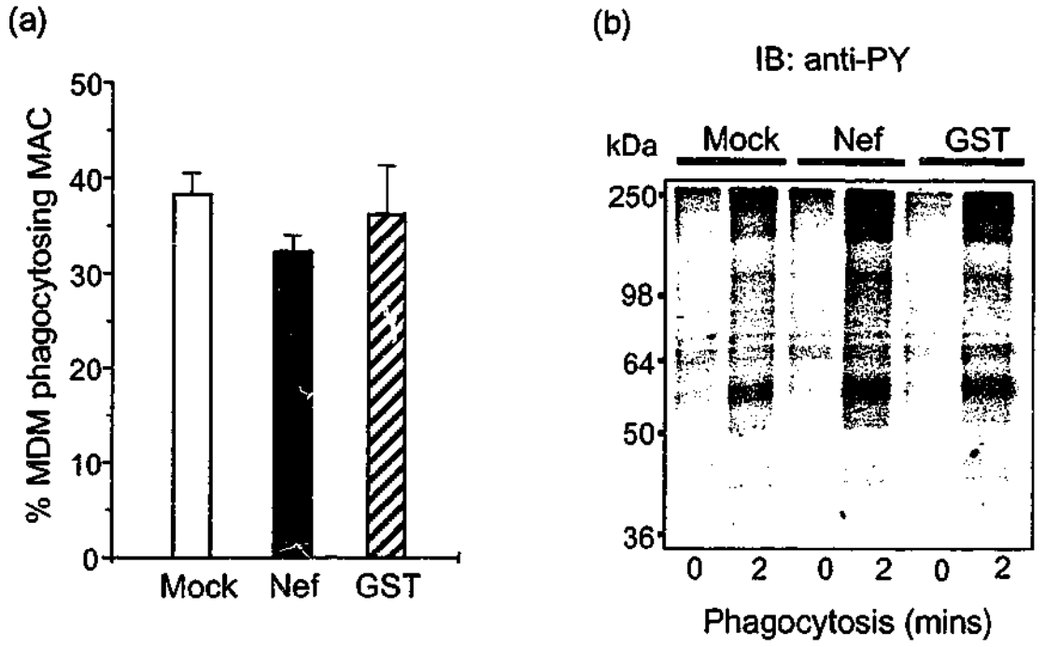

Figure 5.5 Impact of Nef electroporation on phagocytosis of (a) MAC-FITC; (b) IgG-opsonised beads.

(a) Phagocytosis of MAC-FITC by MDM that were either electroporated with mock (open bar), Nef (black bar) or GST (hatched bar) were compared. Shown are mean±SEM of 4 experiments. Student t-test was performed to measure the significance of these results. (b) MDM ($2x10^6$) were incubated with IgG-opsonised latex beads ($2x10^7$) for the indicated times, lysed in Triton-X 100 buffer and samples of lysate containing equal amount of protein (50μg) were resolved by SDS-PAGE, then probed with anti-phosphotyrosine conjugated to horseradish peroxidase (RC20). Data shown are representative of 2 experiments using MDM prepared from different donors. IB: immunoblotting; PY: phosphotyrosine.

Table 5.1 Phagocytosis of *Mycobacterium avium* complex by peripheral blood monocytes in *nef*-deleted SBBC and WT HIV-infected subjects *ex vivo*.

		nef-deleted SBBC			WT HIV-infected controls		
Subject	Expt	Phagocytosis (%Control)	Viral load* (copies/ml)	CD4 count (cells/μl)	Phagocytosis (%Control)	Viral load# (copies/ml)	CD4 count (cells/μl)
D36	(i)	124	3100	361	40	<500	713
	(ii)	101	2600	414	67	<500	50
	(iii)	80	4000	540	32	<500	100
C49	(i)	141	<400	918	23	N/T	N/T
C54	(i)	123	1500	1260	20	<500	20
	(ii)	116	1700	1419	35	<500	363
	(iii)	96	1800	1120	65	600	N/T
	(iv)	96	1600	1152	92	N/T	N/T
C64	(i)	137	<400	805	32	385 500	N/T
	(ii)	123	<400	805	83	N/T	N/T
	(iii)	101	<400	1026	22	95 600	40
	(iv)	100	<400	2331	44	119 800	120
C98	(i)	110	<400	612	57	10 700	400
	(ii)	115	<400	306	13	62 400	247
C135	(i)	105	<400	392	55	26 900	138
	(ii)	220	<400	495	130	900	480
p value		0.808			<0.0001		

The phagocytosis of MAC-FITC by monocytes of SBBC and WT HIV-seropositive subjects was compared to that of uninfected controls and is reported as a percentage of the phagocytosis by seronegative controls for the same MAC:monocyte ratio (% control). The values obtained at each MAC:monocyte ratio for the seronegative controls were standardised as 100% of phagocytic efficiency. Each phagocytosis experiment was performed in duplicate. Viral load was assessed by *RT-PCR, #bDNA assay; N/T: not tested at time of the assay.

CHAPTER 6

GM-CSF INHIBITS HIV-1 REPLICATION AND RESTORES PHAGOCYTIC CAPACITY OF MACROPHAGES AND MONOCYTES FOLLOWING HIV-1 INFECTION.

6.1 ABSTRACT.

Disseminated *Mycobacterium avium* complex (MAC) infection is common in patients with advanced HIV-1 infection. In this study the effect of HIV-1 infection on the ability of monocytes and MDM to phagocytose MAC *in vivo* and *in vitro,* and the effect of GM-CSF on this function were investigated. Using a flow cytometric assay to quantify phagocytosis, HIV-1 infection was found to impair the ability of cultured MDM to phagocytose MAC *in vitro,* whereas GM-CSF markedly augmented this function. Phagocytosis of MAC was not altered by exposure to a mutant form of GM-CSF (E21R) binding only to the alpha chain of the GM-CSF receptor, suggesting that augmentation of phagocytosis by GM-CSF requires binding to the αβ heterodimeric form of the receptor. GM-CSF also consistently suppressed HIV-1 replication in MDM, as assessed by measurement of supernatant RT activity and intracellular p24 antigen. This effect was reversed by the addition of the neutralising anti-GM-CSF monoclonal antibody (4D4). Analysis of intracellular HIV-1 DNA and mRNA suggests that HIV-1 replication is inhibited at or before transcription. A clinical study confirmed the beneficial effect of GM-CSF in a patient with AIDS and disseminated multi-drug resistant MAC infection. GM-CSF therapy improved phagocytosis of MAC by peripheral blood monocytes obtained from this patient, and reduced bacteraemia. These results imply that GM-CSF therapy may be useful in restoring antimycobacterial function by human monocytes and macrophages in the setting of HIV-1 infection.

6.2 INTRODUCTION.

Infection of monocytes and macrophages with HIV-1 has been reported to impair a number of effector functions carried out by these cells, resulting in defective phagocytosis of *Candida albicans* (Crowe *et al.*, 1994), *Aspergillus fumigatus* and *Toxoplasma gondii* (Biggs *et al.*, 1995), impaired intracellular killing of *Candida pseudotropicalis*, *Toxoplasma gondii* (Biggs *et al.*, 1995) and *Mycobacterium avium* complex (MAC) (Crowle *et al.*, 1992; Kallenius *et al.*, 1992; Newman *et al.*, 1993; Ghassemi *et al.*, 1995). These alterations in macrophage function contribute to the pathogenesis of AIDS by allowing the reactivation of opportunistic pathogens (reviewed in Crowe, 1995).

M. avium is a facultative intracellular pathogen that replicates within circulating monocytes and tissue macrophages. MAC can be found in many organs and tissues including lymph nodes, bone marrow, spleen, liver and rectal tissue (Yakrus and Good, 1990), and is associated with high tissue burden (10^9 to 10^{10} mycobacteria/g tissue) in AIDS patients (Shiratsuchi *et al.*, 1994). Disseminated MAC is one of the most common opportunistic infections, diagnosed in up to 40% of patients with advanced HIV-1 infection, and causing significant morbidity and mortality in those individuals (reviewed in Hoy, 1996). Although combinations of antimycobacterial drugs are usually effective in suppressing symptoms, drug resistance leading to persistent clinical symptoms is increasingly being recognised.

GM-CSF, a cytokine regulating proliferation, differentiation and function of granulocytes and macrophages, has been previously shown to augment effector functions of macrophages, increasing their antifungal, antiparasitic, antimycobacterial and antitumor activity (Smith *et al.*, 1990; Eischen *et al.*, 1991; Roilides and Pizzo, 1992; Bermudez *et al.*, 1994; Laursen *et al.*, 1994; Bober *et al.*, 1995). GM-CSF has been also shown to upregulate phagocytosis and intracellular killing of MAC by murine and human MDM

and human alveolar macrophages *in vitro* (Bermudez and Young, 1990; Bermudez *et al.*, 1994; Suzuki *et al.*, 1994; Hsu *et al.*, 1995). A recent report has demonstrated that GM-CSF therapy can also enhance mycobactericidal activity in monocytes obtained from AIDS patients with MAC bacteraemia (Kemper *et al.*, 1998). The effect of GM-CSF on phagocytosis of MAC by HIV-infected monocytes/MDM has not been reported.

There are conflicting reports regarding the effect of GM-CSF on HIV-1 replication in cells of macrophage lineage *in vitro*. Early studies investigating the effect of GM-CSF on HIV-1 replication reported that this growth factor exerts an upregulatory effect on viral production in both MDM (Koyanagi *et al.*, 1988; Perno *et al.*, 1989; Schuitemaker *et al.*, 1990; Perno *et al.*, 1992; Wang *et al.*, 1998) and in the chronically infected promonocytic lines U937 and U1 (Folks *et al.*, 1987; Pomerantz *et al.*, 1990). However, other investigators have reported inconsistent (Kornbluth *et al.*, 1989; Hammer *et al.*, 1990) or inhibitory effects of GM-CSF on HIV-1 entry or replication in MDM (Matsuda *et* al., 1995; Di Marzio *et al.*, 1998) associated with reduced β-chemokine receptor expression. In order to clarify the effects of GM-CSF on HIV-1 replication in MDM *in vitro*, the activity of this cytokine was also examined in this study.

GM-CSF mediates its activities through binding to a heterodimeric receptor comprising a ligand-specific α chain and a common β chain that is shared with IL-3 and IL-5 (reviewed in Crowe and Lopez, 1997; Armitage, 1998). Amino acid 21 within the first alpha helix of GM-CSF has been found to be critical for the biological function of GM-CSF and for the interactions between GM-CSF and the beta chain of the GM-CSF receptor that are necessary for high affinity binding (Lopez *et al.*, 1992). A mutant form of GM-CSF (E21R), which binds only to the α-chain of the receptor (Lopez *et al.*, 1992; Hercus *et al.*, 1994), was used to explore the roles of the α and β chain of the GM-CSF receptor on both phagocytosis of MAC and HIV-1 replication *in vitro*.

In this study the ability of human monocytes and MDM to phagocytose MAC following infection with HIV-1, and the effects of GM-CSF on this function, were investigated. HIV-1 infection of MDM *in vitro* markedly impaired phagocytosis of MAC. GM-CSF treatment of MDM resulted in the restoration of phagocytic activity and consistent inhibition of HIV-1 replication within those cells. In addition, the effect of a course of adjunctive GM-CSF therapy in a patient with advanced HIV-1 infection and disseminated infection with multi-drug resistant MAC was also examined. GM-CSF therapy improved phagocytosis of MAC by this patient's monocytes and prolonged the time required for MAC to be detected in blood culture.

6.3 METHODS.

6.3.1 GM-CSF stimulation of MDM.

Human monocytes were isolated from buffy coats (Section 2.1.1) and cultured in suspension in teflon jars. Freshly isolated monocytes were treated with recombinant human GM-CSF (Genzyme, Cambridge, MA, USA) at varying concentrations (1-100ng/ml). Control cells from the same donors were cultured in the absence of GM-CSF. Experiments were also performed using a mutant form of GM-CSF (E21R; BresaGen, Adelaide, South Australia) with a site mutation within the first alpha helix resulting in a substitution of arginine for glutamic acid (Lopez *et al.*, 1992). E21R GM-CSF binds to the α chain of the GM-CSF receptor with low-affinity and thus it was used at concentrations 10 to 100 times higher than that of wild type GM-CSF. These concentrations were not associated with cellular toxicity (Iversen *et al.*, 1997). To determine the specificity of the effect of GM-CSF on HIV-1 replication in MDM, GM-CSF was incubated with a neutralising anti-human GM-CSF Mab (100 μg/ml; 4D4) or a non-neutralising anti-GM-CSF control Mab (100 μg/ml; 4A12) for 30 min at 4^0C prior to its addition to MDM cultures.

To exclude the possibility of toxicity of GM-CSF on MDM, cells were cultured in suspension with GM-CSF (0.01-100ng/ml) for 21 days. Cell counts were performed at regular intervals and viability was assessed using trypan blue exclusion.

6.3.2 Quantification of HIV-1 replication.

MDM were infected 4 to 6 days after isolation with the macrophage-tropic strain, HIV-$1_{Ba\text{-}L}$ (Section 2.5). After HIV-1 infection cells were washed and resuspended in supplemented Iscove's medium in the presence or absence of GM-CSF or E21R GM-CSF. HIV-1 replication was quantified by monitoring RT activity in culture supernatants obtained 7 days after the infection using a micro RT assay (Section 2.6.1). In selected experiments, HIV-1 replication in MDM cultured in suspension in teflon jars was determined in pre-fixed and permeabilised cells by staining for intracellular p24 and flow cytometric analysis (Section 2.6.3).

To examine effects of GM-CSF on HIV-1 DNA and mRNA levels, MDM were infected with HIV-$1_{Ba\text{-}L}$ then pretreated with 10U of RNase-free DNase (Boehringer Mannheim Australia, Castle Hill, New South Wales) for 20 min at room temperature in the presence of 10mM $MgCl_2$ to remove contaminating viral DNA. Extraction of DNA was performed as described in Section 2.10. Amplification of the HIV-1 *gag* gene was performed using SK38 and SK39 primers and hybridisation with a ^{32}P-labelled probe SK19 (Lewin *et al.*, 1996). Briefly, HIV-1 *gag* gene (115 bp region) was amplified using SK38 primer (ATAATCCACCTATCCCAGTAGGAGAAAT) and SK39 primer: (TTTGGTCCTTGTCTTATGTCCAGAATGC) (Bresatec, Adelaide, South Australia). HLA-DQ alpha (242 bp) was amplified in the same reaction using GH-26 primer (GTGCTGCAGGTGTAAACTTGTACCAG) and GH-27 primer (CACGGATCCGGTAGCAGCGGTAGAGTTG) (Bresatec) to standardise the amount of DNA in the lysates. PCR reaction and amplification were performed as described in

Section 2.11.3.

The SK19 probe: ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTAC for HIV-1, and the GH64 probe: TGGACCTGGAGAGGAAGGAGACTG for HLA-DQ were ^{32}P-labelled for detection of HIV-1 following PCR amplification. The SK19 probe (15pM) and the GH64 probe (30pM) were radiolabelled in separate tubes containing 60µl of reaction mix consisting of 1x kinase buffer (Promega), 20U T4 polynucleotide kinase (PNK) and 50µCi γ-^{32}P-ATP (Amersham) at 37^{0}C for 1 hr, then at 68^{0}C for 10 min. After adding 40µl of 0.75mM NaCl and 10mM EDTA, the unincorporated label was separated through a G25 Sephadex column by centrifugation at 1600xg for 3 min. The probes were pooled and adjusted to a final volume of 400µl with 0.75mM NaCl, 10mM EDTA. For hybridisation, 5µl of the probe mix was added to 15µl of PCR products, denatured at 95^{0}C for 5 min and incubated at 59^{0}C for 15 min. ^{32}P-labelled products were resolved using 10% polyacrylamide gels (Section 2.12.2) and exposed to the X-ray film (Hyperfilm, Amersham) for 18 to 72 hr.

mRNA was extracted from MDM lysates using oligo $(dT)_{25}$ beads and subsequently converted to cDNA as described in Section 2.11.1. Beads\cDNA complexes were resuspended in 100µl TE buffer (pH 8.0) and stored at 4°C in 10-fold dilutions in lysis buffer until used for PCR reactions using *gag*-specific primers and detected by hybridisation with a ^{32}P labelled SK19 probe. Levels of cDNA were standardised according to beta-actin levels (Section 2.11.2) as assessed by laser densitometry. To check for viral DNA contamination samples prepared without AMV-RT were included within each experiment.

6.3.3 GM-CSF treatment of the HIV-1 infected patient.

A 35-year-old male was hospitalised with disseminated MAC infection resistant to ethambutol, clarithromycin and amikacin. He had a history of extensive prior antiretroviral therapy with drug combinations of protease inhibitors and nucleoside and non-nucleoside reverse transcriptase inhibitors. Two weeks prior to commencing GM-CSF therapy his antiretroviral regimen was changed with the addition of two new drugs (nelfinavir and abacavir). On day one of therapy he received recombinant GM-CSF (Schering-Plough, New South Wales, Australia) by sub-cutaneous injection at an initial daily dose of 400μg/day. GM-CSF was suspended on day 7 of therapy due neutrophilia and recommenced at a dose of 200μg/day on day 14 when his neutrophil count declined to $10.2x10^3$/ml. On day 38 he developed central line-related sepsis and multi-organ failure. He died on day 40 of this study due to central-line-associated complications.

Peripheral blood was collected from the patient twice prior to commencing GM-CSF, and 2 to 3 times per week during GM-CSF therapy to assess phagocytosis of MAC by monocytes and plasma HIV-1 RNA (bDNA assay; Chiron Corporation, Emeryville, USA; performed by the Clinical Research Laboratory, Macfarlane Burnet Centre for Medical Research; Melbourne). Blood and duodenal biopsy specimens were cultured for MAC by inoculation into BACTEC 13A media (Becton Dickinson Diagnostic Instrument Systems, MD, USA; performed by Ms Aina Sievers; Victorian Infectious Disease Reference Laboratory, Melbourne) using standard procedures, and cultured at 35°C for up to 8 weeks. A growth index of >20 using the BACTEC 460 system indicated a positive culture. Zeihl-Neelsen and auramine-rhodamine stains were performed on all positive cultures and identification was confirmed by the State Reference Laboratory of Victoria using a DNA probe (Accuprobe, Gen-Probe, San Diego, CA, USA) and biochemical tests.

6.3.4 Phagocytosis assays.

6.3.4.1 Using cultured MDM in vitro.

On day 7 following HIV-1 infection *in vitro*, MDM ($2x10^5$ cells in 100µl of PBS-CMF) were exposed to fluoresceinated MAC, at different MAC to MDM ratios, in duplicate (Section 2.8.1.3). The percentage of MDM that phagocytosed MAC-FITC was quantified by flow cytometry.

6.3.4.2 Using monocytes within blood.

Blood from the HIV-1 infected patient treated with GM-CSF was collected in lithium heparin anticoagulant along with a minimum of 4 HIV-seronegative and seropositive untreated controls. Blood from the same HIV-seronegative controls were used for each experiment. Phagocytosis of MAC-FITC by peripheral blood monocytes was performed as described in Section 2.8.1.2. The percentage of CD14-positive monocytes phagocytosing MAC-FITC was calculated as the % of FITC-labelled cells in the PE-positive cell population.

6.3.5 Statistical analyses.

Multiple linear regression analysis was used to determine the effects of HIV-1 infection and GM-CSF therapy on the phagocytic ability of monocytes. The raw data were linearised by log-transforming the values of the MAC-FITC: monocyte ratios. A regression equation was constructed to estimate % phagocytosis by monocytes as a function of $\log_{10}$ (MAC-FITC: monocytes) and dummy variables introduced to account for elapsed time, HIV-1 infection status and GM-CSF treatment status. Separate regression equations were also constructed to assess the effects of HIV-infection status and GM-CSF treatment status on phagocytic activity on specific days prior to

and during treatment. The analysis was performed using the STATA (Stata, College Station, TX) statistical analysis package.

The significance of the effect of treatment with GM-CSF or the E21R mutant GM-CSF on phagocytosis of MAC and HIV-1 replication was assessed using the Student's t test (paired, 2-tailed).

6.4 RESULTS.

6.4.1 GM-CSF stimulation of monocyte-derived macrophages in vitro.

6.4.1.1 GM-CSF restores phagocytosis of Mycobacterium avium complex.

The effect of HIV-1_{Ba-L} infection of MDM *in vitro* on phagocytosis of MAC-FITC was assessed by flow cytometry using cells from ten different donors and a range of MDM to MAC-FITC ratios (1:12 to 1:75). By 7 days following HIV-1 infection a consistent impairment of phagocytosis compared with uninfected controls (significant at ratios of 1:50; $p<0.05$, and 1:75; $p<0.01$) was found (Figure 6.1). At a ratio of MDM to MAC-FITC of 1:75, a mean of 56.1% of uninfected MDM ingested MAC-FITC compared to 34.4% of MDM in HIV-infected cultures ($p<0.01$) (Table 6.1).

GM-CSF treatment of MDM augmented phagocytosis of MAC-FITC by HIV-1 infected MDM (significant at MDM:MAC ratios of 1:25, 1:50 and 1:75 ($p<0.05$, $p<0.01$, $p<0.01$ respectively) (Figure 6.1). At the highest ratio, HIV-infected, GM-CSF-treated MDM phagocytosed a mean of 55.9% MAC-FITC compared with 34.4% of HIV-infected MDM cultured without GM-CSF (Table 6.1). Furthermore, phagocytosis by HIV-1 infected, GM-CSF-treated MDM was restored to levels comparable to those found in uninfected MDM (at a ratio of 1:75, 55.9% and 56.1% respectively $p=0.487$). The apparent

increase in phagocytosis by GM-CSF treated, uninfected MDM was of borderline significance (at a ratio of 1:75, p = 0.057).

A mutant form of GM-CSF (E21R) binding only to the α chain of the GM-CSF receptor did not augment phagocytosis of MAC by either HIV-infected (p=0.68) or paired uninfected MDM (p=0.54) (Table 6.1).

There was no toxic effect observed in monocytes incubated with GM-CSF (0.01 -100 ng/ml) or the mutant GM-CSF E21R (1μg/ml) immediately after isolation, and maintained in the presence of GM-CSF over 21 days (data not shown).

6.4.1.2 GM-CSF inhibits HIV-1 replication in MDM.

GM-CSF reproducibly suppressed HIV-$1_{Ba\text{-}L}$ replication in MDM as assessed by a number of different techniques. GM-CSF reduced RT activity assessed in the culture supernatant (82%±5.2% SEM inhibition; n=7, p<0.01), whereas E21R GM-CSF did not significantly decrease RT (13.6%±18% SEM inhibition; n=7, p=0.5) (Figure 6.2a). Similarly, GM-CSF dramatically decreased intracellular p24 antigen concentrations quantified by flow cytometry, whereas the mutant GM-CSF E21R did not significantly alter intracellular p24 antigen levels, compared to MDM not exposed to GM-CSF (Figure 6.2b). The net MESF (Molecules of Equivalent Soluble Fluochrome) values were $7.2x10^5$, $1.8x10^5$, $5.2x10^5$ and for intracellular p24 fluorescence of MDM cultured in the absence of GM-CSF, presence of GM-CSF and mutant E21R GM-CSF respectively.

Results of 6 experiments using MDM from different donors confirmed that a concentration of GM-CSF of 0.1ng/ml or greater significantly inhibited HIV-$1_{Ba\text{-}L}$ replication by a mean of 67.7%±4.9% (standard error of the mean, SEM) compared to untreated controls (data not shown). GM-CSF inhibition was blocked by a neutralising

Mab (4D4) against GM-CSF, but not by a non-neutralising anti-GM-CSF MAb (4A12), demonstrating the specificity of the observed effect (Figure 6.3).

To establish whether the decrease in HIV-1 replication occurs before or after transcription, the effect of GM-CSF on HIV-1 DNA and mRNA levels were investigated. There was a minimal 1-3 fold decrease in the level of HIV *gag* DNA in infected cells cultured in the presence of GM-CSF (n=3) when compared with cells not exposed to GM-CSF or treated with E21R GM-CSF (Figure 6.4a). As assessed by laser densitometry, the ratio of *gag* to *DQ* signal was 0.47, 0.18 and 0.47 (mean of the first 3 dilutions, results for a representative experiment) for cells cultured in the absence of GM-CSF, presence of GM-CSF and E21R GM-CSF respectively. Analysis of cell lysates from 3 donors showed a 3 to 10 fold decrease in HIV-1 *gag* mRNA expression in MDM infected with HIV-$1_{Ba\text{-}L}$ that were exposed to GM-CSF and approximately 2-fold inhibition in those exposed to GM-CSF E21R when compared with untreated MDM (Figure 6.4b). Taken together, these results suggest that HIV-1 replication is inhibited by GM-CSF before or at the level of transcription. Densitometry units for *gag* signal standardised to equivalent levels of β-actin levels were 1956, 732 and 957 for MDM cultured in the absence of GM-CSF, presence of GM-CSF and E21R GM-CSF respectively.

6.4.2 GM-CSF therapy of HIV-infected patient.

6.4.2.1 GM-CSF augments phagocytic capacity of peripheral blood monocytes.

Prior to commencing GM-CSF therapy, the phagocytic activity of this patient's monocytes was significantly lower than that of HIV-uninfected control subjects ($p<0.001$) (Figure 6.5a). GM-CSF therapy improved the ability of the patient's monocytes to phagocytose MAC-FITC to levels comparable to those of HIV-uninfected individuals, starting from day 5 after commencement of treatment ($p=0.45$, Figure 6.5b-

d; Figure 6.6a). The phagocytic activity of monocytes from HIV-infected patients not undergoing GM-CSF therapy was significantly lower than that of HIV-uninfected controls and the GM-CSF-treated patient ($p<0.001$) (Figure 6.5c,d).

6.4.2.2 GM-CSF increases time to MAC positive cultures.

The patient was monitored for MAC bacteraemia for more than a year prior to GM-CSF therapy. Over this period, blood samples took a mean of 11.1 days to return positive MAC cultures (n=10). A week prior to GM-CSF treatment MAC was cultured from blood and duodenal biopsy within 6 days and 4 days respectively, whereas after 28 to 34 days of GM-CSF the time was 17-24 days (mean of 20.0 days, n=4) (Figure 6.6b).

6.4.2.3 GM-CSF does not affect plasma viral load.

At commencement of GM-CSF therapy his plasma HIV-1 RNA was $4.0\log_{10}$ copies/ml. The patient experienced only minor fluctuations in plasma HIV-1 RNA levels during therapy with a possibly significant (defined as $>0.5\log_{10}$ copies/ml) increase of $0.7\log_{10}$ copies/ml occurring at only one time point (day 28 of therapy) (Figure 6.6c).

6.5 DISCUSSION.

This study shows that phagocytosis of MAC by human MDM is defective following HIV-1 infection *in vitro*, and that GM-CSF stimulation can largely restore this antimycobacterial function. These data are supported by results from an HIV-infected patient with drug-resistant MAC infection whose *ex vivo* phagocytic capacity was significantly improved during adjunctive therapy with GM-CSF, and was accompanied by a reduction in MAC bacteraemia as measured by the length of time required for blood cultures to become positive. In addition, GM-CSF consistently suppressed HIV-1 replication *in vitro,* and the administration of GM-CSF *in vivo* did not result in significant changes in plasma viral load during 40 days of therapy. The inhibitory effect was GM-

CSF-specific since it was totally reversible by addition of neutralising Mab, 4D4, but not by addition of a non-neutralising anti-GM-CSF control Mab, 4A12. E21R GM-CSF, binding only to the α-chain of the GM-CSF receptor (Lopez *et al.*, 1992), did not affect HIV-1 replication or phagocytosis of MAC *in vitro*.

The effector cells used in the phagocytosis assay employed in this study were MDM infected with HIV-1 following 5 days in culture. A marked degree of donor variation in the ability of human MDM to phagocytose MAC under conditions tested (mean 56%; range 35-82%) was observed. This may reflect the variability in surface expression of CR3 receptor facilitating phagocytosis of MAC or complement levels (required for opsonisation) present within human serum used in the experiments. There was also a variable suppression of phagocytosis resulting from HIV-1 infection of MDM, which may reflect variation in the proportion of MDM infected with HIV-1 resulting from differing levels of surface chemokine co-receptor expression.

The results presented in this study are consistent with data showing that HIV-1 infection of MDM *in vitro* impairs immunologic defences against opportunistic pathogens other than MAC, including *Cryptococcus neoformans*, *Candida albicans* and *Toxoplasma gondii* (Cameron *et al.*, 1993; Crowe *et al.*, 1994; Biggs *et al.*, 1995). Similarly, monocytes from HIV-1 infected persons have been found to be less efficient in terms of phagocytosis and killing (Estevez *et al.*, 1986; Delemarre *et al.*, 1995; Chaturvedi *et al.*, 1995), although there are also reports of normal macrophage function following *in vitro* and *in vivo* infection with HIV-1 (Nottet *et al.*, 1993). There are also inconsistent data regarding the effect of HIV-1 infection on the ability of monocytes and macrophages to control MAC infection, with the data generally indicating reduced antimycobacterial function (Kallenius *et al.*, 1992; Shiratsuchi *et al.*, 1994; Zerlauth *et al.*, 1995). A number of variables may be responsible for the inconsistencies in the literature, including the strain of HIV-1 used *in vitro*, donor variability of MDM regarding

susceptibility to HIV-1 infection, the stage of maturation of monocytes/macrophages at the time of functional assessment, the type of intracellular pathogen, the type of exogenous cytokines used or the nature of the patient cohort.

Recombinant GM-CSF has been found by most investigators to augment the antimycobacterial function (including intracellular killing) of normal human and murine monocytes (reviewed in Armitage, 1998). Earlier studies using impure preparations of GM-CSF reported no improvement in macrophage function following GM-CSF treatment (Nathan *et al.*, 1984). In this study there was clear evidence of improvement in phagocytosis of MAC by MDM infected with HIV-1 as well as in paired uninfected MDM from the same donors. A mutant form of GM-CSF, E21R, binding only to the α chain of the receptor, failed to stimulate phagocytosis of MAC, suggesting that the principal signals controlling phagocytosis occur via the receptor β-chain.

In vitro GM-CSF stimulation of MDM prior to HIV-1 infection not only augmented phagocytosis of MAC by those cells, but also inhibited HIV-1 replication. There is a longstanding controversy in the literature regarding the effect of GM-CSF on HIV-1 replication within cells of macrophage lineage. Most of the early studies report augmentation (Koyanagi *et al.*, 1988; Perno *et al.*, 1989; Schuitemaker *et al.*, 1990; Perno *et al.*, 1992; Wang *et al.*, 1998) or no change (Kornbluth *et al.*, 1989; Hammer *et al.*, 1990) in viral production. However, two recent studies suggest inhibition of HIV-1 replication in MDM by GM-CSF (Matsuda *et al.*, 1995; Di Marzio *et al.*, 1998). The mechanism by which GM-CSF alters HIV-1 replication in MDM and in promonocytic cell lines is also controversial. Wang *et al* (Wang *et al.*, 1998) reported that the GM-CSF-induced increase in HIV-1 replication in MDM was attributable to up-regulation of CCR5 expression. These data are in direct contrast to those of Di Marzio and collegues (Di Marzio *et al.*, 1998) demonstrating that GM-CSF suppressed CCR5 and CD4 expression on MDM, and reduced HIV-1 entry into these cells. The data reported here

suggest minimal inhibition at *gag* DNA level and a 3-10 fold decrease in *gag* mRNA within MDM treated with GM-CSF compared with untreated cells, suggesting that the block to HIV-1 replication occurs at or prior to transcription. The difference in the inhibitory effect between DNA and mRNA levels could be due to the effect of GM-CSF at multiple points, donor variation, or the semi-quantitative nature of the assays. The results reported here are in agreement with Matsuda *et al.* (Matsuda *et al.*, 1995) who have also reported inhibition of HIV-1 replication by GM-CSF with a decrease in pro-viral DNA.

Currently GM-CSF is used rarely for the treatment of HIV-infected patients in part due to the conflicting *in vitro* data leading to concerns regarding potential activation of HIV-1 replication. Similarly, early clinical trials showed that GM-CSF treatment of HIV-infected patients in the absence of antiretroviral therapy resulted in increased serum p24 antigen levels and plasma HIV-1 RNA titres (Kaplan *et al.*, 1991; Lafeuillade *et al.*, 1996). However, when used in combination with effective antiretroviral therapy, GM-CSF has been safely administered to patients without any significant increase in viral load (Yarchoan *et al.*, 1990; Krown *et al.*, 1992). In fact GM-CSF has been found to increase the activity of antiretroviral drugs (Perno *et al.*, 1992). Data from studies of HIV-infected patients receiving antiretroviral therapy and GM-CSF have shown that patients experienced a decrease in viral load and an increase in CD4 counts (Skowron *et al.*, 1999; Brites *et al.*, 2000).

The clinical study reported in this thesis demonstrates a beneficial effect of GM-CSF on antimycobacterial activity without a significant increase in viral load in the HIV-infected patient. The improvement in phagocytosis of MAC and lengthening of time to positive MAC blood culture in the single patient with drug-resistant MAC adjunctively treated with GM-CSF support *in vitro* findings reported in this thesis. Similarly, a recent report by Kemper *et al* (1998) showed enhanced activation and mycobactericidal activity of blood

monocytes in four AIDS patients with newly diagnosed MAC receiving azithromycin combined with GM-CSF, as compared to patients receiving azithromycin alone (Kemper *et al.*, 1998). However, the authors of this study found no significant difference between both groups in the reduction of mycobacteraemia, which may reflect the efficacy of azithromycin on previously untreated MAC. This study reports not only increased phagocytosis of MAC but also improvement in bacteraemia, as measured by the length of time required for blood cultures to become positive, in an HIV-infected patient with multi drug-resistant disseminated MAC.

Clinical improvement and augmented monocyte phagocytic function following GM-CSF therapy has similarly been reported in patients with advanced HIV-1 infection and fluconazole-resistant *Candida albicans* infection, again without an increase in viral load (Vazquez *et al.*, 1998). Augmentation of other aspects of macrophage function has been also found in other immunosuppressed patients (Williams *et al.*, 1997). Data from the study in which AIDS patients received nucleoside therapy has shown that GM-CSF administered twice weekly for 6 months not only reduced the frequency of opportunistic infections but also significantly decreased viral load and increased CD4 counts in those individuals (Brites *et al.*, 2000).

Taken together, these data suggest that adjunctive GM-CSF therapy may be useful to augment macrophage function and to restore antimycobacterial activity in HIV-1 infected patients with opportunistic infections.

6.6 RELEVANT PUBLICATIONS.

The following publications have arisen from the work presented in this chapter:

1. **Kedzierska, K.,** Mak, J., Mijch, A., Cooke, I., Rainbird, M., Roberts, S., Paukovics, G., Jolley, D., Lopez, A. and Crowe, S.M. (2000) Granulocyte-macrophage colony stimulating fa :tor augments phagocytosis of *Mycobacterium avium* complex by HIV-1 infected monocyte/macrophages in vitro and in vivo. *J Inf Dis*, **181**:390-394.

I have performed the experiments presented in this manuscript, with some technical assistance from Melissa Rainbird regarding the E21R/*in vitro* phagocytosis data. I have analysed the results and written the manuscript with input from Prof. Suzanne Crowe and Dr. Johnson Mak. A/Prof. Ian Cooke and Dr. Damien Jolley have provided their expertise with statistical analyses. Geza Paukovics has provided his assistance with flow cytometry. The clinical study was performed in collaboration with Drs Anne Mijch and Sally Roberts. Prof. Angel Lopez has provided the E21R GM-CSF and constructive criticism of the manuscript.

2. **Kedzierska, K.,** Maerz, A., Warby, T., Jaworowski, A., Chan, H., Mak, J., Sonza, S., Lopez, A. and Crowe, S.M. (2000) Granulocyte-macrophage colony stimulating factor inhibits HIV-1 replication in monocyte-derived macrophages. *AIDS*, **14**:1739-1748.

I have performed the experiments included in this Chapter from the above-mentioned manuscript, analysed results and written the manuscript with input from my supervisors Prof. Suzanne Crowe and Dr. Anthony Jaworowski. Drs. Johnson Mak and Secondo Sonza have provided their expertise with HIV-1 mRNA and DNA experiments. Prof. Angel Lopez has provided the E21R GM-CSF and constructive criticism of the manuscript. Anne Maerz, Tammra Warby and Hiu-Tat Chan have provided their

technical assistance with Figure 2 and Table 2 of the manuscript, which are not included in my thesis (a partial requirement for Ms. Warby's PhD thesis).

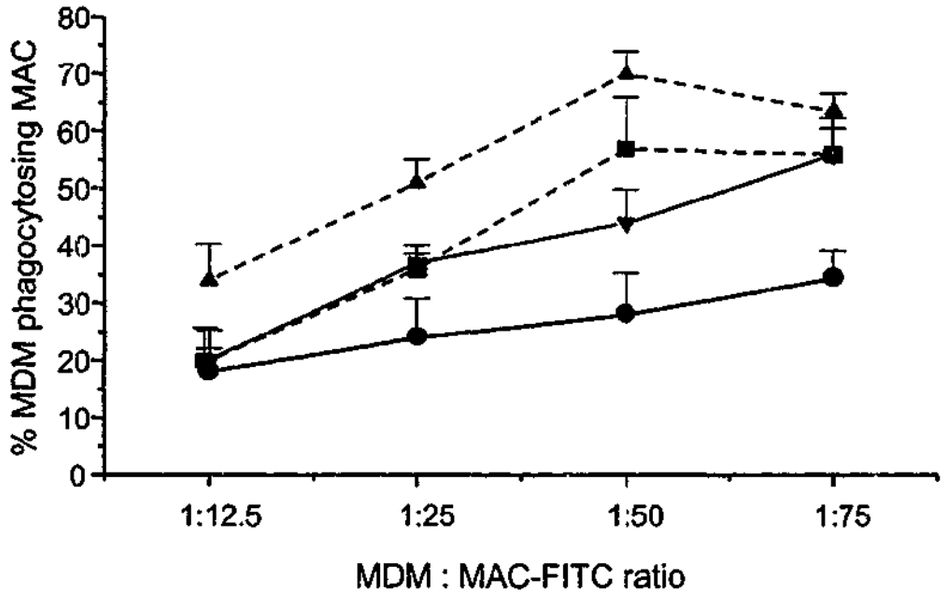

Figure 6.1 Comparison of phagocytosis of MAC-FITC by HIV-1 infected and uninfected MDM cultured in the presence or absence of GM-CSF.

Freshly isolated monocytes were treated with GM-CSF at a concentration of 100ng/ml (blue lines) or left unexposed to this cytokine (black lines). MDM on day 5 post-isolation were either mock-infected (dotted lines) or infected with HIV_{Ba-L} (solid lines). Phagocytosis of MAC-FITC by MDM was assessed by flow cytometric analysis on day 7 post-infection. The proportion of MDM phagocytosing MAC labelled with FITC (mean±SEM) is shown using MDM to MAC-FITC ratios of 1:12.5, 1:25, 1:50 and 1:75. These data are the means compiled from results from 4 donors using MDM to MAC-FITC ratios of 1:12.5-1:50 and 10 donors using a ratio of 1:75.

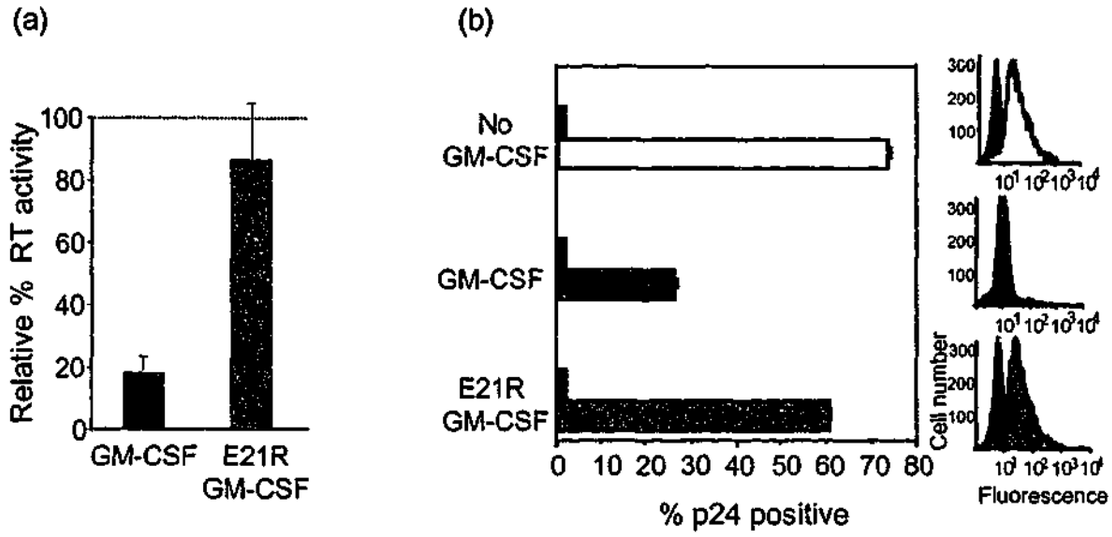

Figure 6.2 Wild type GM-CSF but not E21R GM-CSF inhibits HIV-1 replication in MDM.

Freshly isolated monocytes were pretreated with GM-CSF (1ng/ml; blue bars) or a mutant E21R GM-CSF (100ng/ml; green bars) for 5 days prior to HIV-1 infection. MDM from the same donor not exposed to GM-CSF were used as controls (white bars). Cytokine was replenished in cultures during the course of the experiment. On day 10 post-infection HIV-1 replication was quantified by (a) measuring RT activity in culture supernatants by RT micro assay. Results were compared with those from supernatants of HIV-1 infected control MDM cultured in the absence of GM-CSF that were standardised to represent 100% (n=7); (b) intracellular HIV-1 p24 antigen level assessed by staining cells with anti-p24 monoclonal antibody (white, blue and green histograms) or isotype-matched control (black histograms) and quantified by flow cytometry. Histograms were unimodal. Results from a representative donor are shown. The level of p24 staining or RT activity in mock-infected MDM cultures was below the level of detection of those assays.

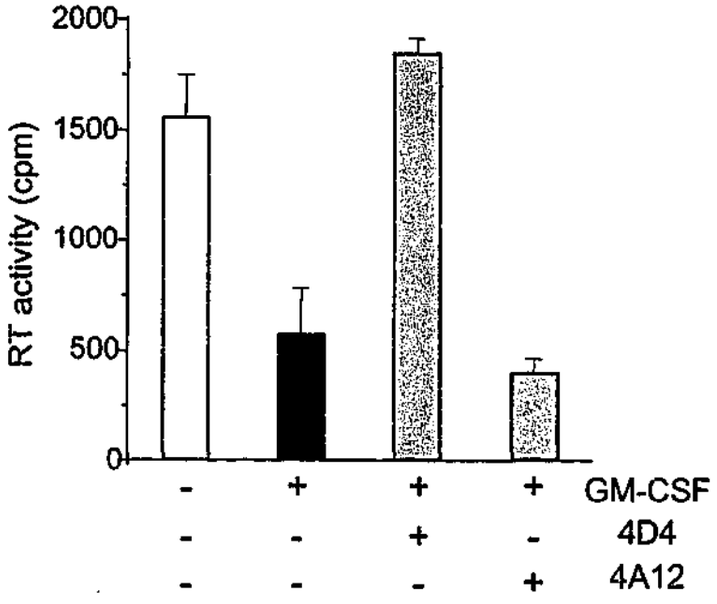

Figure 6.3 Specificity of the GM-CSF-induced inhibition of HIV-1 replication in MDM.

MDM were cultured in the presence of GM-CSF alone (Genetics Institute; 1ng/ml; blue bar) or with GM-CSF pretreated with the specific anti-GM-CSF blocking monoclonal antibody, 4D4 (100μg/ml; red bar), or anti-GM-CSF non-blocking monoclonal antibody, 4A12 (100μg/ml; red bar). Control cells from the same donors were cultured in the absence of the cytokine (white bar). GM-CSF and monoclonal antibodies were maintained in all treated cultures after HIV-1 infection. Replication of HIV-1 was quantified by RT activity assay using culture supernatants obtained 10 days following infection. Results shown are representative from two experiments using different MDM donors.

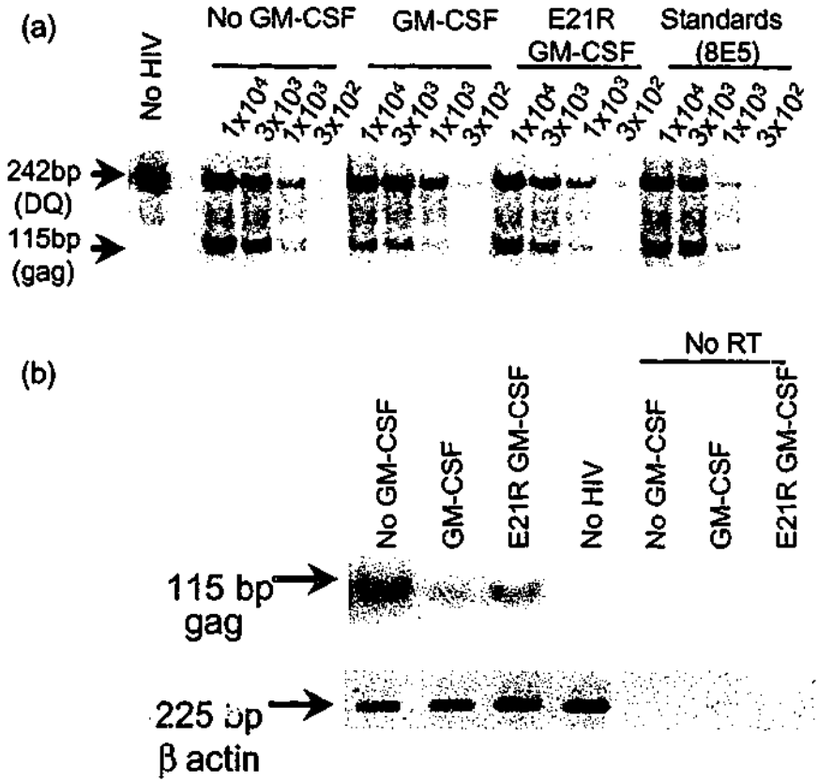

Figure 6.4 Effects of GM-CSF on HIV-1 *gag* DNA and mRNA.

Monocytes were cultured for 5 days in presence or absence of GM-CSF (Genzyme) or mutant GM-CSF E21R, infected on day 5, and DNA and mRNA extractions were performed 7 days after infection. a) 3-fold dilutions of cell extracts were prepared from HIV-infected MDM cultured in the presence or absence of GM-CSF or GM-CSF E21R for PCR of DNA using *DQ* and *gag* primers, detected by hybridisation with a ^{32}P-labelled probe GH 26 for HLA-DQ and SK19 for *gag*. Dilutions were similarly prepared from cell lysates of 8E5 cells (containing 1 HIV-1 provirus per cell) and amplified concurrently to serve as standards. b) cDNA was synthesised from mRNA and PCR was performed using *gag*-specific primers and detected by hybridisation with a ^{32}P-labelled probe SK19. Levels of cDNA were standardised according to beta-actin levels. PCR on samples prepared without reverse transcriptase were negative.

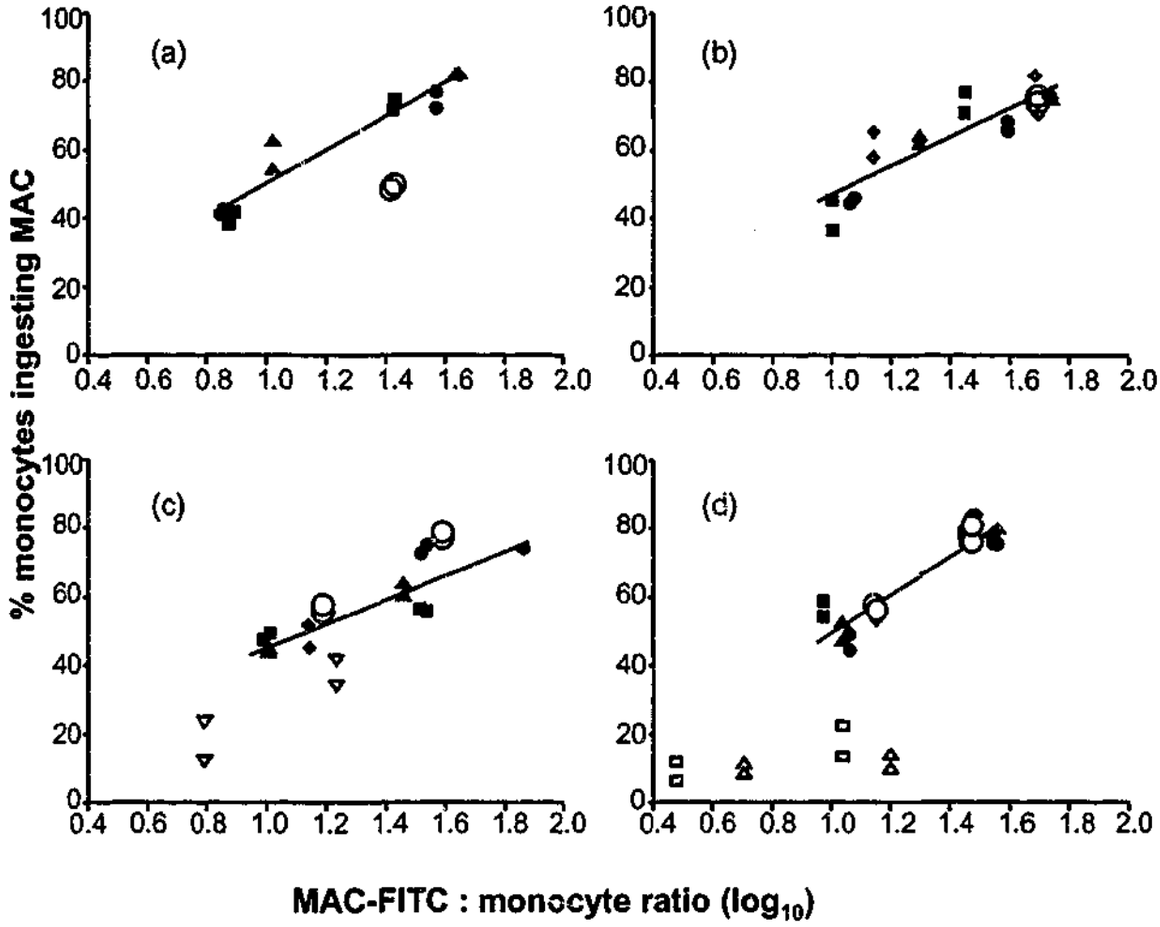

Figure 6.5 Quantitation of MAC-FITC phagocytosis by monocytes in an *ex vivo* assay.

Phagocytosis of MAC-FITC by monocytes obtained from an HIV-infected patient undergoing GM-CSF therapy (○) was compared with phagocytosis by monocytes obtained from HIV-uninfected controls (▲, ●, ■, ◆) and HIV-1 infected patients, not receiving GM-CSF (▽,△,□). Shown here are representative results from the GM-CSF-treated patient obtained a) prior to GM-CSF treatment b) 17 days, c) 21 days, d) 30 days after commencing GM-CSF. The regression lines shown in each graph were fitted to the data points obtained from HIV-uninfected controls.

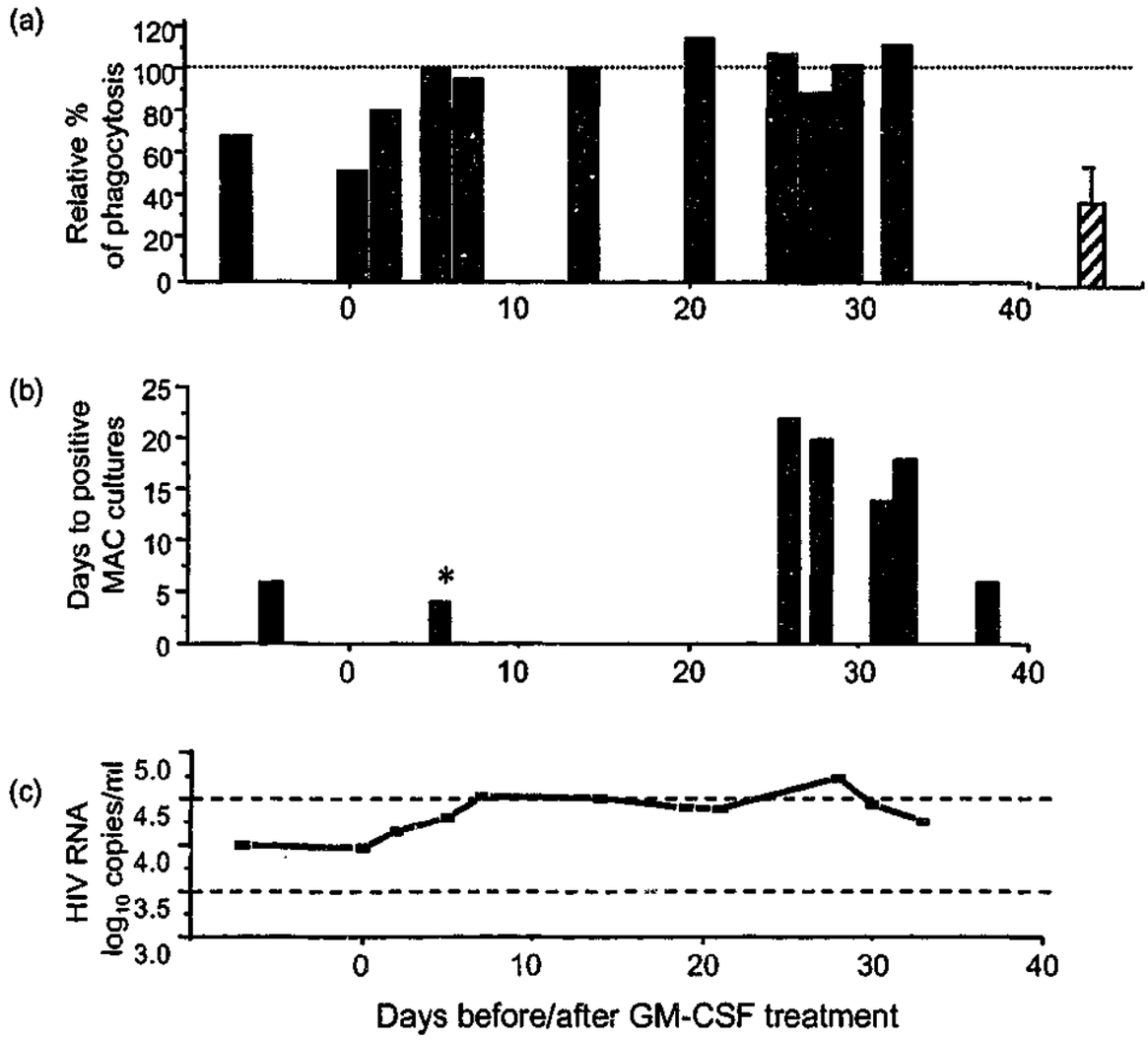

Figure 6.6 Effect of GM-CSF therapy on phagocytosis of MAC-FITC, bacteraemia and plasma viral load.

The effect of GM-CSF therapy (blue bars) on a) phagocytosis of MAC-FITC by monocytes within whole blood of an HIV-infected patient receiving GM-CSF, compared to that of uninfected controls (standardised as 100% for a given monocyte: MAC-FITC ratio). Patient's blood was collected twice prior to commencing GM-CSF treatment (black bars). Results for the mean and standard deviation of phagocytosis by monocytes within blood of 6 HIV-infected controls not receiving GM-CSF (a striped bar) are shown, b) time to positive culture of MAC from blood and duodenal biopsy(*) c) plasma HIV-1 RNA levels. The phagocytosis of MAC by monocytes of the patient and HIV-seropositive controls are reported as a percentage of the phagocytosis by seronegative controls for the same MAC:monocyte ratio. The values obtained at each MAC:monocyte ratio for the seronegative controls were standardised as 100% of phagocytic efficiency.

Table 6.1 Effects of GM-CSF/HIV-1 on phagocytosis of *Mycobacterium avium* complex by monocyte-derived macrophages *in vitro*.

Percent of macrophages phagocytosing *Mycobacterium avium* complex						
	Mock-infected (mean ± SD)			HIV-infected (mean ± SD)		
No. of expt*	Control	GM-CSF	E21R GM-CSF	Control	GM-CSF	E21R GM-CSF
10	56.1±4.5	63.6±3.1	-	34.4±4.8	55.9±4.8	-
5	64.1±6.2	69.7±3.5	41.6±11.0	30.9±5.9	54.7±4.0	28.5±7.0

On the day of isolation, monocytes were treated with GM-CSF (100ng/ml), E21R GM-CSF (1μg/ml) or cultured unexposed to any growth factors. MDM were infected with HIV-1_{Ba-L} on day 5 after isolation. Phagocytosis of MAC-FITC by MDM was assessed by flow cytometric analysis on day 7 post-infection. *Experiments were performed in duplicate using MDM from different donors. Results show the percentage of macrophages containing MAC-FITC at 1:75 MDM to MAC-FITC ratio.

CHAPTER 7

GENERAL DISCUSSION

An understanding of the mechanisms by which HIV-1 impairs cell-mediated immunity leading to degeneration of the immune system is critical for the full control of HIV-1 disease progression. Defects in immunological function of cells of the macrophage lineage contribute considerably to the pathogenesis of HIV-1 infection. In immunocompetent individuals peripheral blood monocytes and tissue macrophages mediate the cell-mediated response to a variety of pathogens such as bacteria (*Mycobacterium avium* complex, *Mycobacterium tuberculosis, Rhodococcus equi*), parasites (*Toxoplasma gondii,* microsporidia) and fungi (*Candida albicans, Cryptococcus neoformans, Pneumocystis carinii*) (Drancourt *et al.*, 1992; Wehle *et al.*, 1993; Doultree *et al.*, 1995). As these pathogens are the cause of opportunistic infections in HIV-infected individuals at late stages of disease, their inefficient control as a result of defective macrophage function is considered to be the main mechanism underlying the development of AIDS-related opportunistic infections.

My doctoral studies were aimed to investigate the mechanisms underlying defective monocyte and macrophage function following HIV-1 infection. Impairment of critical effector functions carried out by cells of macrophage lineage, including phagocytosis, intracellular killing and cytokine production has been reviewed in the General Introduction to this thesis (Section 1.4.3). Impairment of phagocytosis of pathogens such as *Toxoplasma gondii, Candida pseudotropicalis, Porphyromonas gingivalis, Fusobacterium nucleatum* and *Cryptococcus neoformans* by peripheral blood monocytes, alveolar macrophages and MDM have been reported following HIV-1 infection *in vivo* as well as MDM infected with HIV-1 *in vitro*. However, the underlying mechanism whereby HIV-1 impairs phagocytosis by cells of macrophage lineage remained unknown.

In my studies defective phagocytosis by both peripheral blood monocytes obtained from HIV-infected individuals and MDM infected with HIV-1 *in vitro* was observed. A number of different phagocytic receptors (Section 1.6.1) mediate engulfment of opportunistic pathogens, in particular complement receptors (C'R) for MAC uptake (Schlesinger and Horwitz, 1991) and Fcγ receptors (FcγR) for *Toxoplasma gondii* phagocytosis (Joiner *et al.*, 1990). The effect of HIV-1 on the surface expression of these phagocytic receptors has previously been investigated by others. The majority of reports show that HIV-1 infection results in either elevated or unchanged surface expression of FcγRs or C'Rs (Petit *et al.*, 1988; Capsoni *et al.*, 1992; Nottet *et al.*, 1993; Capsoni *et al.*, 1994; Stent *et al.*, 1995; Dunne *et al.*, 1996). The results presented in this thesis are in agreement with those reports and suggest that the inhibition of phagocytosis by HIV-1 in both monocytes and macrophages occurs during or after receptor binding.

Monocytes and macrophages are distinct cell populations that differ in the expression of surface receptors (including CD4, CCR5 and phagocytic receptors) (Sonza *et al.*, 1995; Fear *et al.*, 1998) cytokine/chemokine production (reviewed in Fantuzzi *et al.*, 2000) and their susceptibility to HIV-1 infection (monocytes being highly refractory, whereas macrophages are fully permissive to HIV-1) (Sonza *et al.*, 1996). It is therefore possible that different mechanisms underlie defective phagocytosis in those two cell populations. Since only a small proportion of blood monocytes (0.001% to 1%) is infected with HIV-1, the high level of inhibition of phagocytosis in monocytes obtained from HIV-infected individuals may reflect an indirect consequence of infection. Conversely, a high proportion of MDM in cultures infected *in vitro* under conditions used in this study expressed HIV-1 antigens, in the range of 20 to 70% (Figure 6.2b), a proportion similar to for that reported for some populations of tissue macrophages in HIV-infected individuals (Orenstein *et al.*, 1997). This suggests that a direct effect of HIV-1 on phagocytosis by the host macrophages is possible. This is supported by a

previous report from our laboratory demonstrating defective phagocytosis of *Toxoplasma gondii* predominantly in MDM shown by flow cytometric analysis to be positive for p24 antigen (Biggs *et al.*, 1995), and by evidence that heat-killed HIV-1 did not impair phagocytosis of *Candida albicans* (Crowe *et al.*, 1994).

To investigate the mechanism of defective phagocytosis in tissue macrophages following HIV-1 infection *in vivo*, MDM infected with HIV-1 *in vitro* have been used as a model in my studies. MDM differentiated from peripheral blood monocytes *in vitro* are highly susceptible to HIV-1 infection, as are tissue macrophages. They produce HIV-1 for several weeks, and exhibit few cytopathic effects on infection (Crowe *et al.*, 1987; Sonza *et al.*, 1996). MDM are therefore a very useful model for tissue macrophages. Some investigators have used promonocytic leukemic cell lines such as U937 cells (Sundstrom and Nilsson, 1976) or U1 cells (a clone of chronically infected U937 cells) (Folks *et al.*, 1988) as a model for HIV-infected macrophages. However, a few considerable differences between primary macrophages and these cell lines have been reported including their proliferation and differentiation status as well as susceptibility to HIV-1 infection. Primary macrophages are generally considered to be non-replicating and terminally differentiated cells, whereas promonocytic cell lines are capable of replication as well as differentiation upon stimulation with phorbol 12-myristate 13-acetate (PMA) or retinoic acid (Clapham *et al.*, 1987; Pautrat *et al.*, 1990). Furthermore, monocytic cell lines such as U937 cells are not permissive to M-tropic strains of HIV-1 commonly used in laboratories such as HIV-1_{Ba-L}, but are susceptible to certain T cell-tropic strains of HIV-1 (Schuitemaker *et al.*, 1992a). For these reasons, such cell lines were not used in my studies. Furthermore, infection of MDM with HIV-1 results in a heterogenous population of cells comprising of both HIV-infected and uninfected MDM. Such inherent variability within the primary cell culture models is more relevant to the *in vivo* situation.

Phagocytosis mediated via the different phagocytic receptors utilises different signalling pathways to promote cytoskeletal rearrangement and engulfment of the target (Allen and Aderem, 1996). Most earlier reports delineating the signalling events during FcγR-mediated phagocytosis were performed using murine macrophages or cell lines transfected with FcγR, with only two reported studies using either human macrophages or monocytes (reviewed in Section 1.6.3). The mechanism underlying defective phagocytosis mediated via FcγRs by HIV-infected MDM was a focus of my studies. The results presented in this thesis show that the early signalling events during FcγR-mediated phagocytosis in human MDM are similar to those previously characterised in murine macrophages. In both species, tyrosine phosphorylation of a number of cellular proteins which localise around the phagocytic cup is seen, and phagocytosis is blocked by a protein tyrosine kinase inhibitor, genistein. The key protein tyrosine kinases, Hck (a macrophage-specific Src family kinase) and Syk (a docking site for γ signalling subunit of FcγR) are activated during this process. In addition, my study also provides the first evidence for activation of Pyk2 and redistribution of the phosphorylated form of paxillin (the actin-binding protein) between the cytoplasm and cytoskeleton during FcγR-mediated phagocytosis.

Having determined several key signalling molecules involved in FcγR-mediated phagocytosis by human MDM, the effect of HIV-1 on these signalling pathways was investigated. HIV-1 infection of MDM *in vitro* was shown to lead to a significant impairment of FcγR-mediated phagocytosis, associated with the inhibition of tyrosine phosphorylation of cellular proteins stimulated during phagocytosis. The strong inhibition of tyrosine phosphorylation of a wide variety of cellular proteins following stimulation with IgG-opsonised targets in HIV-infected MDM suggested dysfunction at an early stage in FcγR-mediated signalling events. Further results confirmed this and demonstrated impaired phosphorylation of the tyrosine kinases Hck and Syk during

phagocytosis by HIV-infected MDM. Impaired Syk phosphorylation would inhibit Syk-mediated activation of substrates required for actin polymerisation and cytoskeletal rearrangement essential for FcγR-mediated phagocytosis. Consistent with this data, HIV-infected MDM displayed reduced phosphorylation of paxillin, one of Syk downstream effector molecules. Similarly, decreased phosphorylation of Pyk-2, the FAK-related kinase, during FcγR-mediated phagocytosis in HIV-infected MDM has also been observed, although this work is ongoing in the laboratory. The activation status of other downstream substrates of Syk such as PI-3 kinase, Vav or Rho family GTPases during FcγR-mediated phagocytosis in HIV-infected MDM has not been investigated in this study, although they are likely to be reduced as well.

In seeking to understand why activation of Syk and Hck is impaired when expression of FcγRs is normal, the levels of associated γ-signalling subunit was investigated, since this is the only known intermediate between FcγRs and Src family kinases. Decreased protein expression of this molecule was found in HIV-infected MDM. As the γ signalling subunit is critical for Syk activation and subsequent signalling events during FcγR-mediated phagocytosis (Park *et al.*, 1993; Indik *et al.*, 1995b), reduced protein levels of the ITAM-containing γ-signalling subunit in HIV-infected MDM are likely to be responsible for the defective phagocytosis by these cells. To my knowledge, this is the first study reporting inhibition of tyrosine phosphorylation and signalling events underlying defective effector functions in HIV-infected macrophages. Since mRNA levels were normal, it is concluded that HIV-1 inhibits γ chain expression at a post-transcriptional level, indicating that protein synthesis, transport or degradation might be affected by HIV-1 infection. To determine the exact site of HIV-induced downmodulation of γ signalling subunit, further experiments such as pulse-chase studies of γ chain synthesis need to be performed.

Since the HIV-1 encoded protein, Nef, interacts with cellular proteins and kinases

which are potentially involved in FcγR-mediated phagocytosis, the effect of Nef was investigated. Surprisingly, it was found that *in vitro* electroporation of Nef into uninfected MDM did not inhibit the level of tyrosine phosphorylation during FcγR-mediated phagocytosis. Similarly, *in vitro* infection of MDM with Δ*nef* strains of HIV-1 impaired phagocytosis to the same degree as infection with wild type HIV-1, indicating that inhibition of phagocytosis by MDM *in vitro* occurs independent of Nef in this system. Infection of MDM with a *nef*-deleted HIV-1 expressing the γ-chain of FcγR resulted in a lower inhibition of FcγR-mediated phagocytosis, supporting the hypothesis that reduced expression of the γ-signalling subunit of FcγR may be responsible for impaired FcγR-mediated phagocytosis in HIV-infected MDM. Further experiments to correlate phagocytic efficiency of macrophages and γ chain levels are planned using the HIV-1 construct described in this thesis. This study therefore provides the first possible mechanism of defective cellular activation in HIV-infected macrophages not only during phagocytosis but potentially also underlying other functions mediated via FcγR. Impaired FcγR-mediated signalling may explain why HIV-infected macrophages fail to control opportunistic pathogens such as *Toxoplasma gondii* (Joiner *et al.*, 1990). The proposed mechanism is specific for FcγR-mediated phagocytosis and the events underlying defective phagocytosis mediated via other receptors by HIV-infected macrophages need to be investigated independently.

The mechanism underlying defective phagocytosis by peripheral blood monocytes obtained from HIV-infected individuals could not be investigated using similar biochemical approaches due to limitations in the amount of blood available from HIV-infected patients. For such studies a rapid whole blood assay has been developed in our laboratory, using only 100μl of blood coupled with flow cytometric analysis (Hewish *et al.*, 1996). Opportunistic pathogens relevant to HIV-1 pathogenesis such as *Mycobacterium avium* complex and *Toxoplasoma gondii* conjugated to FITC were used

as phagocytic targets in my research. Phagocytosis of MAC (utilising predominantly C'R for phagocytosis (Schlesinger and Horwitz, 1991)) and *Toxoplasma gondii* (utilising FcγRII; (Joiner *et al.*, 1990)) were impaired in peripheral blood monocytes from HIV-infected individuals compared to monocytes from uninfected controls. There appears to be no correlation between the observed inhibition of phagocytosis and either viral load or CD4 counts in individuals infected with HIV-1, suggesting that defective phagocytosis in HIV-infected individuals is not due to either of those factors, which is consistent with previous reports (Roilides *et al.*, 1993).

To investigate the mechanism underlying defective FcγR-mediated phagocytosis by blood monocytes obtained from HIV-infected individuals, a whole blood phagocytosis assay using FITC-tagged phalloidin to measure the level of actin polymerisation within monocytes was developed in this study. Using this assay inhibition of phagocytosis by monocytes from HIV-infected individuals was associated with dysregulated actin polymerisation within those cells. Increased basal levels of F-actin in monocytes from HIV-infected individuals, at different stages of disease, when compared to uninfected subjects has been demonstrated previously (Elbim *et al.*, 1999). The results, using a modification of the assay to assess FcγR-mediated phagocytosis and presented in this thesis, confirmed findings by Elbim *et al* and have further shown that monocytes from HIV-infected individuals display either minimal or no elevation of F-actin above their basal level when presented with IgG-opsonised targets. This is in contrast to significant net increases from the basal F-actin level in uninfected controls. As actin polymerisation plays a critical role in the formation of the phagocytic cup and ingestion of phagocytosed particles, defective actin remodelling is a potential mechanism underlying impaired phagocytic function in HIV-infected individuals. To determine if this HIV-induced effect occurs specifically at the actin polymerisation level or rather as a consequence of altered signalling events after engagement of FcγRs, the early signalling pathways during FcγR-mediated phagocytosis need to be investigated. The

whole blood assay developed in this study to assess the level of actin polymerisation during phagocytosis might be further modified to determine the level of tyrosine phosphorylation during FcγR-mediated phagocytosis. Similarly, the phosphorylation status of Hck or Syk could be determined by flow cytometric analysis, provided that suitable monoclonal antibodies against phosphorylated forms of those kinases are available.

In circulating blood monocytes, in contrast to tissue macrophages, the viral burden is very low (0.01 to 1% of cells are infected), suggesting that a defective phagocytosis in those cells could be rather explained by an indirect effect of HIV-1 infection. Impaired phagocytosis might therefore reflect dysregulation of cytokine/chemokine production by monocytes and/or other cells present in blood (reviewed in Section 1.3.4). In support of this proposal, defective phagocytosis by neutrophils (not targets for HIV-1 infection) from HIV-positive individuals has also been reported (Dobmeyer *et al.*, 1995). Phagocytosis might be also impaired by viral proteins present in the circulation. HIV-1 glycoprotein, gp120, has been previously shown to inhibit phagocytosis of *Cryptococcus neoformans* by bronchoalveolar macrophages (Wagner *et al.*, 1992).

Phagocytosis of MAC and *Toxoplasma gondii* is not impaired in peripheral blood monocytes from members of the Sydney Blood Bank Cohort (SBBC) infected with an attenuated strain of HIV-1 containing deletions within the *nef* gene and deletions and duplications within the overlapping long terminal repeat overlap (Deacon *et al.*, 1995). Normal phagocytic efficiency of monocytes in the blood of SBBC members is therefore consistent with their long term non/slow progression and in all but one, the absence of opportunistic infections (Learmont *et al.*, 1999). Since monocytes from individuals infected with *nef*-deleted HIV-1 displayed normal phagocytic efficiency, Nef might be responsible for the observed impairment of phagocytosis by monocytes following HIV-1 infection. However, as the attenuated virus from this group also has deletions and

rearrangements within the LTR (Deacon *et al.*, 1995), a tight correlation of phagocytic efficiency and *nef*-deletion is not possible. Since the inhibition of phagocytosis does not correlate with viral load or CD4 counts (discussed above), the normal CD4 levels seen in the cohort members is not likely to be a reason why phagocytosis is unaltered. This study demonstrates for the first time normal phagocytic activity of monocytes obtained from long-term non-progressors, and provides a mechanism, at least in part, as to why these cohort members remain asymptomatic. As inhibition of phagocytosis by MDM infected with HIV-1 *in vitro* was independent of Nef, these results suggest that if HIV-1 Nef protein has an effect on monocyte and macrophage phagocytic function, it is mediated through a complex indirect effect only evident *in vivo.* Given that the impairment of phagocytosis in monocytes from HIV-infected individuals is associated with defective actin rearrangements, it would be interesting to determine the F-actin content in monocytes from individuals infected with the attenuated strain of HIV-1 containing deletions in *nef* gene. To date this has not been possible due to receiving blood samples of very small volumes.

The ultimate aim of this thesis was to propose a potential adjunctive immunotherapy which would help to control opportunistic infections *in vivo.* As GM-CSF is a well-known immunomodulator augmenting monocyte and macrophage function of human and murine monocytes/macrophages (reviewed in Section 1.5.3.2), the ability of this cytokine to restore phagocytosis following HIV-1 infection was investigated. This study provides clear evidence of improvement of phagocytosis of MAC by MDM infected with HIV-1 as well as in uninfected MDM from the same donors. A mutant form of GM-CSF, E21R, binding only to the α chain of the receptor, failed to stimulate phagocytosis of MAC, suggesting that the principal signals controlling phagocytosis occur via the β-chain of the receptor.

GM-CSF stimulation of MDM prior to HIV-1 infection not only augmented phagocytosis of MAC by those cells, but also inhibited HIV-1 replication. The effect of GM-CSF on HIV-1 replication in macrophages has been controversial. Most of the early studies report augmentation or no change in viral production, with two recent studies suggesting inhibition of HIV-1 replication in MDM by GM-CSF (reviewed in Section 1.5.3.2). The results presented in this thesis demonstrate that this observed inhibitory effect is GM-CSF-specific since it is totally reversible by addition of neutralising monoclonal antibody, 4D4, but not by addition of a non-neutralising anti-GM-CSF control monoclonal antibody, 4A12. Furthermore, E21R GM-CSF, binding only to the α-chain of the GM-CSF receptor (Lopez *et al.*, 1992), does not affect HIV-1 replication. The inhibition of HIV-1 replication was found to occur at or before transcription of viral genes, in agreement with results reported previously by Matsuda *et al.* (Matsuda *et al.*, 1995).

Due to concerns regarding the potential activation of HIV-1 replication, GM-CSF is used rarely for the treatment of HIV-infected patients. Early clinical trials show that GM-CSF treatment of HIV-infected patients in the absence of antiretroviral therapy may result in increased serum p24 antigen levels and plasma HIV-1 RNA titres (Kaplan *et al.*, 1991; Lafeuillade *et al.*, 1996). However, when used in combination with effective antiretroviral therapy, GM-CSF has been safely administered to patients without any significant increase in viral load (Yarchoan *et al.*, 1990; Krown *et al.*, 1992). In addition GM-CSF has been found to augment the antiretroviral activity of 2',3'-dideoxy-3'-azidothymidine (AZT), concurrent with an increased anabolism of AZT to its active 5'-triphosphate moieties (Perno *et al.*, 1992). Data from a recent clinical study of HIV-infected patients receiving antiretroviral therapy and GM-CSF have shown that patients experienced a decrease in viral load and an increase in CD4 counts (Skowron *et al.*, 1999; Brites *et al.*, 2000).

The clinical study presented in this thesis demonstrates a beneficial effect of GM-CSF therapy on antimycobacterial activity without a significant increase in viral load. The augmentation of phagocytosis of MAC and improvement in bacteraemia (measured by the length of time required for MAC blood cultures to become positive) was seen in the single HIV-infected patient with multi drug-resistant disseminated MAC adjunctively treated with GM-CSF. Clinical improvement and augmented monocyte phagocytic function following GM-CSF therapy has similarly been reported in patients with advanced HIV-1 infection and other opportunistic infections, including *Candida albicans* (Vazquez *et al.*, 1998; Brites *et al.*, 2000). Taken together, these data suggest that adjunctive GM-CSF therapy may be useful to augment monocyte/macrophage functions and to restore activity against a variety of opportunistic pathogens in HIV-1 infected individuals.

The mechanism whereby GM-CSF restores phagocytosis by monocytes and macrophages following HIV-1 infection remains unknown. The results presented in this thesis demonstrate that short-term stimulation of uninfected MDM with GM-CSF augments FcγR-mediated phagocytosis and increases the levels of tyrosine phosphorylation in phagocytosing MDM cultures, indicating tyrosine kinase-mediated activation. Since GM-CSF treatment of MDM did not enhance phosphorylation of Syk, I postulate that GM-CSF exerts its stimulatory effect on FcγR-phagocytosis by acting either distal to Syk kinase in FcγR-mediated signalling cascade or in a parallel pathway mediated via Fcγ receptor. A study by Rossman *et al* (1993) shows that stimulation of human monocytes with GM-CSF for 48 hours results in increased expression of FcγR II, and increased binding of IgG-opsonised particles (Rossman *et al.*, 1993). Therefore, GM-CSF may augment FcγR-mediated phagocytosis by several mechanisms, i.e. by stimulating the level of Fcγ receptor expression as well as directly affecting FcγR signalling pathways. GM-CSF augments phagocytosis mediated via both FcγR and C'Rs (Bermudez and Young, 1990; Capsoni *et al.*, 1992), therefore it is likely that this

cytokine acts on the distal signalling events common to phagocytosis mediated via different receptors, such as mechanisms involved in cytoskeletal rearrangements.

Collectively, the results obtained in this thesis clearly demonstrate that HIV-1 inhibits phagocytosis by both monocytes and macrophages. Such impaired phagocytosis following HIV-1 infection is likely to directly contribute to HIV-1 pathogenesis, by allowing reactivation of opportunistic pathogens normally controlled by cells of macrophage lineage. The specific mechanisms underlying FcγR-mediated phagocytosis by human MDM have been characterised and involve the key tyrosine kinases Hck, Syk and Pyk2, and the actin-binding protein, paxillin (Kedzierska *et al.*, 2001b). Impaired FcγR-mediated phagocytosis by HIV-infected MDM results from the strong inhibition of tyrosine phosphorylation of cellular proteins, specifically at the early signalling events such as Hck, Syk phosphorylation. The reduced protein level of the γ chain of FcγRs (upstream of Hck and Syk) found in HIV-infected MDM is likely to be responsible for defective phagocytosis by those cells. Defective phagocytosis by peripheral blood monocytes obtained from HIV-infected individuals is associated with dysregulated actin polymerisation (Kedzierska *et al.*, 2001a). SBBC members infected with attenuated strains of HIV-1 display normal monocyte functions, suggesting that *nef*/LTR region might be responsible for inhibition of phagocytosis by monocytes *in vivo* (Kedzierska *et al.*, 2001a). GM-CSF restores phagocytosis of MAC by MDM infected with HIV-1 *in vitro* as well as by monocytes within the peripheral blood of HIV-infected patient (Kedzierska *et al.*, 2000b). GM-CSF also inhibits HIV-1 replication in MDM *in vitro* (Kedzierska *et al.*, 2000a). These results indicate that adjunctive GM-CSF therapy may be useful to augment monocyte and macrophage function and to restore antimycobacterial activity in HIV-1 infected patients with opportunistic infections. Therefore, this thesis combines basic research with some supporting clinical evidence to provide a scientific rationale for using GM-CSF treatment in the setting of HIV-1 infection. Since opportunistic infections are the major problem for patients failing HAART and are likely to increase as the

resistance to antiretroviral drugs becomes more widespread, such adjunctive immunotherapy able to control opportunistic pathogens is urgently needed.

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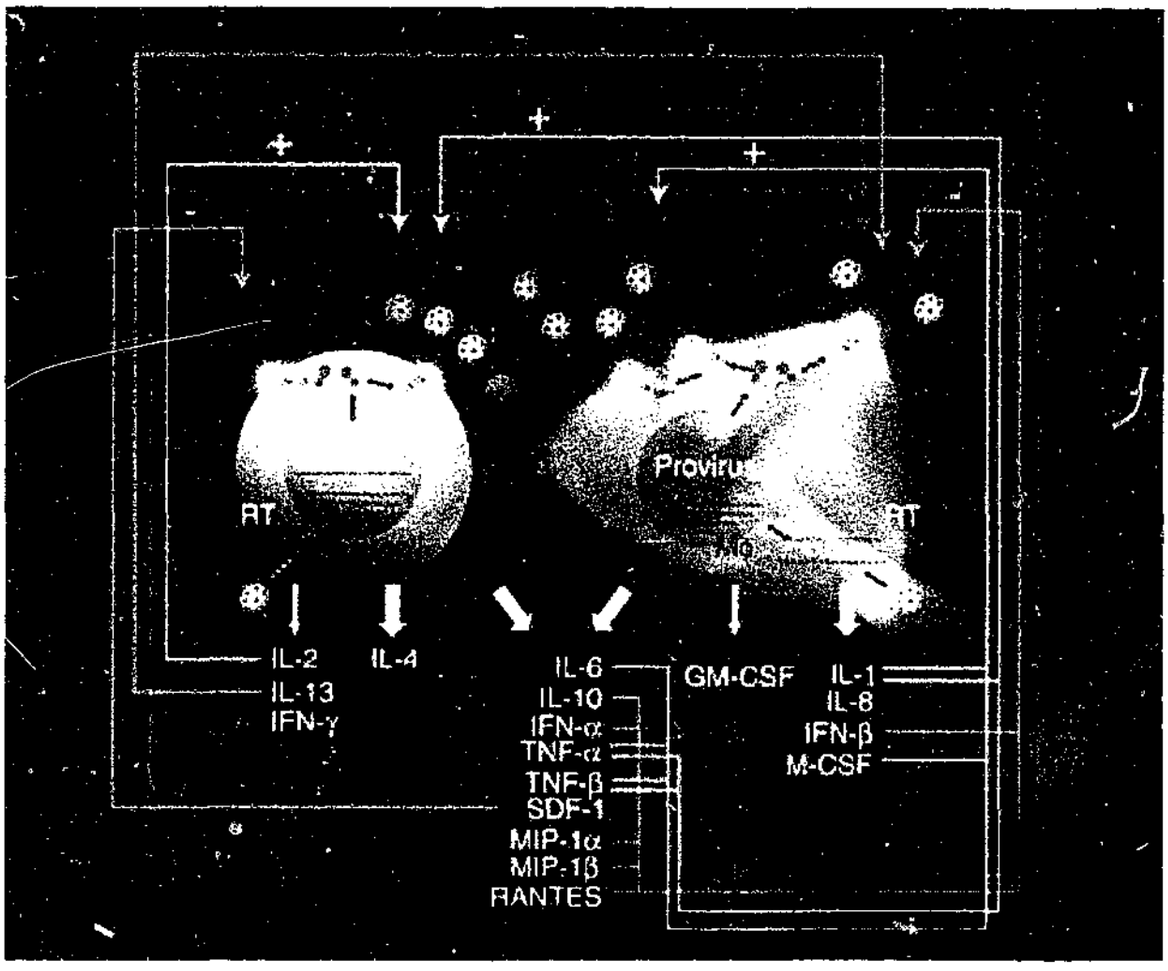

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Review
Cytokines and HIV-1: interactions and clinical implications

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Cytokines play an important role in controlling the homoeostasis of the immune system. Infection with HIV results in dysregulation of the cytokine profile *in vivo* and *in vitro*. During the course of HIV-1 infection secretion of T-helper type 1 (Th1) cytokines, such as interleukin (IL)-2, and antiviral interferon (IFN)-γ, is generally decreased, whereas production of T helper type 2 (Th2) cytokines, IL-4, IL-10, proinflammatory cytokines (IL-1, IL-6, IL-8) and tumour necrosis factor (TNF)-α, is increased. Such abnormal cytokine production contributes to the pathogenesis of the disease by impairing cell-mediated immunity. A number of cytokines have been shown to modulate *in vitro* HIV-1 infection and replication in both CD4 T lymphocytes and cells of macrophage lineage. HIV-inductive cytokines include: TNF-α, TNF-β, IL-1 and IL-6, which stimulate HIV-1 replication in T cells and monocyte-derived macrophages (MDM), IL-2, IL-7 and IL-15, which upregulate HIV-1 in T cells, and macrophage-colony stimulating factor, which stimulates HIV-1 in MDM. HIV-suppressive cytokines include: IFN-α, IFN-β and IL-16, which inhibit HIV-1 replication in T cells and MDM, and IL-10 and IL-13, which inhibit HIV-1 in MDM. Bifunctional cytokines such as IFN-γ, IL-4 and granulocyte-macrophage colony-stimulating factor have been shown to have both inhibitory and stimulatory effects on HIV-1. The β-chemokines, macrophage-inflammatory protein (MIP)-1α, MIP-1β and RANTES are important inhibitors of macrophage-tropic strains of HIV-1, whereas the α-chemokine stromal-derived factor-1 suppresses infection of T-tropic strains of HIV-1. This review outlines the interactions between cytokines and HIV-1, and presents clinical applications of cytokine therapy combined with highly active antiretroviral therapy or vaccines.

Keywords: HIV-1, cytokines, chemokines, macrophages, T cells

Introduction

HIV, a member of the lentivirus subfamily, causes progressive degeneration of the immune system, leading to the development of AIDS (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983). The main cellular targets of HIV are cells expressing the CD4 molecule, mainly the CD4 subset of T cells and cells of macrophage lineage (both peripheral blood monocytes and tissue macrophages). These cell types express chemokine coreceptors for HIV entry, which are important for the cellular tropism of HIV. CCR5 is the major co-receptor for macrophage (M)-tropic (R5) strains of HIV-1, and CXCR4 is the main coreceptor for T cell (T)-tropic (X4) strains (Alkhatib *et al.*, 1996a). HIV-associated immune deficiency results from a decline in the number of CD4 T lymphocytes, defective immunological function of both T cells and macrophages as well as dysregulation of cytokine production (Diamond *et al.*, 1988; Biggs *et al.*, 1995; Kedzierska *et al.*, 2000b). Several groups have demonstrated that HIV infection is associated with a switch from a predominantly T-helper type 1 (Th1) to T-helper type 2 (Th2) response, that is, decreased production of interleukin (IL)-2 and interferon (IFN)-γ and increased levels of IL-4 and IL-10 secretion (Clerici & Shearer, 1993), and increased production of proinflammatory cytokines IL-1, IL-6, IL-8 and tumour necrosis factor (TNF)-α (Esser *et al.*, 1991).

The susceptibility of cells to productive HIV-1 infection depends on the level of T-lymphocyte activation and the stage of monocyte/macrophage differentiation (Zack *et al.*, 1990; Sonza *et al.*, 1996), suggesting that cytokines and growth factors that promote activation and differentiation of cells of the immune system can also modulate both HIV-1 infection and replication. Effects of cytokines on

HIV-1 can be either inhibitory, stimulatory or bifunctional (that is, both inhibitory and stimulatory), indicating that HIV-1 infection and replication *in vivo* and *in vitro* is under the continuous regulation of a complex cytokine network produced by a variety of cells. This review outlines the overall interactions between cytokines and HIV-1, considers the mechanisms by which cytokines may contribute to HIV-1 pathogenesis and presents examples of clinical applications of cytokine therapy combined with highly active antiretroviral therapy (HAART) or vaccines.

HIV-1 alters cytokine secretion *in vitro* and *in vivo*

HIV-1 infection is associated with dysregulation of cytokine production *in vivo*. Decreased secretion of specific cytokines and increased production of others contributes to the progression of HIV-associated immune deficiency (Clerici & Shearer, 1993). Using different experimental approaches, numerous investigators have examined cytokine production by cells from HIV-infected individuals as well as within cultures infected with HIV-1 *in vitro* (Table 1).

Interleukins

IL-1. IL-1, a 17 kDa protein, is a proinflammatory cytokine produced predominantly by cells of macrophage lineage, and to a lesser extent by B cells, endothelial cells and fibroblasts in response to infections and inflammation (Dinarello, 1997). There are two biologically active forms of IL-1 (IL-1α and IL-1β). *In vitro* infection of monocyte-derived macrophages (MDM) with HIV-1 results in upregulation and constitutive secretion of IL-1 in culture supernatants (Esser *et al.*, 1991, 1996, 1998). Similarly, monocytes obtained from HIV-infected individuals display constitutive production of biologically active IL-1α and IL-1β proteins, which is suppressed during the course of antiretroviral therapy (Sadeghi *et al.*, 1995; Baqui *et al.*, 2000).

IL-2. IL-2, a 14–17 kDa monomeric glycoprotein comprising four α-helices, is a Th1 cytokine produced by CD4 and CD8 T cells. The primary role of IL-2 is to stimulate the proliferation of activated T cells, cytotoxic activity of CD8 T cells and natural killer (NK) cells, as well as inducing B cells and monocyte function (Smith, 1988). *In vitro* HIV-1 infection of CD4 T cells results in a significant downmodulation of IL-2 mRNA expression that can be reversed by culturing cells in the presence of antiretroviral drugs (Fan *et al.*, 1997). Stimulation of peripheral blood mononuclear cells (PBMC), CD8 T cell-depleted PBMC and CD4 T cell lines with HIV-1 envelope glycoprotein (gp160) also reduces IL-2 secretion *in vitro* (Hu *et al.*, 1994). Similarly, deficiency of IL-2 production is one of the first immunological defects that can be demonstrated in HIV-infected individuals (Kinter & Fauci, 1996). IL-2 production by *in vitro* activated PBMC, isolated from HIV-infected subjects at various stages of disease, is significantly decreased when compared with PBMC obtained from uninfected subjects (Barcellini *et al.*, 1994; Meroni *et al.*, 1996; Meyaard *et al.*, 1996). IL-2 production is related to CD4 T cell counts and clinical status of the patients. It is significantly decreased in patients with CD4 cell counts of <200 cells/mm^3 compared to those with higher CD4 counts (Hong *et al.*, 1998).

IL-4. IL-4, an 18 kDa monomeric protein, is a Th2 cytokine produced predominantly by activated CD4 T cells, but also by NK cells, mast cells and basophils. It stimulates B-cell activation and differentiation, secretion of IgG1 and IgE, T-cell activation and MHC II expression on B cells and macrophages (Paul, 1991). Several studies have shown that increased secretion of IL-4 is associated with HIV-1 infection *in vivo*. IL-4 production is upregulated in mitogen-stimulated PBMC and CD4 T cells obtained from HIV-infected patients at various stages of disease, including recently infected patients (Barcellini *et al.*, 1994; Maggi *et al.*, 1994). Unstimulated PBMC from HIV-infected individuals not receiving antiretroviral therapy have detectable IL-4 mRNA expression, which falls to undetectable levels once HAART is commenced (Imami *et al.*, 1999). Enhanced secretion of IL-4 by HIV-seropositive individuals receiving no antiviral therapy has been recently confirmed by investigators using a sensitive three-colour flow cytometric assay to quantify intracellular cytokine production at the single cell level (Breytenbach *et al.*, 2001). However, there are conflicting reports regarding the effect of HIV-1 infection on IL-4 production, as reduced or normal IL-4 levels have also been reported in supernatants of cultured PBMC and purified CD4 T cells obtained from HIV-infected individuals (Re *et al.*, 1992; Meyaard *et al.*, 1996).

IL-6. IL-6, a 26 kDa protein, is a proinflammatory cytokine produced by a variety of cells, including T cells, B cells, macrophages, fibroblasts and endothelial cells, in response to viral or bacterial infection. It has a wide spectrum of activities including B-cell stimulation, monocyte differentiation and induction of IL-4 producing cells (Rincon *et al.*, 1997). *In vitro* HIV-1 infection of MDM induces upregulation and constitutive secretion of IL-6 in culture supernatants (Esser *et al.*, 1991, 1996, 1998; Foli *et al.*, 1997). Stimulation with HIV-1 glycoproteins gp41 or gp120 increases IL-6 production by the human monocytic cell line, THP-1, and mitogen-activated PBMC, respectively (Takeshita *et al.*, 1995; Capobianchi, 1996). Similarly, IL-6

Table 1. Effects of HIV-1 infection on cytokine production *in vivo* and *in vitro*

Cytokine	Secretion level	Source of cells	HIV infection	Reference
IL-1	↑	MDM	*In vitro*	Esser *et al.*, 1991,1996,1998
	↑	monocytes	*In vivo*	Sadeghi *et al.*, 1995; Baqui *et al.*, 2000
IL-2	↓	CD4 T cells	*In vitro*	Fan et al., 1997
	↓	PBMC, CD4 T cells	*In vitro* - gp160 stimulation	Hu *et al.*, 1994
	↓	PBMC	*In vivo*	Barcellini *et al.*, 1994; Meroni *et al.*, 1996; Meyaard *et al.*, 1996; Hong *et al.*, 1998
IL-4	↑	PBMC, CD4 T cells	*In vivo*	Barcellini *et al.*, 1994; Maggi *et al.*, 1994; Imami *et al.*, 1999
	No change	CD4 T cells	*In vivo*	Meyaard *et al.*, 1996
	↓	CD4 T cells	*In vivo*	Re *et al.*, 1992
IL-6	↑	MDM	*In vitro*	Esser *et al.*, 1991,1996, 1998; Foli *et al.*, 1997
	↑	THP-1 cell line, PBMC	*In vitro* - gp41, gp120 stimulation	Takeshita *et al.*, 1995; Capobianchi *et al.*, 1996
	↑	Serum	*In vivo*	Birx *et al.*, 1990; Breen *et al.*, 1990
IL-8	↑	MDM	*In vitro*	Esser *et al.*, 1991,1996,1998
	↑	Serum, bronchoalveolar fluid	*In vivo*	Denis *et al.*, 1994a
IL-10	↑	Monocytes, MDM	*In vitro*	Borghi *et al.*, 1995
	↑	PBMC, Monocytes, MDM	*In vitro* - gp41, gp120 stimulation	Borghi *et al.*, 1995; Capobianchi *et al.*, 1996; Schols *et al.*, 1996; Gessani *et al.*, 1997; Taoufic *et al.*, 1997; Barcova *et al.*, 1998
	↑	PBMC, Bronchoalveolar fluid	*In vivo*	Ameglio *et al.*, 1994; Barcellini *et al.*, 1994; Denis *et al.*, 1994; Meroni *et al.*, 1996; Rizzardi *et al.*, 1998; Imami *et al.*, 1999
IL-12	↑	Monocytes, MDM	*In vitro* - gp120 stimulation	Fantuzzi *et al.*, 1996
	↓	PBMC	*In vivo*	Marshall *et al.*, 1999
IL-13	↓	PBMC, CD4, CD8 T cells	*In vivo*	Bailer *et al.*, 1999; Zou *et al.*, 1997
FN-α	↓	Monocytes, MDM	*In vitro*	Gendelman *et al.*, 1990; Perno *et al.*, 1994
	↑	CD4 T cells, Monocytes	*In vitro* - gp120 stimulation	Capobianchi *et al.*, 1996
	↑	Serum	*In vivo*	Ambrus *et al.*, 1989; Francis *et al.*, 1992; Bizzini *et al.*, 1999
IFN-β	↑	Monocytes, MDM	*In vitro* - gp120 stimulation	Gessani *et al.*, 1994a,1997
IFN-γ	↓	CD4 T cells	*In vitro*	Fan *et al.*, 1997
	↑	CD4 T cells	*In vivo*	Vyakarnam *et al.*, 1990
	↓	PBMC	*In vitro*	Meroni *et al.*, 1996; Meyaard *et al.*, 1996; Bailer *et al.*, 1999
GM-CSF	↓	MDM	*In vitro*	Esser *et al.*, 1996,1998
	No change	Monocytes	*In vivo*	Delemarre *et al.*, 1998
M-CSF	↑	MDM	*In vitro*	Gruber *et al.*, 1995; Kutza *et al.*, 2000
	↑	Cerebrospinal fluid	*In vivo*	Gallo *et al.*, 1994
TNF-α	↑	PBMC, CD4 T cells, MDM	*In vitro*	Vyakarnam *et al.*, 1990; Esser *et al.*, 1991,1996; Foli *et al.*, 1997
	↑	Monocytes, PBMC, Serum	*In vivo*	Lahdevirta *et al.*, 1988; Rizzardi *et al.*, 1998; Baqui *et al.*, 2000
TNF-β	↑	PBMC, CD4 T cells	*In vitro*	Vyakarnam et *al.*, 1990
	↑	Cerebrospinal fluid	*In vivo*	Jassoy *et al.*, 1993
SDF-1	↑	PBMC	*In vivo*	Ohashi *et al.*, 1998
MIP-1α,-β, RANTES	↑	CD4 T cells, MDM	*In vitro*	Greco et *al.*, 1999
	↑	PBMC, Plasma	*In vivo*	Cocchi *et al.*, 1995; Malnati *et al.*, 1997; Garzino-Demo *et al.*, 1999; Fantuzzi *et al.*, 2001

IL, interleukin; MDM, monocyte-derived macrophages; PBMC, peripheral blood mononuclear cells; ↑, increase; ↓ decrease; SDF, stromal-derived factor; MIP, macrophage inflammatory protein.

secretion is increased in the sera of HIV-infected individuals, particularly at the early stages of infection, when compared with HIV-seronegative subjects (Birx *et al.*, 1990; Breen *et al.*, 1990). CD4 T cells and monocytes from HIV-infected subjects, stimulated with phytohaemagglutinin (PHA) or lipopolysaccaride (LPS), respectively, show enhanced secretion of IL-6 compared with cells from uninfected individuals (Cayota *et al.*, 1992; Baqui *et al.*, 2000). The spontaneous production of IL-6 has also been observed in B cells obtained from HIV-infected individuals, suggesting that HIV is able to induce IL-6 production from a variety of cells of the immune system (Kehrl *et al.*, 1992).

IL-8. The inflammatory and antimicrobial cytokine, IL-8, is produced by macrophages, T cells, neutrophils and endothelial cells in acute and chronic inflammatory states. It is a potent chemotactic factor for T cells, NK cells, neutrophils and basophils (reviewed in (Harada *et al.*, 1994).

IL-8 is upregulated and constitutively secreted following *in vitro* HIV-infection of MDM (Esser *et al.*, 1991, 1996, 1998), but reduced in the chronically infected U937 monocytic cell line (Ohashi *et al.*, 1994). Elevated levels of IL-8 have been also observed in patients with HIV-1 infection, both in patients' serum (Matsumoto *et al.*, 1993) and bronchoalveolar fluid (Denis & Ghadirian, 1994a).

IL-10. IL-10, an 18kDa protein, is a Th2 cytokine produced by activated T and B cells, monocytes, macrophages and keratinocytes. It inhibits T cell proliferation, predominantly by suppressing synthesis of Th1 cytokines (including IL-2, IFN-γ), and inhibits macrophage activation and secretion of pro-inflammatory cytokines (IL-1, IL-6, IL-8, IL-12, TNF-α) (reviewed in Moore *et al.*, 2001).

In vitro HIV-1 infection as well as gp120 or gp41 treatment of PBMC, monocytes and MDM results in a significant increase in IL-10 secretion and IL-10 mRNA levels in comparison to uninfected or untreated cells (Borghi *et al.*, 1995; Capobianchi *et al.*, 1996; Schols and De Clerq, 1996; Gessani *et al.*, 1997; Taoufik *et al.*, 1997; Barcova *et al.*, 1998). However, HIV-1 infection of MDM with M-tropic strains of HIV-1 at various multiplicities of infection has also been shown not to induce significant IL-10 secretion or IL-10 mRNA overexpression (Dereuddre-Bosquet *et al.*, 1997).

The production of IL-10 increases with HIV-1 infection *in vivo*. There is increased IL-10 secretion (Ameglio *et al.*, 1994; Barcellini *et al.*, 1994; Meroni *et al.*, 1996) and mRNA expression (Imami *et al.*, 1999) in PBMC obtained from HIV-infected patients at all stages of HIV-1 infection compared to uninfected controls. In patients with advanced HIV-1 infection elevated IL-10 production correlates with increased levels of the pro-inflammatory cytokine, TNF-α, suggesting that a combination of those two cytokines has immunopathological consequences and is related to progression to disease (Rizzardi *et al.*, 1998). Increased levels of IL-10 have been also observed in bronchoalveolar fluid from HIV-infected patients at all stages of infection when compared to levels in uninfected persons (Denis & Ghadirian, 1994a).

IL-12. IL-12, a 70 kDa heterodimeric protein consisting of two polypeptide chains, p40 and p35, produced predominantly by cells of macrophage lineage, plays a significant role in cell-mediated immunity against infections by regulating IFN-γ production and modulating responses of cytotoxic T lymphocytes (and macrophages (Ma & Montaner, 2000). HIV-1 tion induces production of IL-12 by macrophages, subsequently leading to enhanced production of IFN-γ by T cells and NK cells (Trinchieri & Scott, 1999). Stimulation of monocytes and macrophages with gp120 induces IL-12 secretion in the supernatant and expression of IL-12 mRNA of both p35 and p40 subunits (Fantuzzi *et al.*, 1996). However, PBMC isolated from HIV-infected subjects are deficient in their ability to produce IL-12 when compared with uninfected donors, and unable to upregulate the IL-12 receptor β2 chain in response to mitogenic stimuli, suggesting that IL-12 secretion is modulated differently in monocyte/macrophages infected with HIV-1 *in vitro* and in PBMC during HIV-1 infection *in vivo* (Marshall *et al.*, 1999).

IL-13. IL-13, a Th2 cytokine, is produced by T cells and dendritic cells. It shares a receptor subunit and immunoregulatory functions with IL-4 on B cells, monocytes, dendritic cells and fibroblasts, but not T cells, due to the absence of IL-13 receptors on those cells. IL-13 is required for optimal IgE production by B cells, induces MHC II expression on antigen presenting cells (APC) and regulates inflammation by inhibiting proinflammatory cytokine and chemokine production (reviewed in (de Vries, 1998). PBMC from HIV-infected individuals activated *in vitro* via CD3/CD28, secrete significantly lower amounts of IL-13 than uninfected controls (Bailer *et al.*, 1999). This defect is observed in both CD4 and CD8 T-cell subsets, concurrent with downmodulation of IL-13 mRNA level, and correlates positively with the patient's CD4 T cell count and negatively with HIV-1 viral load (Zou *et al.*, 1997; Bailer *et al.*, 1999).

IL-16. IL-16, a 16 kDa homotetrameric cytokine, is produced by CD4 T cells, mast cells and eosinophils in response to stimuli such as mitogens, histamine and serotonin. It chemoattracts T cells, monocytes and eosinophils, and modulates T-cell function by enhancing adhesion and IL-2 receptor α and HLA-DR expression (Krakauer *et al.*,

1998). IL-16 is a natural ligand for the CD4 receptor and inhibits HIV-1 replication (Maciaszek *et al.*, 1997). Serum levels of IL-16 in asymptomatic HIV-infected individuals are within the normal range or slightly increased, although a significant reduction in IL-16 secretion occurs with progression of HIV-1 infection when compared with levels in uninfected subjects (Amiel *et al.*, 1999; De Paoli *et al.*, 1999). IL-2 treatment of HIV-infected individuals significantly enhances production of IL-16 after 2 weeks of therapy (De Paoli *et al.*, 1999).

Interferons

Interferons (IFN-α, -β and -γ) have a wide variety of antiviral, antiproliferative and immunomodulatory effects, and are capable of inhibiting viral infection in a non-specific manner. IFN-α (produced by leukocytes) and IFN-β (produced by fibroblasts and lymphocytes) are 18 kDa monomers, share a common type I IFN receptor, activate indistinguishable signalling pathways and have the same biological actions (Bazan, 1990). Their functions include stimulation of CTL-mediated killing by increasing MHC I expression, stimulation of NK cell lytic function, inhibition of viral replication and cell proliferation. IFN-γ, a homodimer glycoprotein consisting of 21–24 kDa subunits, is produced by activated T and NK cells and binds to a distinct type II IFN receptor (Bazan, 1990). Apart from shared activities with IFN-α and -β, this cytokine also promotes T- and B-cell proliferation, activates mononuclear phagocytes and neutrophils, increases MHC II expression and suppresses IL-4 responses (Le Page *et al.*, 2000).

IFN-α. Productive HIV-infection of monocytes *in vitro* results in reduced production of IFN-α (Gendelman *et al.*, 1990; Perno *et al.*, 1994), although binding of CD4 on T cells or monocytes with HIV-1 envelope glycoprotein, gp120, has been reported to induce IFN-α production in both cell types (Capobianchi, 1996). Increased levels of IFN-α in serum (Ambrus *et al.*, 1989; Francis *et al.*, 1992; Bizzini *et al.*, 1999) and cerebrospinal fluid have been reported in HIV-infected individuals with dementia compared with non-demented and uninfected individuals, suggesting that this cytokine may play a role in the pathogenesis of HIV-related dementia (Rho *et al.*, 1995).

IFN-β. *In vitro* HIV-1 infection or gp120 treatment of monocytes of MDM results in the induction of low levels of IFN-β compared with uninfected/untreated cells (Gessani *et al.*, 1997). HIV-1 infection of MDM in the presence of antibodies against IFN-β results in increased levels of p24 antigen in the culture supernatant, suggesting that IFN-β secreted by MDM after HIV-1 infection treatment effectively inhibits HIV-1 replication (Gessani *et al.*, 1994a).

IFN-γ. The literature provides conflicting evidence regarding the effect of HIV-1 infection on IFN-γ production. *In vitro* HIV-1 infection of CD4 T cells results in a significant downmodulation of IFN-γ mRNA expression, blocked by zidovudine treatment (Fan *et al.*, 1997), but other investigators report enhanced secretion of IFN-γ in the culture supernatant (Vyakarnam *et al.*, 1990). The discrepancies may be explained by chemokine receptor usage of the HIV-1 strain, because increased production of IFN-γ by HIV-infected CD4 T cells *in vitro* has been shown to occur in cells infected with only non-syncytium inducing R5 strains of HIV-1, and not with syncytium inducing X4 strains (Greco *et al.*, 1999). The production of IFN-γ in HIV-infected individuals decreases with disease progression (Clerici & Shearer, 1993; Hong *et al.*, 1998). IFN-γ secretion is significantly reduced in the supernatant of PBMC obtained from HIV-infected subjects, and is activated *in vitro* when compared with those from uninfected persons (Meroni *et al.*, 1996; Meyaard *et al.*, 1996), and correlates with the patient's CD4 count and negatively with HIV-1 plasma viral load (Bailer *et al.*, 1999). Downmodulation of IFN-γ production is observed in both CD4 and CD8 T cell subsets, and is associated with reduced IFN-γ mRNA levels (Imami *et al.*, 1999), as well as reduced numbers of IFN-γ-producing cells (Meyaard *et al.*, 1996). Treatment of patients who have late stage HIV-1 infection with antiretroviral therapy, including protease inhibitors, results in a significant increase in IFN-γ production, when compared with untreated patients (Kelleher *et al.*, 1999). However, there are also reports of increased IFN-γ production by cytotoxic T cells derived from blood and cerebrospinal fluid of HIV-infected individuals (Jassoy *et al.*, 1993).

Colony-stimulating factors

Granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF, a 23–28 kDa glycoprotein comprising a two-stranded antiparallel β-sheet and four α-helices (Lopez *et al.*, 1992), is produced by a variety of cell types, including activated T cells, endothelial cells, macrophages and fibroblasts. This haemopoietic growth factor is required for survival, proliferation and differentiation of granulocyte/macrophage progenitor cells and the function of their mature progeny (Armitage, 1998). *In vitro* infection of MDM with HIV-1 results in decreased secretion of GM-CSF in comparison with uninfected cells (Esser *et al.*, 1996). Reduced GM-CSF production occurs in all the cells present in HIV-infected cultures, regardless of their level of infection, as determined by immunocytochemical staining for p24 antigen, which suggests that dysregulation of cytokine production occurs not only in HIV-infected MDM but also in non-infected cells via an indirect mechanism (Esser *et al.*, 1998). The production of GM-CSF by non-stimulated monocytes from AIDS patients is similar

to that of uninfected individuals, although it increases after LPS stimulation (Delemarre *et al.*, 1998).

Macrophage colony-stimulating factor (M-CSF). M-CSF, a 40 kDa polyprotein produced by fibroblasts and endothelial cells, specifically stimulates the growth and differentiation of cells of macrophage lineage. *In vitro* HIV-1 infection induces M-CSF production. MDM productively infected with HIV-1 *in vitro* produce 5- to 24-fold higher levels of M-CSF than uninfected cells (Gruber *et al.*, 1995; Kutza *et al.*, 2000). Increased levels of M-CSF have been detected in the cerebrospinal fluid of HIV-infected patients, presumably synthesized by cells of macrophage lineage within the brain (Gallo *et al.*, 1994).

TNF. TNF (TNF-α and TNF-β), a 17 kDa protein, is a proinflammatory cytokine forming a homotrimer capable of cross-linking TNF receptors (Smith & Baglioni, 1987). TNF-α is produced by a wide variety of cells, including monocytes, macrophages, T cells, B cells, NK cells, neutrophils and microglia cells, whereas TNF-β secretion is restricted to B cells and T cells. Both TNF-α and TNF-β mediate a broad spectrum of inflammatory and immune responses, although the effects of TNF-β are relatively weaker than TNF-α (Armitage, 1994).

TNF-α. TNF-α is increased in association with HIV-1 infection. HIV-1 infection of PBMC and CD4 T cells *in vitro* enhances their secretion of TNF-α (Vyakarnam *et al.*, 1990). Exposure of T cells to anti-TNF-α neutralizing antibody, decreases HIV-1 production (as assessed by p24 antigen release), prevents HIV-induced CD4 T cell depletion *in vitro* and inhibits NFκB activation, suggesting that autocrine secretion of TNF-α controls HIV-1 replication in primary T cells via NFκB activation (Munoz-Fernandez *et al.*, 1997). TNF-α is upregulated and constitutively secreted after *in vitro* HIV-infection of MDM, and also augments the infectivity of macrophages in an autocrine fashion (Esser *et al.*, 1991, 1996, 1998; Foli *et al.*, 1997; Esser *et al.*, 1998). Increased levels of TNF-α are found in the sera of HIV-infected individuals, monocytes and PBMC obtained from HIV-infected subjects and cultured *in vitro* (Lahdevirta *et al.*, 1988; Rizzardi *et al.*, 1998; Baqui *et al.*, 2000).

TNF-β. HIV-1 replication in PBMC and CD4 T cells is associated with enhanced secretion of TNF-β, similar to that of TNF-α (Vyakarnam *et al.*, 1990). HIV-specific CTL clones derived from peripheral blood and cerebrospinal fluid of HIV-infected individuals release TNF-β following stimulation with specific antigens (Gag, Env, RT and Nef) (Jassoy *et al.*, 1993), suggesting that increased production of TNF-β *in vivo* is from HIV-specific CTL.

Chemokines

α-Chemokines. CXC chemokines, known as α-chemokines, belong to one of three subfamilies of chemokines defined by their structure and the position of cysteine residues. α-chemokines are distinquished by possessing a non-conserved amino acid separating the terminal cysteine residues. The α-chemokine stromal-derived factor (SDF)-1 is a natural ligand for CXCR4. SDF-1 is a chemotactic factor for resting human T cells and monocytes that is expressed on a variety of tissues including heart, lung, liver, spleen, kidney, brain and muscle. It binds to CXCR4, promotes calcium mobilization and down-modulation of CXCR4 (Bleul *et al*, 1996). There are two splice variants of the SDF-1 gene: SDF-1α and SDF-1β, which possesses four extra amino acids at the carboxyl terminus. PBMC obtained from HIV-infected individuals, at both sympomatic and asymptomatic stages of disease, display higher levels of SDF-1 mRNA expression than cells from uninfected individuals (Ohashi *et al*, 1998).

β-chemokines. The CC chemokine β subset is characterized by two unseparated terminal cysteine residues. β-chemokines are secreted by a wide variety of cells, including T cells, monocytes, macrophages, fibroblasts and mast cells, bind to seven transmembrane domain receptors and promote chemotaxis, leukocyte trafficking, inflammation and haematopoiesis (Rollins, 1997). Three of the β-chemokines, macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES have been identified as natural ligands for CCR5, a major co-receptor for M-tropic strains of HIV-1 (Cocchi *et al.*, 1995).

In vitro HIV-1 infection of CD4 T cells results in increased secretion of MIP-1α and MIP-1β by M-tropic strains of HIV-1, and increased production of RANTES by both M- and T-tropic isolates (Greco *et al.*, 1999). HIV-1 infection also induces MDM to secrete MIP-1α and MIP-1β, subsequently enhancing chemotaxis and activating resting T cells (Schmidtmayerova *et al.*, 1996; Swingler *et al.*, 1999). Exposure of monocytes and MDM to gp120 or interactions between soluble CD40 ligand and CD40 on MDM, also stimulate the production of MIP-1α, MIP-1β and RANTES in a dose-dependent manner (Kornbluth *et al.*, 1998; Fantuzzi *et al.*, 2001). Similarly, activated CD8 T cells or PBMC obtained from HIV-infected individuals produce increased levels of MIP-1α, MIP-1β and RANTES (Cocchi *et al.*, 1995, 2000; Garzino-Demo *et al.*, 1999). Elevated levels of β-chemokines are found in the plasma of HIV-infected individuals at different stages of infection (Malnati *et al.*, 1997; Vicenzi *et al.*, 1997), although other investigators report no difference in serum concentrations of all three β-chemokines between HIV-infected and uninfected individuals (Kreuzer *et al.*, 2000).

Table 2. Effects of cytokines on HIV-1 replication

Cytokine	Replication level	Source of cells	HIV infection	Reference
IL-1	↑	PBMC, MDM, U1	*In vitro*	Poli *et al.*, 1994b; Granowitz *et al.*, 1995; Kinter *et al.*, 1995
IL-2	↑	PBMC, T cell line	*In vitro*	Poli *et al.*, 1995; Weissman *et al.*, 1996; Al-Harthi *et al.*, 1998
	↑	PBMC	*In vivo*	Kinter *et al.*, 1995
IL-4	↑	Monocytes, MDM, PBMC, U937	*In vitro*	Kazazi *et al.*, 1992; Naif *et al.*, 1994,1997; Poli *et al.*, 1995
	↓	Monocytes, MDM	*In vitro*	Novak *et al.*, 1990; Schuitemaker *et al.*, 1992; Wang *et al.*, 1998
IL-6	↑	PBMC, MDM, U1	*In vitro*	Poli *et al.*, 1990,1994b; Poli & Fauci, 1992
	No change	Plasma	*In vivo*	Marfaing-Koka *et al.*, 1996
IL-7	↑	PBMC	*In vitro*	Moran *et al.*, 1993
	↑	PBMC	*In vivo*	Smithgall *et al.*, 1996
IL-10	↓	MDM, U1	*In vitro*	Weissman *et al.*, 1994,1995; Kootstra *et al.*, 1994; Wang *et al.*, 1998
IL-12	↑	PBMC, T cell lines	*In vitro*	Poli *et al.*, 1995; Al-Harthi *et al.*, 1998
	↓	MDM	*In vitro*	Akridge & Reed, 1996
IL-13	↓	MDM	*In vitro*	Montaner *et al.*, 1993,1997; Denis & Ghadirian, 1994b; Wang *et al.*, 1998
IL-15	↑	PBMC, T cell lines	*In vitro*	Lucey *et al.*, 1997; Al-Harthi *et al.*, 1998
	↑	PBMC	*In vivo*	Lucey *et al.*, 1997
IL-16	↓	CD4 T cells, PBMC, MDM, dendritic cells	*In vitro*	Maciaszek *et al.*, 1997; Amiel *et al.*, 1999; Truong *et al.*, 1999
IL-18	↑	U1	*In vitro*	Shapiro *et al.*, 1998
IFN-α, IFN-β	↓	Monocytes, MDM, U937, T cells	*In vitro*	Poli *et al.*, 1989,1994b; Shirazi & Pitha, 1992; Fan *et al.*, 1994; Gessani *et al.*, 1994
	↓	Plasma	*In vivo*	Schnittman *et al.*, 1994
IFN-γ	↓	Monocytes, MDM	*In vitro*	Denis & Ghadirian, 1994b; Fan *et al.*, 1994; Dhawan *et al.*, 1995; Hariharan *et al.*, 1999
	↑	U1, U937, PBMC, CD4 T cells	*In vitro*	Vyakarnam *et al.*, 1990; Biswas *et al.*, 1992; Han *et al.*, 1996
GM-CSF	↑ Variable	MDM, U937	*In vitro*	Koyanagi *et al.*, 1988; Schuitemaker *et al.*, 1990; Pomerantz *et al.*, 1990; Perno *et al.*, 1992
	↓	MDM	*In vitro*	Kornbluth *et al.*, 1989; Hammer *et al.*, 1990
	↑	MDM	*In vitro*	Matsuda *et al.*, 1995; Di Marzio *et al.*, 1998; Kedzierska *et al.*, 2000a
M-CSF	↑	MDM	*In vitro*	Kornbluth *et al.*, 1989; Kalter *et al.*, 1991; Perno *et al.*, 1992; Matsuda *et al.*, 1995; Wang *et al.*, 1998
TNF-α, TNF-β	↑	T cells, MDM, U1	*In vitro*	Folks *et al.*, 1989; Kornbluth *et al.*, 1989; Novak *et al.*, 1990; Naif *et al.*, 1994; Han *et al.*, 1996
SDF-1	↓	PBMC, HeLa-CD4 cells	*In vitro*	Oberlin *et al.*, 1996; Bleul *et al.*, 1996; Schols *et al.*, 1997; Yang *et al.*, 1999
MIP-1α, MIP-1β, RANTES	↓	Monocyte, MDM, PBMC, migroglial cells	*In vitro*	Cocchi, 1995; Alhatib *et al.*, 1996; Cocchi *et al.*, 1996; Paxton *et al.*, 1996; Dragic *et al.*, 1996; Coffey *et al.*, 1997; Capobianchi *et al.*, 1998; Vicenzi *et al.*, 2000; Kitai *et al.*, 2000

IL, interleukin; MDM, monocyte-derived macrophages; PBMC, peripheral blood mononuclear cells; ↑ increase; ↓decrease; SDF, stromal-derived factor; MIP, macrophage inflammatory protein.

Cytokines alter HIV-1 replication *in vitro* and *in vivo*

HIV-1 replication is controlled by both viral and host factors. A variety of cytokines have been reported to modulate HIV-1 replication at multiple stages of its life cycle, from binding to the CD4 receptor to budding of virions from the infected cells. Cytokines exert both stimulatory and inhibitory effects on HIV-1 infection and replication in both CD4 T cells and cells of macrophage lineage (Table 2).

Interleukins

IL-1. IL-1 has been shown to enhance HIV-1 replication in IL-2-stimulated PBMC, MDM, and the U1 latently infected cell line derived from U937 (Poli *et al.*, 1994b; Granowitz *et al.*, 1995; Kinter *et al.*, 1995b).

IL-2. IL-2 is one of the most potent HIV-inducing cytokines (reviewed in Poli & Fauci, 1992). In mitogen-stimulated PBMC, but not in unstimulated cells, IL-2 induces production of HIV-1 over 7 days in culture compared with cells unexposed to this cytokine (Foli *et al.*, 1995). IL-2-induced upregulation of HIV-1 replication in PBMC and in IL-2 dependent T cell lines is mediated predominantly through a post-transcriptional mechanism, independent of direct HIV-1 long terminal repeat (LTR) activation (Al-Harthi *et al.*, 1998). The stimulatory effect of IL-2 on HIV-1 replication can be reversed by antibodies to IL-2 and by IL-10, which blocks IL-2 secretion (Weissman *et al.*, 1996). Removal of CD8 T cells results in a potent stimulation of the HIV-inductive ability of IL-2 in PBMC or lymph node mononuclear cells obtained from HIV-seropositive donors, suggesting that IL-2 induces CD8 T cells to suppress HIV-1 replication (Kinter *et al.*, 1995a). IL-2 also inhibits apoptosis induced by cross-linking of gp120 and CD4 receptor in CD4 T cells (Randrizzani *et al.*, 1995; Tuosto *et al.*, 1995).

IL-2 is used in clinical trials in combination with antiviral agents to increase the efficacy of anti-HIV therapy by modification of the immune response. Recent studies show that co-administration of HAART and IL-2 results in increased CD4 T cell numbers without an increase in viral load (Emery *et al.*, 2000; Pandolfi *et al.*, 2000; Pett & Emery, 2001; Tambussi *et al.*, 2001). Virus cannot be isolated from either the blood or lymph nodes obtained from HIV-infected patients on HAART/IL-2 therapy (Chun *et al.*, 1999). IL-2/HAART also results in a significant reduction in latently infected resting peripheral blood CD4 T cells (a major reservoir of HIV-1 *in vivo*), in comparison with patients receiving HAART alone, although prolonged therapy for 2 or more years does not eradicate HIV-1 (Zanussi & De Paoli, 2000). A combination of HAART/IL-2 with IFN-γ in patients with early HIV-1 infection reduces proviral load and shows evidence of immune reconstitution, but again without eradication of viral reservoirs (Lafeuillade *et al.*, 2001). IL-2 is also used experimentally in combination with DNA vaccines to augment cellular immune responses. Co-administration of HIV-1 and SIV in DNA vaccines with an IL-2 plasmid in rhesus monkeys results in controlled viremia and prevention of clinical AIDS in contrast to sham-vaccinated monkeys who experience rapid downregulation of CD4 cell counts, high viral load and disease progression (Barouch *et al.*, 2000).

IL-4. IL-4 displays both stimulatory and inhibitory effects on HIV-1 replication. IL-4 stimulates HIV-1 production in mitogen-stimulated PBMC, monocytes, MDM and promonocytic U937 cell lines (Kazazi *et al.*, 1992; Naif *et al.*, 1994, 1997; Foli *et al.*, 1995). IL-4 induces upregulation of HIV-1 replication in MDM when added at the time of HIV-1 infection, which can be reversed by antibodies to IL-4 (Kazazi *et al.*, 1992). However, IL-4 treatment of freshly isolated monocytes prior to HIV-1 infection downmodulates HIV-1 entry and subsequent replication, possibly as a result of reduced surface expression of the CCR5 co-receptor for M-tropic strains of HIV-1 (Schuitemaker *et al.*, 1992; Wang *et al.*, 1998). These data suggest that the state of maturation of monocytes into macrophages may determine the stimulatory or inhibitory effect of IL-4 on HIV-1 replication, although even this finding is not clear. Others have shown that pretreatment of monocytes with IL-4 for 5 days prior to HIV-1 infection enhances HIV-1 replication, inducing p24 antigen levels up to 230 times that of cells cultured in the absence of IL-4 (Novak *et al.*, 1990). The reasons for these discrepancies are not known.

IL-6. IL-6 has been reported to enhance HIV-1 replication in IL-2-stimulated PBMC, MDM and the U1 latently infected cell line derived from U937 (Poli *et al.*, 1990; Poli & Fauci, 1992). IL-6 synergizes with TNF-α in upregulation of HIV-1 production and potentiates the TNF-α-induced transcription of NFκB (Poli *et al.*, 1990, 1994b). Treatment of HIV-infected patients with lymphoma using an anti-IL-6 monoclonal antibody had no significant effect on plasma viral load, but reduced B cell-induced production of IgG and IgA (Marfaing-Koka *et al.*, 1996).

IL-7. In vitro treatment of CD3-stimulated PBMC with IL-7 results in a significant increase in HIV-1 production (Moran *et al.*, 1993). Similarly, exogenous IL-7 induces HIV-1 replication in PBMC from asymptomatic HIV-positive patients at proviral HIV-1 DNA and mRNA levels (Smithgall *et al.*, 1996). Co-vaccination of IL-7 with gp160 in HIV-infected individuals results in expansion of T-cell proliferative responses to HIV-1 Env (Kim *et al.*,

1997). IL-7 also increases activation and expansion of specific anti-Gag CTL precursors when co-administered to HIV-infected individuals with a vaccinia/HIV-1 based vaccine, suggesting IL-7-mediated upregulation of cytotoxic immune responses in HIV-infected individuals may potentially be of therapeutic value (Ferrari *et al.*, 1995).

IL-10. Exposure of primary MDM and the chronically infected U1 cell line to IL-10 decreases HIV-1 replication (Weissman *et al.*, 1994, 1995; Wang *et al.*, 1998). This effect of IL-10 is macrophage-specific, because treatment of T cells with IL-10 does not alter HIV-1 replication (Kootstra *et al.*, 1994). The inhibitory effect of IL-10 on HIV-1 replication is associated with its ability to down-modulate production of TNF-α and IL-6 (Weissman *et al.*, 1994). Immunoblotting demonstrates an accumulation of Gag precursor protein combined with an absence of mature p24 or gp120, suggesting than inhibitory effect of IL-10 occurs at the level of protein processing (Kootstra *et al.*, 1994).

IL-12. IL-12 upregulates HIV-1 replication in mitogen-stimulated PBMC and the IL-2 dependent T cell lines when compared with untreated cells, predominantly through a post-transcriptional mechanism that is independent of direct HIV-1 LTR activation (Foli *et al.*, 1995; Al-Harthi *et al.*, 1998). Similar to IL-2 treatment, IL-12 inhibits gp120-induced apoptosis in IL-2-dependent Th1 clone (Randrizzani *et al.*, 1995). However, IL-12 treatment of HIV-infected MDM decreases reverse transcriptase (RT) activity in culture supernatants, suggesting that IL-12 directly suppresses HIV-1 replication in purified MDM cultures (Akridge & Reed, 1996). IL-12 is currently being employed as an immunomodulator in HIV vaccine trials to enhance cell-mediated immunity (Ma & Montaner, 2000; Gherardi *et al.*, 2001).

IL-13. IL-13 is a potent suppressor of HIV-1 replication in MDM. This cytokine inhibits both virus infectivity and virus production by down modulating CCR5 expression on MDM, blocking the completion of RT and inhibiting replication at the post-transcriptional level (Montaner *et al.*, 1993, 1997; Denis & Ghadirian, 1994b; Wang *et al.*, 1998). IL-13 in the presence of TNF-α reduces HIV-1 replication of a dual-tropic strain of HIV-1 in MDM, concurrent with down modulation of surface expression of CD4, CCR5 and CXCR4 as well as post-entry viral gene expression (Bailer *et al.*, 2000).

IL-15. IL-15 upregulates HIV-1 replication in PBMC and IL-2-dependent T cell lines *in vitro* (Lucey *et al.*, 1997; Al-Harthi *et al.*, 1998). IL-15 also enhances HIV-1 production in PBMC obtained from HIV-seropositive individuals (Lucey *et al.*, 1997). Similar to IL-12, an IL-15 expression plasmid has been found to increase cell-mediated immunity, including CTL activity and HIV-specific DTH responses, induced by an HIV-1 envelope DNA vaccine when co-administered intranasally to mice (Xin *et al.*, 1999).

IL-16. IL-16 inhibits HIV-1 entry and replication in CD4 T cells, MDM and dendritic cells (Truong *et al.*, 1999). IL-16 treatment of CD8-depleted mitogen-activated lymphocytes, obtained from HIV-infected individuals, results in significant inhibition of secreted p24 antigen (Amiel *et al.*, 1999), and reduces apoptosis of PBMC (Idziorek *et al.*, 1998). Pretreatment of CD4 T cells with IL-16 results in up to a 60-fold suppression of HIV-1 promoter activity and prevents Tat activation (Maciaszek *et al.*, 1997). Antiviral effects of IL-16 suggest a potentially beneficial role on the inhibition of HIV-1 replication *in vivo*, although there are currently no data to support this.

IL-18. IL-18 stimulates HIV-1 production by up to 30-fold in the chronically infected monocytic cell line, U1, and is associated with nuclear translocation of NFκB (Shapiro *et al.*, 1998). Co-delivery of IL-18 (with or without IFN-γ) with DNA vaccine constructs enhances antigen-specific humoral and cellular responses in rhesus macaques and mice (Kim *et al.*, 1999; Billaut-Mulot *et al.*, 2001).

Interferons

Interferons (IFN-α, IFN-β and IFN-γ) are generally considered to protect MDM from productive infection by HIV-1 *in vitro*, with minimal or no p24 antigen accumulation in the supernatant, and no HIV-1 RNA or DNA in cell lysates (Kornbluth *et al.*, 1989; Denis & Ghadirian, 1994b; Fan *et al.*, 1994; Dhawan *et al.*, 1995).

IFN-α. IFN-α is up to 10–20 times less effective than IFN-γ in inhibiting HIV-1 replication in monocytes and in the U937 promonocytic cell line *in vitro* (Shirazi & Pitha, 1992; Fan *et al.*, 1994) measured by viral protein levels in the culture supernatant and in cell lysates (Shirazi & Pitha, 1992). Electron microscopy suggests an altered virus assembly, with the production of morphologically altered virus particles (Dianzani *et al.*, 1998) and the inhibition of budding of newly formed virions from the cells surface (Poli *et al.*, 1989). IFN-α inhibits HIV-1 replication at multiple levels, suppressing RT and inhibiting the production of late products of RT (Shirazi & Pitha, 1992), and preventing transcription of integrated provirus (Poli *et al.*, 1994a). The level of inhibition of IFN-α depends on the cytokine concentration, duration of exposure to cells in culture, viral strain and multiplicity of infection (Baca-Regen *et al.*, 1994).

IFN-α was initially used in the treatment of HIV-1 disease, particularly in patients affected by Kaposi's sarcoma (Lane *et al.*, 1988). However, relatively high doses of IFN-α are required for anti-tumour activity and are not well tolerated by patients. In patients receiving IFN-α, drug intolerance, neutropenia and anaemia were commonly observed (Haas *et al.*, 2000). IFN-α therapy transiently increases CD4 T cell proliferation and reduces HIV titres. The latter effect is prominent in HIV-infected patients receiving a combination of IFN-α and IL-2 treatment (Schnittman *et al.*, 1994). Combination therapy of IFN-α2a with zidovudine and zalcitabine transiently enhances plasma viral load decreases but increases toxicity (Fischl *et al.*, 1997).

IFN-β. IFN-β is also a potent inhibitory antiviral agent in MDM infected with HIV-1 *in vitro.* HIV-1 infection of MDM in the presence of antibodies to IFN-β results in a significant enhancement in p24 release, suggesting that HIV-1 infection of MDM induces production of IFN-β in the culture, which in turn downregulates HIV-1 replication in MDM (Gessani *et al.*, 1994b). Macrophages derived from umbilical cord blood, transduced with a vector carrying the IFN-β coding sequence, are resistant to infection with a M-tropic strain of HIV-1, demonstrating the efficacy on IFN-β-mediated inhibition of HIV-1 replication (Cremer *et al.*, 2000). Constitutive low production of IFN-β in peripheral blood lymphocytes by transduction of an IFN-β retroviral vector into lymphocytes from HIV-infected individuals also inhibits viral production (Vieillard *et al.*, 1997).

IFN-γ. IFN-γ reportedly exerts both stimulatory and inhibitory effects on HIV-1 replication in MDM, depending on whether this cytokine is added before or after infection (Koyanagi *et al.*, 1988; Poli & Fauci, 1992; Poli *et al.*, 1994a). A number of investigators have shown the inhibitory effect of IFN-γ on HIV-1 replication in monocytes and MDM, with reduced p24 antigen in supernatant as well as HIV-1 DNA and mRNA levels (Fan *et al.*, 1994; Denis & Ghadirian, 1994b; Dhawan *et al.*, 1995). IFN-γ also inhibits viral entry, possibly via downmodulation of surface expression of CD4, the major receptor for HIV-1, although it upregulates the expression of CCR5 and CCR3 co-receptors used by M-tropic strains of HIV-1 (Dhawan *et al.*, 1995; Hariharan *et al.*, 1999). In U937-derived clones, IFN-γ exerts its inhibitory effect on HIV-1 replication via inhibition of the JAK/STAT signalling pathway in these cells (Bovolenta *et al.*, 1999).

However, there are also a number of studies in which a stimulatory effect of IFN-γ on HIV-1 infection has been found. Exposure of chronically infected U1 cells to IFN-γ reportedly activates HIV-1 replication, as measured by culture supernatant RT activity, and cell-associated HIV-1 protein and mRNA (Biswas *et al.*, 1992). Treatment of U937 cells with IFN-γ modestly increases HIV-1 expression, whereas when added in the presence of TNF-α, IFN-γ synergistically potentiates the stimulatory effect of TNF-α (Han *et al.*, 1996). IFN-γ has also been found to increase HIV-1-induced syncytium formation by up to 10000-fold in PBMC and CD4 T cells infected with HIV-1 *in vitro* (Vyakarnam *et al.*, 1990).

In vivo IFN-γ administration is generally well tolerated and augments the anti-bacterial, -fungal and -parasite activities of monocytes, tissue macrophages and neutrophils (Murray, 1996). IFN-γ treatment of HIV-infected patients has been used successfully as adjunctive therapy in individuals with drug-resistant tuberculosis or Kaposi's sarcoma (Heagy *et al.*, 1990; Murray, 1996). IFN-γ is currently used experimentally as an adjuvant in vaccine preparations. Co-administration of IFN-γ with DNA vaccine constructs increases antigen-specific antibody responses to HIV-1 in rhesus macaques (Kim *et al.*, 1999).

Colony-stimulating factors

GM-CSF. Early studies investigating the effect of GM-CSF on HIV-1 replication reported upregulation of HIV-1 production in both MDM (Koyanagi *et al.*, 1988; Perno *et al.*, 1989; Schuitemaker *et al.*, 1990; Perno *et al.*, 1992; Wang *et al.*, 1998) as well as promonocytic cell lines U937 and U1 (Folks *et al.*, 1987; Pomerantz *et al.*, 1990). However, other investigators have found either inconsistent (Kornbluth *et al.*, 1989; Hammer *et al.*, 1990) or inhibitory effects of GM-CSF on HIV-1 entry or replication in MDM (Matsuda *et al.*, 1995; Di Marzio *et al.*, 1998). Recently, we have reported that GM-CSF inhibits HIV-1 replication in MDM *in vitro*, measured as reduced RT and p24 in the culture supernatant and reduced mRNA expression (Kedzierska *et al.*, 2000a). Although a number of laboratory variables could contribute to the conflicting data, potential confounders have been extensively examined, with reproduction of assay conditions used by other investigators (Crowe and Lopez, 1997; Kedzierska *et al.*, 1998), including strain of HIV-1, source/concentration of GM-CSF, timing of incubation with cytokine, maturity (monocyte versus MDM) and strain of HIV-1 used for infection. Regardless of experimental conditions, reduced replication of HIV-1 has been consistently observed, and GM-CSF-mediated inhibition of HIV-1 replication can be reversed by the addition of a neutralizing anti-GM-CSF monoclonal antibody (Kedzierska *et al.*, 2000a). Analysis of CD4 and CCR5 surface expression on MDM using a flow cytometry, suggests that the inhibition of HIV-1 does not occur at the entry level, rather inhibition is at or before the level of transcription. A mutant form of GM-CSF, E21R, which binds only to the α-chain of the GM-CSF receptor (Lopez *et al.*, 1992) does not inhibit HIV-1 replication in

MDM, suggesting that GM-CSF inhibits HIV-1 replication via binding to the β-chain of the GM-CSF receptor (Kedzierska *et al.*, 2000a) and subsequent signalling events in the cell (T Warby, unpublished data).

Currently, GM-CSF is rarely used for the treatment of HIV-infected patients due to the conflicting *in vitro* data, and thus concern regarding potential activation of HIV-1 replication. GM-CSF treatment of HIV-infected patients in the absence of antiretroviral therapy results in increased serum p24 antigen levels and plasma HIV RNA titres (Kaplan *et al.*, 1991; Lafeuillade *et al.*, 1996). However, when used in combination with effective antiretroviral therapy, GM-CSF has been safely administered to patients without any significant increase in viral load (Yarchoan *et al.*, 1990; Krown *et al.*, 1992; Kedzierska *et al.*, 2000b). In fact, GM-CSF has been found to increase the activity of antiretroviral drugs (Perno *et al.*, 1992). Data from two studies of HIV-infected patients receiving antiretroviral therapy and GM-CSF have shown that patients experienced a decrease in viral load and an increase in CD4 counts (Skowron *et al*; 1999; Brites *et al.*, 2000). Clinical improvement and augmented monocyte phagocytic function against mycobacteria and fungal infections, again without an increase in viral load, has also been reported in patients with advanced HIV-1 infection and drug-resistant opportunistic infections treated with GM-CSF (Vazquez *et al.*, 1998; Kedzierska *et al.*, 2000b). These data suggest that GM-CSF might be useful in augmenting macrophage function, especially in HIV-infected patients with drug-resistant opportunistic infections.

M-CSF. Pretreatment of MDM with recombinant M-CSF significantly increases HIV-1 production, measured by RT and viral antigen in the culture supernatant on days 7–10 after infection (Kornbluth *et al.*, 1989; Perno *et al.*, 1992). MDM exposed to M-CSF are at least 400-fold more susceptible to HIV-1 infection than cells cultured in its absence (Kalter *et al.*, 1991). M-CSF treatment of MDM results in increased HIV-1 entry and replication, possibly as a result of increasing the surface expression of both CD4 and the CCR5 co-receptor for M-tropic strains of HIV-1 (Wang *et al.*, 1998). M-CSF exposure also results in increased syncytial formation and increased levels of proviral DNA compared with GM-CSF-treated MDM, which inhibited HIV-1 replication (Matsuda *et al.*, 1995). M-CSF increases HIV-1 production in bone marrow stem cells infected with an M-tropic strain of HIV-1, as assessed by p24 antigen levels in the culture supernatant (Kitano *et al.*, 1991). Addition of an M-CSF antagonist, anti-M-CSF monoclonal or polyclonal antibodies, or soluble M-CSF receptors dramatically reduces HIV-1 replication in HIV-infected MDM, suggesting that an M-CSF antagonist may prevent infection of monocytes and macrophages *in vivo* (Kutza *et al.*, 2000).

Tumour necrosis factors

Both TNF-α and TNF-β are potent activators of HIV-1 replication in T cells and cells of macrophage lineage (Folks *et al.*, 1989; Kornbluth *et al.*, 1989; Novak *et al.*, 1990; Naif *et al.*, 1994) via the activation of the cellular transcription factor, NF-κB (Griffin *et al.*, 1991). The exposure of PHA-activated T lymphocytes to TNF-α before HIV-1 infection does not modify HIV-1 entry (Herbein *et al.*, 1996). TNF-α upregulates production of HIV-1 mRNA in MDM and in the U1 HIV-infected clone of the U937 cell line, peaking 3–4 days after infection (Naif *et al.*, 1994). The effect of TNF-α is potentiated by IFN-γ (Han *et al.*, 1996). TNF-α and TNF-β increase HIV-1-induced syncytium formation by up to 10000-fold in PBMC and CD4 T cells infected with HIV-1 *in vitro* (Vyakarnam *et al.*, 1990). However, an inhibitory role of TNF-α on HIV-1 entry in MDM has also been reported with TNF-α pretreatment for 3 days before infection, resulting in approximately 75% inhibition of viral entry (Herbein *et al.*, 1996).

Chemokines

α-Chemokines. SDF-1 suppresses cell fusion and infection of T-tropic strains of HIV-1 in PBMC and HeLa-CD4 cells when compared with uninfected cells (Bleul *et al.*, 1996; Oberlin *et al.*, 1996), but does not affect infection by M-tropic or dual tropic strains of HIV-1 *in vitro* (Oberlin *et al*, 1996). SDF-1 exerts its inhibitory effect via binding to CXCR4 (Schols *et al.*, 1997) and downmodulation of CXCR4 (Yang *et al.*, 1999).

β-Chemokines. β-Chemokines play an important role in modulation of HIV-1 infection (Choe *et al.*, 1996). MIP-1α, MIP-1β and RANTES are inhibitors of HIV-1 replication in cells of macrophage lineage (Cocchi *et al.*, 1995, 1996; Alkhatib *et al.*, 1996b; Coffey *et al.*, 1997), PBMC (Coffey *et al.*, 1997; Vicenzi *et al.*, 2000) and microglial cells (Kitai *et al.*, 2000), suppressing M-tropic isolates, but having no effect on T-tropic strains (Cocchi *et al.*, 2000). Treatment of MDM or T cells with β-chemokines inhibits HIV-1 entry (Cocchi *et al.*, 1995; Dragic *et al.*, 1996; Paxton *et al.*, 1996; Capobianchi *et al.*, 1998), viral production and uncoating as well as CCR5 expression (Jiang & Jolly, 1999).

HIV-1 and cytokines: summary and conclusions

HIV-1 infection at all stages of the disease is associated with chronic immune activation and dysfunctional cytokine production. During HIV-1 infection a shift of Th cell function from Th1 (decreased production of IL-2 and IFN-γ) to Th2 (increased IL-4 and IL-10 serum levels) is

thought to play a central role in the immunopathogenesis of the disease. Increased production of proinflammatory cytokines (IL-1, IL-6, IL-8 and TNF-α) are also thought to activate HIV-1 replication and maintain active HIV-1 expression via binding of NF-κB to LTR. This abnormal cytokine profile can be partially reversed with HAART, resulting in increases in IL-2 and IFN-γ secretion (but not IL-4 and IL-10), paralleled by suppression of HIV-1 replication and restored CD4 T cell counts. The use of cytokine therapy coupled to antiretroviral treatment shows clinical benefit in some circumstances and restoration of immune responses.

The studies presented in this review demonstrate that HIV-1 replication is under regulation of a complex and dysregulated network of both HIV-inductive and -suppressive cytokines, together with a variety of viral and cellular factors. IFN-α, IFN-β, IL-10, IL-13 and IL-16 are clearly the HIV-suppressive cytokines, whereas TNF-α, TNF-β, M-CSF, IL-1, IL-2, IL-6, IL-7, IL-15 and IL-18 augment HIV-1 replication. Bifunctional cytokines, such as IFN-γ, GM-CSF and IL-4, reportedly may exert suppressive or inductive effects on HIV-1 infection and replication, possibly depending on the state of differentiation of monocytes and the experimental protocols used by various laboratories. HIV-1 infection and replication can also be modulated by the presence of LPS in cultures, and results should be carefully examined with this in mind. The presence of LPS has been shown to decrease HIV-1 production by MDM infected by HIV-1 *in vitro* (Kornbluth *et al.*, 1989), increase viral infectivity of CD4-enriched PBMC population infected with a T tropic but not an M-tropic strain of HIV-1 (Juffermans *et al.*, 2000) as well as upregulate HIV-1 co-receptors CXCR4 and CCR5 on CD4 T cells and induce the secretion of proinflammatory cytokines IL-1, IL-6, IL-8 and TNF-α (Goletti *et al.*, 1996). Therefore, there is a complex interplay between cytokines *in vivo*. The modulation of infection by exposure of cells to specific cytokines *in vitro*, a reductionist approach, may oversimplify the biological situation.

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References

Akridge RE & Reed SG (1996) Interleukin-12 decreases human immunodeficiency virus type 1 replication in human macrophage cultures reconstituted with autologous peripheral blood mononuclear cells. *Journal of Infectious Diseases* **173**:559–564.

Al-Harthi L, Roebuck K & Landay A (1998) Induction of HIV-1 replication by type 1-like cytokines, interleukin (IL)-12 and IL-15: effect on viral transcriptional activation, cellular proliferation, and endogenous cytokine production. *Journal of Clini . Immunology* **18**:124–131.

Alkhatib G, Broder CC & Berger EA (1996a) Cell type specific fusion cofactors determine human immunodeficiency virus type 1 tropism for T cell lines versus primary macrophages. *Journal of Virology* **70**:5487–5494.

Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM & Berger EA (1996b) CC CKR5; a RANTES, MIP-1 alpha, MIP-1beta receptor as a fusion cofactor for macrophage tropic HIV-1. *Science* **272**:1955–1958.

Ambrus J, Poiesz B, Lillie M, Stadler I, Di Berardino L & Chadha K (1989) Interferon and interferon inhibitor levels in patients infected with varicella-zoster virus, acquired immunodeficiency syndrome, acquired immunodeficiency syndrome-related complex, or Kaposi's sarcoma, and in normal individuals. *American Journal of Medicine* **87**:405–407.

Ameglio F, Cordiali Fei P, Solmone M, Bonifati C, Prignano G, Giglio A, Caprilli F, Gentili G & Capobianchi MR (1994) Serum IL-10 levels in HIV-positive subjects: correlation with CDC stages. *Journal of Biological Regulators and Homeostatic Agents* **8**:48–52.

Amiel C, Darcissac E, Truong M, Dewulf J, Lovens M, Mouton Y, Capron A & Bahr G (1999) Interleukin-16 (IL-16) inhibits human immunodeficiency virus replication in cells from infected subjects, and serum IL-16 levels drop with disease progression. *Journal of Infectious Diseases* **179**:83–91.

Armitage J (1998) Emerging applications of recombinant human granulocyte-macrophage colony stimulating factor. *Blood* **92**:4491–4508.

Armitage R (1994) Tumor necrosis factor receptor superfamily members and their ligands. *Current Opinion in Immunology* **6**:407–413.

Baca-Regen L, Heinzinger N, Stevenson M & Gendelman HE (1994) Alpha interferon-induced antiretroviral activities: restriction of viral nucleic acid synthesis and progeny virion production in human immunodeficiency virus type 1-infected monocytes. *Journal of Virology* **68**:7559–7565.

Bailer R, Holloway A, Sun J, Margolick J, Martin M, Kostman J & Montaner L (1999) IL-13 and IFN-gamma secretion by activated T cells in HIV-1 infection associated with viral suppression and a lack of disease progression. *Journal of Immunology* **162**:7534–7542.

Bailer RT, Lee B & Montaner LJ (2000) IL-13 and TNF-alpha inhibit dual-tropic HIV-1 in primary macrophages by reduction of surface expression of CD4, chemokine receptors CCR5, CXCR4 and post-entry viral gene expression. *European Journal of Immunology* **30**:1340–1349.

Baqui A, Jabra-Rizk M, Kelley J, Zhang M, Falkler WJ & Meiller T (2000) Enhanced interleukin-1beta, interleukin-6 and tumor necrosis factor-alpha production by LPS stimulated human monocytes isolated from HIV+ patients. *Immunopharmacology and Immunotoxicology* **22**:401–421.

Barcellini W, Rizzardi G, Borghi M, Fain C, Lazzarin A & Meroni P (1994) TH1 and TH2 cytokine production by peripheral blood mononuclear cells from HIV-infected patients. *AIDS* **8**:757–762.

Barcova M, Kacani L, Speth C & Dierich M (1998) gp41 envelope protein of human immunodeficiency virus induces interleukin (IL)-10 in monocytes, but not in B, T, or NK cells, leading to reduced IL-2 and interferon-gamma production. *Journal Infectious Diseases* **177**:905–13.

Barouch D, Santra S, Schmitz J, Kuroda M, Fu T, Wagner W, Bilska M, Craiu A, Zheng X, Krivulka G, Beaudry K, Lifton M, Nickerson C, Trigona W, Punt K, Freed D, Guan L, Dubey S, Casimiro D, Simon A, Davies M, Chastain M, Strom T, Gelman R, Montefiori D & Lewis M (2000) Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* **290**:486–492.

Barre-Sinoussi F, Chermann J, Rey F, Nugeyre M, Chamaret S,

Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W & Montagnier L. (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**:868–871.

Bazan J (1990) Shared architecture of hormone binding domains in type I and II interferon receptors. *Cell* **61**:753–754.

Biggs BA, Hewish M, Kent S, Hayes K & Crowe SM (1995) HIV-1 infection of human macrophages impairs phagocytosis and killing of Toxoplasma gondii. *Journal of Immunology* **154**:6132–6139.

Billaut-Mulot O, Idziorek T, Loyens M, Capron A & Bahr GM (2001) Modulation of cellular and humoral immune responses to a multiepitopic HIV-1 DNA vaccine by interleukin-18 DNA immunization/viral protein boost. *Vaccine* **19**:2803–2811.

Birx D, Redfield R, Tencer K, Fowler A, Burke D. & Tosato G (1990) Induction of interleukin-6 during human immunodeficiency virus infection. *Blood* **76**:2303–2310.

Biswas P, Poli G, Kinter A, Justement J, Stanley S, Maury W, Bressler P, Orenstein J & Fauci A (1992) Interferon gamma induces the expression of human immunodeficiency virus in persistently infected promonocytic cells (U1) and redirects the production of virions to intracytoplasmic vacuoles in phorbol myristate acetate-differentiated U1 cells. *Journal of Experimental Medicine* **176**:739–750.

Bizzini B, Volpato I, Lachgar A, Cohen P & Gringeri A (1999) IFN alpha kinoid vaccine in conjunction with tritherapy, a weapon to combat immunopathogenesis in AIDS. *Biomedical Pharmacotherapy* **53**:87–89.

Bleul C, Farzan M, Choe H, Parolin C, Clark-Lewis I, Sodrowski J & Springer T (1996) The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* **382**:829–833.

Borghi P, Fantuzzi L, Varano B, Gessani S, Puddu P, Conti L, Capobianchi MS, Ameglio F & Belardelli F (1995) Induction of interleukin-10 by human immunodeficiency virus type 1 and its gp120 protein in human monocyte/macrophages. *Journal of Virology* **69**:1284–1287.

Bovolenta C, Lorini AL, Mantelli B, Camorali L, Novelli F, Biswas P & Poli G (1999) A selective defect of IFN-gamma- but not of IFN-alpha-induced JAK/STAT pathway in a subset of U937 clones prevents the antiretroviral effect of IFN-gamma against HIV-1. *Journal of Immunology* **162**:323–330.

Breen E, Rezai A, Nakajima K, Beall G, Mitsuyasu R, Hirano T, Kishimoto T & Martinez-Maza O (1990) Infection with HIV is associated with elevated IL-6 levels and production. *Journal of Immunology* **144**:480–484.

Breytenbach U, Clark A, Lamprecht J & Bouic P (2001) Flow cytometric analysis of the Th1-Th2 balance in healthy individuals and patients infected with the human immunodeficiency virus (HIV) receiving a plant sterol/sterolin mixture. *Cell Biology International* **25**:3–9.

Brites C, Gilbert MJ, Pedral-Sampaio D, Bahia F, Pedroso C, Alcantara AP, Sasaki MD, Matos J, Renjifo B, Essex M, Whitmore JB, Agosti JM & Badaro R (2000) A randomized, placebo-controlled trial of granulocyte-macrophage colony- stimulating factor and nucleoside analogue therapy in AIDS. *Journal of Infectious Diseases* **182**:1531–1535.

Capobianchi M (1996) Induction of lymphomonocyte activation by HIV-1 glycoprotein gp120. Possible role in AIDS pathogenesis. *Journal of Biological Regulators and Homeostatic Agents* **10**:83–91.

Capobianchi M, Abbate I, Antonelli G, Turriziani O, Dolei A & Dianzani F (1998) Inhibition of HIV type 1 BaL replication by MIP-1alpha, MIP-1beta, and RANTES in macrophages. *AIDS Research and Human Retroviruses* **14**:233–240.

Cayota A, Vuillier F, Scott-Algara D, Feuillie V & Dighiero G (1992) Impaired proliferative capacity and abnormal cytokine profile of naive and memory CD4 T cells from HIV-seropositive patients. *Clinical and Experimental Immunology* **88**:478–483.

Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, Gerard N, Gerard C & Sodroski J (1996) The beta chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**:1135–1148.

Chun T, Engel D, Mizell S, Hallahan C, Fischette M, Park S, Davey RJ, Dybul M, Kovacs J, Metcalf J, Mican J, Berrey M, Corey L, Lane H & Fauci A (1999) Effect of interleukin-2 on the pool of latently infected, resting CD4+ T cells in HIV-1-infected patients receiving highly active anti-retroviral therapy. *Nature Medicine* **5**:651–655.

Clerici M & Shearer G (1993) A TH1—>TH2 switch is a critical step in the etiology of HIV infection. *Immunology Today* **14**:107–111.

Cocchi F, DeVico A, Garzino-Demo A, Arya S, Gallo R & Lusso P (1995) Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **270**:1811–1815.

Cocchi F, DeVico A, Garzino-Demo A, Cara A, Gallo R & Lusso P (1996) The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection. *Nature Medicine* **2**:1244–1247.

Cocchi F, DeVico AL, Yarchoan R, Redfield R, Cleghorn F, Blattner WA, Garzino-Demo A, Colombini-Hatch S, Margolis D & Gallo RC (2000) Higher macrophage inflammatory protein (MIP)-1alpha and MIP-1beta levels from CD8+ T cells are associated with asymptomatic HIV-1 infection. *Proceeding of the National Academy of Science, USA* **97**:13812–13817.

Coffey M, Woffendin C, Phare S, Strieter R & Markovitz D (1997) RANTES inhibits HIV-1 replication in human peripheral blood monocytes and alveolar macrophages. *American Journal of Physiology* **272**:1025–1029.

Cremer I, Vieillard V and De Maeyer E (2000) Retrovirally mediated IFN-beta transduction of macrophages induces resistance to HIV, correlated with up-regulation of RANTES production and down-regulation of C-C chemokine receptor-5 expression. *Journal of Immunology* **164**:1582–1587.

Crowe SM & Lopez A (1997) GM-CSF and its effects on replication of HIV-1 in cells of macrophage lineage. *Journal of Leukocyte Biology* **62**:41–48.

De Paoli P, Zanussi S, Caggiari L, Bortolin M, D'Andrea M, Simonelli C & Tirelli U (1999) Kinetics of lymphokine production in HIV+ patients treated with highly active antiretroviral therapy and interleukin 2. *Journal of Clinical Immunology* **19**:317–325.

de Vries J (1998) The role of IL-13 and its receptor in allergy and inflammatory responses. *Journal of Allergy and Clinical Immunology* **102**:165–169.

Delemarre F, Stevenhagen A, Kroon F & van Furth R (1998) Reduced toxoplasmastatic activity of monocytes from AIDS patients: a role for granulocyte-macrophage colony-stimulating factor. *Scandinavian Journal of Immunology* **47**:163–166.

Denis M & Ghadirian E (1994a) Dysregulation of interleukin 8, interleukin 10, and interleukin 12 release by alveolar macrophages from HIV type 1-infected subjects. *AIDS Research and Human Retroviruses* **10**:1619–1627.

Denis M & Ghadirian E (1994b) Interleukin 13 and interleukin 4 protect bronchoalveolar macrophages from productive infection with human immunodeficiency virus type 1. *AIDS Research and Human Retroviruses* **10**:795–802.

Dereuddre-Bosquet N, Clayette P, Martin M, Benveniste O, Fretier P, Jaccard P, Vaslin B, Lebeaut A and Dormont D (1997) Lack of interleukin 10 expression in monocyte-derived macrophages in response to *in vitro* infection by HIV type 1 isolates. *AIDS Research and Human Retroviruses* **13**:961–966.

Dhawan S, Heredia A, Wahl LM, Epstein JS, Meltzer MS &

Hewlett IK (1995) Interferon-gamma-induced downregulation of CD4 inhibits the entry of human immunodeficiency virus type-1 in primary monocytes. *Pathobiology* **63**:93–99.

Di Marzio, Tse J & Landau NR (1998) Chemokine regulation and HIV type 1 tropism in monocyte-macrophages. *AIDS Research and Human Retroviruses* **14**:129–138.

Diamond DC, Sleckman BP, Gregory T, Lasky LA, Greenstein JL & Burakoff SJ (1988) Inhibition of CD4+ T cell function by the HIV envelope protein, gp120. *Journal of Immunology* **141**:3715–3717.

Dianzani F, Castilletti C, Gentile M, Gelderblom HR, Frezza F & Capobianchi MR (1998) Effects of IFN alpha on late stages of HIV-1 replication cycle. *Biochimie* **80**:745–754.

Dinarello C (1997) Interleukin-1. *Cytokine Growth Factor Reviews* **8**:253–265.

Dragic T, Litwin V, Allaway G, Martin S, Huang Y, Nagashima K, Cayanan C, Maddon P, Koup R, Moore J & Paxton W (1996) HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature*, **381**:667–673.

Emery S, Capra W, Cooper D, Mitsuyasu R, Kovacs J, Vig P, Smolskis M, Saravolatz L, Lane H, Fyfe G & Curtin P (2000) Pooled analysis of 3 randomized, controlled trials of interleukin-2 therapy in adult human immunodeficiency virus type 1 disease. *Journal of Infectious Diseases* **182**:28–34.

Esser R, von Briesen H, Brugger M, Ceska M, Glienke W, Muller S, Rehm A, Rubsamen-Waigmann H & Andreesen R (1991) Secretory repertoire of HIV-infected human monocytes/macrophages. *Pathobiology* **59**:219–222.

Esser R, Glienke W, von Briesen H, Rubsamen-Waigmann H & Andreesen R (1996) Differential regulation of proinflammat. y and hematopoietic cytokines in human macrophages after infec. with human immunodeficiency virus. *Blood* **88**:3474–3481.

Esser R, Glienke W, Andreesen R, Unger R, Kreutz M, Rubsamen-Waigmann H & von Briesen H (1998) Individual cell analysis of the cytokine repertoire in human immunodeficiency virus-1-infected monocytes/macrophages by a combination of immunocytochemistry and in situ hybridization. *Blood* **91**:4752–4760.

Fan SX, Turpin JA, Aronovitz JR & Meltzer MS (1994) Interferon-gamma protects primary monocytes against infection with human immunodeficiency virus type 1. *Journal of Leukocyte Biology* **56**:362–368.

Fan J, Li P, Kok T & Burrell C (1997) AZT blocks down-regulation of IL-2 and IFN-gamma gene expression in HIV acutely infected cells. *Archives of Virology* **142**:1035–1043.

Fantuzzi L, Gessani S, Borghi P, Varano B, Conti L, Puddu P & Belardelli F (1996) Induction of interleukin-12 (IL-12) by recombinant glycoprotein gp120 of human immunodeficiency virus type 1 in human monocytes/macrophages: requirement of gamma interferon for IL-12 secretion. *Journal of Virology* **70**:4121–4124.

Fantuzzi L, Canini I, Belardelli F & Gessani S (2001) HIV-1 gp120 stimulates the production of beta-chemokines in human peripheral blood monocytes through a CD4-independent mechanism. *Journal of Immunology* **166**:5381–5387.

Ferrari G, King K, Rathbun K, Place CA, Packard MV, Bartlett JA, Bolognesi DP & Weinhold KJ (1995) IL-7 enhancement of antigen-driven activation/expansion of HIV-1- specific cytotoxic T lymphocyte precursors (CTLp). *Clinical and Experimental Immunology* **101**:239–248.

Fischl MA, Richman DD, Saag M, Meng TC, Squires KE, Holden-Wiltse J. & Meehan PM (1997) Safety and antiviral activity of combination therapy with zidovudine, zalcitabine, and two doses of interferon-alpha2a in patients with HIV. AIDS Clinical Trials Group Study 197. *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology* **16**:247–253.

Foli A, Saville M, Baseler M & Yarchoan R (1995) Effects of the Th1 and Th2 stimulatory cytokines interleukin-12 and interleukin-4 on human immunodeficiency virus replication. *Blood* **85**:2114–2123.

Foli A, Saville M, May L, Webb D & Yarchoan R (1997) Effects of human immunodeficiency virus and colony-stimulating factors on the production of interleukin 6 and tumor necrosis factor alpha by monocyte/macrophages. *AIDS Research and Human Retroviruses* **13**:829–839.

Folks TM, Justement J, Kinter A, Dinarello CA & Fauci AS (1987) Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science* **238**:8[illegible]02.

Folks T, Clouse K, Justement J, Rabson A, Duh E, Kehrl J & Fauci A (1989) Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proceeding of the National Academy of Science, USA* **86**:2365–2368.

Francis M, Meltzer M & Gendelman H (1992) Interferons in the persistence, pathogenesis, and treatment of HIV infection. *AIDS Research and Human Retroviruses* **8**:199–207.

Gallo RC, Sarin PS, Gelmann EP, Robert-Guroff M, Richardson E, Kalyanaraman VS, Mann D & Sidhu GD (1983) Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science* **220**:865–867.

Gallo P, De Rossi A, Sivieri S, Chieco-Bianchi L & Tavolato B (1994) M-CSF production by HIV-1-infected monocytes and its intrathecal synthesis. Implications for neurological HIV-1-related disease. *Journal of Neuroimmunology* **51**:193–198.

Garzino-Demo A, Moss RB, Margolick JB, Cleghorn F, Sill A, Blattner WA, Cocchi F, Carlo DJ, DeVico AL & Gallo RC (1999) Spontaneous and antigen-induced production of HIV-inhibitory beta- chemokines are associated with AIDS-free status. *Proceeding of the National Academy of Science, USA* **96**:11986–19991.

Gendelman H, Friedman R, Joe S, Baca L, Turpin J, Dveksler G, Meltzer M & Dieffenbach C (1990) A selective defect of interferon alpha production in human immunodeficiency virus-infected monocytes. *Journal of Experimental Medicine* **172**:1433–1442.

Gessani S, Puddu P, Varano B, Borghi P, Conti L, Fantuzzi L & Belardelli F (1994a) Induction of beta interferon by human immunodeficiency virus type 1 and its gp120 protein in human monocytes-macrophages: role of beta interferon in restriction of virus replication. *Journal of Virology* **68**:1983–1986.

Gessani S, Puddu P, Varano B, Borghi P, Conti L, Fantuzzi L, Gherardi G & Belardelli F (1994b) Role of endogenous interferon-beta in the restriction of HIV replication in human monocyte/macrophages. *Journal of Leukocyte Biology* **56**:358–361.

Gessani S, Borghi P, Fantuzzi L, Varano B, Conti L, Puddu P & Belardelli F (1997) Induction of cytokines by HIV-1 and its gp120 protein in human peripheral blood monocyte/macrophages and modulation of cytokine response during differentiation. *Journal of Leukocyte Biology* **62**:49–53.

Gherardi MM, Ramirez JC & Esteban M (2001) Towards a new generation of vaccines: the cytokine IL-12 as an adjuvant to enhance cellular immune responses to pathogens during prime-booster vaccination regimens. *Histology and Histopathology* **16**:655–667.

Goletti D, Kinter A, Hardy E, Poli G & Fauci A (1996) Modulation of endogenous IL-1 beta and IL-1 receptor antagonist results in opposing effects on HIV expression in chronically infected monocytic cells. *Journal of Immunology* **156**:3501–3508.

Granowitz E, Saget B, Wang M, Dinarello C & Skolnik P (1995) Interleukin 1 induces HIV-1 expression in chronically infected U1 cells: blockade by interleukin 1 receptor antagonist and tumor necrosis factor binding protein type 1. *Molecular Medicine* **1**:667–677.

Greco G, Fujimura S, Mourich D & Levy J (1999) Differential effects of human immunodeficiency virus isolates on beta-

chemokine and gamma interferon production and on cell proliferation. *Journal of Virology* **73**:1528–1534.

Griffin G, Leung K, Folks T, Kunkel S & Nabel G (1991) Induction of NF-kappa B during monocyte differentiation is associated with activation of HIV-gene expression. *Research in Virology* **142**:233–238.

Gruber M, Weih K, Boone E, Smith P & Clouse K (1995) Endogenous macrophage CSF production is associated with viral replication in HIV-1-infected human monocyte-derived macrophages. *Journal of Immunology* **154**:5528–5535.

Haas DW, Lavelle J, Nadler JP, Greenberg SB, Frame P, Mustafa N, St Clair M, McKinnis R, Dix L, Elkins M & Rooney J (2000) A randomized trial of interferon alpha therapy for HIV type 1 infection. *AIDS Research and Human Retroviruses* **16**:183–190.

Hammer SM, Gillis JM, Pinkston P & Rose RM (1990) Effect of zidovudine and granulocyte-macrophage colony-stimulating factor on human immunodeficiency virus replication in alveolar macrophages. *Blood* **75**:1215–1219.

Han X, Becker K, Degen H, Jablonowski H & Strohmeyer G (1996) Synergistic stimulatory effects of tumour necrosis factor alpha and interferon gamma on replication of human immunodeficiency virus type 1 and on apoptosis of HIV-1-infected host cells. *European Journal of Clinical Investigation* **26**:286–292.

Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N & Matsushima K (1994) Essential involvement of interleukin-8 (IL-8) in acute inflammation. *Journal of Leukocyte Biology* **56**:559–564.

Hariharan D, Douglas SD, Lee B, Lai JP, Campbell DE and Ho WZ (1999) Interferon-gamma upregulates CCR5 expression in cord and adult blood mononuclear phagocytes. *Blood* **93**:1137–1144.

Heagy W, Groopman J, Schindler J & Finberg R (1990) Use of IFN-gamma in patients with AIDS. *Journal of Acquired Immune Deficiency Syndromes* **3**:584–590.

Herbein G, Montaner L & Gordon S (1996) Tumor necrosis factor alpha inhibits entry of human immunodeficiency virus type 1 into primary human macrophages: a selective role for the 75-kilodalton receptor. *Journal of Virology* **70**:7388–7397.

Hong M, Wakim V, Salomao S, Camargo L, Casseb J & Duarte A (1998) IL-2 and IFN-gamma, but not IL-4 secretion by peripheral blood mononuclear cells (PBMC) are related to CD4+ T cells and clinical status in Brazilian HIV-1-infected subjects. *Revista do Instituto de Medicina Tropical de Sao Paulo* **40**:351–354.

Hu R, Oyaizu N, Kalyanaraman V & Pahwa S (1994) HIV-1 gp160 as a modifier of Th1 and Th2 cytokine response: gp160 suppresses interferon-gamma and interleukin-2 production concomitantly with enhanced interleukin-4 production *in vitro*. *Clinical Immunology and Immunopathology* **73**:245–251.

Idziorek T, Khalife J, Billaut-Mulot O, Hermann E, Aumercier M, Mouton Y, Capron A & Bahr GM (1998) Recombinant human IL-16 inhibits HIV-1 replication and protects against activation-induced cell death (AICD). *Clinical and Experimental Immunology* **112**:84–91.

Imami N, Antonopoulos C, Hardy G, Gazzard B & Gotch F (1999) Assessment of type 1 and type 2 cytokines in HIV type 1-infected individuals: impact of highly active antiretroviral therapy. *AIDS Research and Human Retroviruses* **15**:1499–14508.

Jassoy C, Harrer T, Rosenthal T, Navia B, Worth J, Johnson R & Walker B (1993) Human immunodeficiency virus type 1-specific cytotoxic T lymphocytes release gamma interferon, tumor necrosis factor alpha (TNF-alpha), and TNF-beta when they encounter their target antigens. *Journal of Virology* **67**:2844–2852.

Jiang Y & Jolly P (1999) Effect of beta-chemokines on human immunodeficiency virus type 1 replication, binding, uncoating, and CCR5 receptor expression in human monocyte-derived macrophages. *Journal of Human Virology* **2**:123–132.

Jeffermans N, Paxton W, Dekkers P, Verbon A, de Jonge E, Speelman P, van Deventer S & van der Poll T (2000) Up-regulation of HIV coreceptors CXCR4 and CCR5 on CD4(+) T cells during human endotoxemia and after stimulation with (myco)bacterial antigens: the role of cytokines. *Blood* **96**:2649–2654.

Kalter DC, Nakamura M, Turpin JA, Baca LM, Hoover DL, Dieffenbach C, Ralph P, Gendelman HE & Meltzer MS (1991) Enhanced HIV replication in macrophage colony stimulating factor treated monocytes. *Journal of Immunology* **146**:298–306.

Kaplan L, Kahn J, Crowe S, Northfelt D, Neville P, Grossberg H, Abrams D, Tracey J, Mills J & Volberding P (1991) Clinical and virologic effects of recombinant human granulocyte macrophage colony stimulating factor (rGM-CSF) in patients receiving chemotherapy for HIV associated non-Hodgkin's lymphoma; results of a randomized trial. *Journal of Clinical Oncology* **9**:929–940.

Kazazi F, Mathijs JM, Chang J, Malafiej P, Lopez A, Dowton D, Sorrell TC, Vadas MA & Cunningham AL (1992) Recombinant interleukin 4 stimulates human immunodeficiency virus production by infected monocytes and macrophages. *Journal of General Virology* **73**:941–949.

Kedzierska K, Rainbird M, Lopez A & Crowe S (1998) Effect of GM-CSF on HIV-1 replication in monocytes/macrophages *In vivo* and *in vitro*; a review. *Veterinary Immunology and Immunopathology* **63**:111–121.

Kedzierska K, Maerz A, Warby T, Jaworowski A, Chan H, Mak J, Sonza S, Lopez A & Crowe S (2000a) Granulocyte-macrophage colony stimulating factor inhibits HIV-1 replication in monocyte-derived macrophages. *AIDS* **14**:1739–1748.

Kedzierska K, Mak J, Mijch A, Cooke I, Rainbird M, Roberts S, Paukovics G, Jolley D, Lopez A & Crowe SM (2000b) Granulocyte-macrophage colony-stimulating factor augments phagocytosis of Mycobacterium avium complex by human immunodeficiency virus type 1- infected monocytes/macrophages *in vitro* and *in vivo*. *Journal of Infectious Diseases* **181**:390–394.

Kehrl J, Rieckmann P, Kozlow E & Fauci AS (1992) Lymphokine production by B cells from normal and HIV-infected individuals. *Annals of the New York Academy of Sciences* **651**:220–227.

Kelleher A, Sewell W & Cooper D (1999) Effect of protease therapy on cytokine secretion by peripheral blood mononuclear cells (PBMC) from HIV-infected subjects. *Clinical and Experimental Immunology* **115**:147–152.

Kim JH, Loveland JE, Sitz KV, Ratto Kim S, McLinden RJ, Tencer K, Davis K, Burke DS, Boswell RN, Redfield RR & Birx DL (1997) Expansion of restricted cellular immune responses to HIV-1 envelope by vaccination: IL-7 and IL-12 differentially augment cellular proliferative responses to HIV-1. *Clinical and Experimental Immunology* **108**:243–250.

Kim JJ, Nottingham LK, Tsai A, Lee DJ, Maguire HC, Oh J, Dentchev T, Manson KH, Wyand MS, Agadjanyan MG, Ugen KE & Weiner DB (1999) Antigen-specific humoral and cellular immune responses can be modulated in rhesus macaques through the use of IFN-gamma, IL-12, IL-18 gene adjuvants. *Journal of Medical Primatology* **28**:214–223.

Kinter A, Bende S, Hardy E, Jackson R & Fauci A (1995a) Interleukin 2 induces CD8+ T cell-mediated suppression of human immunodeficiency virus replication in CD4+ T cells and this effect overrides its ability to stimulate virus expression. *Proceedings National Academy Science, USA* **92**:10985–10989.

Kinter A, Poli G, Fox L, Hardy E & Fauci A (1995b) HIV replication in IL-2-stimulated peripheral blood mononuclear cells is driven in an autocrine/paracrine manner by endogenous cytokines. *Journal of Immunology* **154**:2448–2459.

Kinter A & Fauci A (1996) Interleukin-2 and human immunodeficiency virus infection: pathogenic mechanisms and potential for immunologic enhancement. *Immunological Research* **15**:1–15.

Kitai R, Zhao M, Zhang N, Hua L & Lee S (2000) Role of MIP-1beta and RANTES in HIV-1 infection of microglia: inhibition of infection and induction by IFNbeta. *Journal of Neuroimmunology* **110**:230–239.

Kitano K, Abboud CN, Ryan DH, Quan SG, Baldwin GC & Golde DW (1991) Macrophage active colony stimulating factors enhance human immunodeficiency virus type 1 infection in bone marrow stem cells. *Blood* **77**:1696–1705.

Koopstra NA, van 't Wout A, Huisman HG, Miedema F & Schuitemaker H (1994) Interference of interleukin-10 with human immunodeficiency virus type 1 replication in primary monocyte-derived macrophages. *Journal of Virology* **68**:6967–6975.

Kornbluth RS, Oh PS, Munis JR, Cleveland PH & Richman DD (1989) Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by human immunodeficiency virus *in vitro*. *Journal of Experimental Medicine* **169**:1137–1151.

Kornbluth R, Kee K & Richman D (1998) CD40 ligand (CD154) stimulation of macrophages to produce HIV-1-suppressive beta-chemokines. *Proceedings of the National Academy of Science, USA* **95**:5205–5210.

Kovanagi Y, O'Brien WA, Zhao JQ, Golde DW, Gasson JC & Chen IS (1988) Cytokines alter production of HIV-1 from primary mononuclear phagocytes. *Science* **241**:1673–1675.

Krakauer T, Vilcek J & Oppenheim J (1998) Proinflammatory cytokines. In *Fundamental Immunology* pp. 773–811. Edited by W Paul. Philadelphia:Lippincott-Raven.

Kreuzer K, Rockstroh J, Kantfer B & Spengler U (2000) Endogenous levels of C-C chemokines MIP-1alpha, MIP-1beta, and RANTES do not reflect the disease course in HIV-seropositive individuals. *International Journal of Clinical Laboratory Research* **30**:163–168.

Krown SE, Paredes J, Bundow D, Polsky B, Gold JW & Fiorentiberg N (1992) Interferon-alpha, zidovudine, and granulocyte-macrophage colony-stimulating factor: a phase I AIDS Clinical Trials Group study in patients with Kaposi's sarcoma associated with AIDS. *Journal Clinical Oncology* **10**:1344–1351.

Kurtz J, Crim L, Feldman S, Hayes M, Gruber M, Beeler J & Clouse K (2000) Macrophage colony-stimulating factor antagonists inhibit replication of HIV-1 in human macrophages. *Journal of Immunology* **164**:4955–4960.

Lafeuillade A, Poggi C, Chadapaud S, Hittinger G, Chouraqui M, Pisapia M & Delbeke E (2001) Pilot study of a combination of highly active antiretroviral therapy and cytokines to induce HIV-1 remission. *Journal of Acquired Immune Deficiency Syndromes* **26**:44–55.

Lafeuillade A, Poggi C & Tamalet C (1996) GM-CSF increases HIV-1 load. *Lancet* **347**:1123–1124.

Landervirta J, Maury C, Teppo A and Repo H (1988) Elevated levels of circulating cachectin/tumor necrosis factor in patients with acquired immunodeficiency syndrome. *American Journal of Medicine* **85**:289–291.

Lane HC, Kovacs JA, Feinberg J, Herpin B, Davey V, Walker R, Deyton L, Metcalf JA, Baseler M, Salzman N, *et al.* (1988) Antiretroviral effects of interferon-alpha in AIDS-associated Kaposi's sarcoma. *Lancet* **2**:1218–1222.

Le Page C, Genin P, Baines M & Hiscott J (2000) Interferon activation and innate immunity. *Reviews in Immunogenetics* **2**:374–386.

Lopez A, Shannon M, Hercus T, Nicola N, Cambareri B, Dottore M, Layton M, Eglington L & Vadas M (1992) Residue 23 of human granulocyte macrophage colony stimulating factor is critical for biological activity and for high but not low affinity binding. *EMBO Journal* **11**:909–916.

Lucey DR, Pinto LA, Berlike FR, Rasnak J, Melcher GP, Hashemi FN, Landay AL, Kessler HA, Patron RJ, Greisvesen K & Shearer GM (1997) *In vitro* immunologic and virologic effects of interleukin 15 on peripheral blood mononuclear cells from normal donors and human immunodeficiency virus type 1-infected patients. *Clinical and Diagnostic Laboratory Immunology* **4**:43–48.

Ma X & Montaner L (2000) Proinflammatory response and IL-12 expression in HIV-1 infection. *Journal of Leukocyte Biology* **68**:83–90.

Maciaszek JW, Parada NA, Cruikshank WW, Center DM, Kornfeld H & Viglianti GA (1997) IL-16 represses HIV-1 promoter activity. *Journal of Immunology* **158**:5–8.

Maggi E, Mazzetti M, Ravina A, Annunziato F, de Carli M, Piccinni M, Manetti R, Carbonari M, Pesce A & del Prete GA (1994) Ability of HIV to promote a TH1 to TH0 shift and to replicate preferentially in TH2 and TH0 cells. *Science* **265**:244–248.

Malnati M, Tambussi G, Clerici E, Pozo S, Algeri M, Nardese V, Furci L, Lazzarin A & Lusso P (1997) Increased plasma levels of the C-C chemokine RANTES in patients with primary HIV-1 infection. *Journal of Biological Regulators and Homeostatic Agents* **11**:40–42.

Marching-Koka A, Aubin JT, Grangeot-Keros L, Perrier A, Benarrar C, Merrien D, Agut H, Autouturier P, Autran B, Wildanes J, *et al.* (1996) *In vivo* role of IL-6 on the viral load and on immunological abnormalities of HIV-infected patients. *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology* **11**:59–68.

Marshall J, Chehimi J, Gri G, Kostman J, Montaner L & Trinchieri G (1990) The interleukin-12-mediated pathway of immune events is dysfunctional in human immunodeficiency virus-infected individuals. *Blood* **94**:1003–1011.

Matsuda S, Akagawa K, Honda M, Yokota Y, Takebe Y & Takemon T (1995) Suppression of HIV replication in human monocyte derived macrophages induced by granulocyte macrophage colony stimulating factor. *AIDS Research and Human Retroviruses* **11**:1031–1038.

Matsumoto T, Miike T, Nelson R, Trudeau W, Lockey R & Yodoi J (1993) Elevated serum levels of IL-8 in patients with HIV infection. *Clinical and Experimental Immunology* **93**:130–131.

Meroni L, Trabattoni D, Balotta C, Riva C, Gori A, Moroni M, Luisa Villa M, Clerici M & Galli M (1996) Evidence for type 2 cytokine production and lymphocyte activation in the early phases of HIV-1 infection. *AIDS* **10**:23–30.

Meyaard L, Hovenkamp E, Keet I, Hoonbrink B, de Jong I, Otto S & Miedema F (1996) Single cell analysis of IL-4 and IFN-gamma production by T cells from HIV-infected individuals: decreased IFN-gamma in the presence of preserved IL-4 production. *Journal of Immunology* **157**:2712–2718.

Montaner LJ, Doyle AG, Collin M, Herbein G, Elei P, James W, Mintz A, Caput D, Ferrara P & Gordon S (1993) Interleukin 13 inhibits human immunodeficiency virus type 1 production in primary blood-derived human macrophages *in vitro*. *Journal of Experimental Medicine* **178**:743–747.

Montaner LJ, Bailer RT & Gordon S (1997) IL-13 acts on macrophages to block the completion of reverse transcription, inhibit virus production, and reduce virus infectivity. *Journal of Leukocyte Biology* **62**:126–132.

Moore K, de Waal Malefyt R, Coffman R & O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. *Annual Review Of Immunology* **19**:683–765.

Moran PA, Diegel ML, Sias JC, Leitherer JA & Zarling JM (1995) Regulation of HIV production by blood mononuclear cells from HIV-infected donors: I. Lack of correlation between HIV-1 production and T cell activation. *AIDS Research and Human Retroviruses* **9**:455–464.

Munoz-Fernandez M, Navarro J, Garcia A, Pamzon C, Fernandez-Cruz E & Fresno M (1997) Replication of human immunodeficiency virus-1 in primary human T cells is dependent on the autocrine secretion of tumor necrosis factor through the control of nuclear factor-kappa B activation. *Journal of Allergy and Clinical*

Immunology **100**:838–845.

Murray H (1996) Current and future clinical applications of interferon-gamma in host antimicrobial defense. *Intensive Care Medicine* **4**:S456–S461.

Naif H, Ho-Shon M, Chang J & Cunningham AL (1994) Molecular mechanisms of IL-4 effect on HIV expression in promonocytic cell lines and primary human monocytes. *Journal of Leukocyte Biology* **56**:335–339.

Naif HM, Li S, Ho-Shon M, Mathijs JM, Williamson P & Cunningham AL (1997) The state of maturation of monocytes into macrophages determines the effects of IL-4 and IL-13 on HIV replication. *Journal of Immunology* **158**:501–511.

Novak RM, Holzer TJ, Kennedy MM, Heynen CA & Dawson G (1990) The effect of interleukin 4 (BSF-1) on infection of peripheral blood monocyte-derived macrophages with HIV-1. *AIDS Research and Human Retroviruses* **6**:973–976.

Oberlin E, Amara A, Bachelerie FM, Bussia C, Virelizier JL, Schwartz O, Arenzana-Seisdedos F, Schwartz O, Heard JM, Clark-Lewis I, Legler DF, Loetscher M, Baggiolini M & Moser B (1996). The CXC chemokine SDF-1 is the ligand for LESTR/fusinn and prevents infectonT-cell-line-adapted lab. *Nature* **382**:833–835.

Ohashi K, Akazawa R & Kurimoto M (1994) Effects of interferon-alpha on a reduced release of interleukin-8 from latently HIV-1-infected monocytic cell line U937 cells. *Journal of Interferon Research* **14**:129–132.

Ohashi T, Arai M, Kato H, Kubo M, Fuji M, Yamamoto N, Iwamoto A & Kannagi M (1998). High SDF-1 expression in HIV-1 carriers does not correlate with CD8+ T-cell-mediated suppression of viral replication. *Virology* **244**:467–472.

Pandolfi F, Pierdominici M, Marziali M, Livia Bernardi M, Antonelli G, Galati V, D'Offizi G & Aiuti F (2000) Low-dose IL-2 reduces lymphocyte apoptosis and increases naive CD4 cells in HIV-1 patients treated with HAART. *Clinical Immunology* **94**:153–159.

Paul W (1991) Interleukin-4: a prototypic immunoregulatory lymphokine. *Blood* **77**:1859–1870.

Paxton W, Martin S, Tse D, O'Brien T, Skurnick J, VanDevanter N, Padian N, Braun J, Kotler D, Wolinsky S & Koup R (1996) Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposure. *Nature Medicine* **2**:412–417.

Perno CF, Yarchoan R, Cooney DA, Hartman NR, Webb DS, Hao Z, Mitsuya H, Johns DG & Broder S (1989) Replication of human immunodeficiency virus in monocytes. Granulocyte/macrophage colony-stimulating factor (GM-CSF) potentiates viral production yet enhances the antiviral effect mediated by 3'-azido- 2'3'-dideoxythymidine (AZT) and other dideoxynucleoside congeners of thymidine. *Journal of Experimental Medicine* **169**:933–951.

Perno CF, Cooney DA, Gao WY, Hao Z, Johns DG, Foli A, Hartman NR, Calio R, Broder S & Yarchoan R (1992) Effects of bone marrow stimulatory cytokines on human immunodeficiency virus replication and the antiviral activity of dideoxynucleosides in cultures of monocyte/macrophages. *Blood* **80**:995–1003.

Perno C, Aquaro S, Rosenwirth B, Balestra E, Peichl P, Billich A, Villani N & Calio R (1994) *In vitro* activity of inhibitors of late stages of the replication of HIV in chronically infected macrophages. *Journal of Leukocyte Biology* **56**:381–386.

Pett S & Emery S (2001) Immunomodulators as adjunctive therapy for HIV-1 infection. *Journal of Clinical Virology* (in press).

Poli G, Orenstein JM, Kinter A, Folks TM & Fauci AS (1989) Interferon-alpha but not AZT suppresses HIV expression in chronically infected cell lines. *Science* **244**:575–577.

Poli G, Bressler P, Kinter A, Duh E, Timmer WC, Rabson A, Justement JS, Stanley S & Fauci AS (1990) Interleukin 6 induces human immunodeficiency virus expression in infected monocytic cells alone and in synergy with tumor necrosis factor alpha by transcriptional and post-transcriptional mechanisms. *Journal of Experimental Medicine* **172**:151–158.

Poli G & Fauci A (1992) The effect of cytokines and pharmacologic agents on chronic HIV infection. *AIDS Research and Human Retroviruses* **8**:191–197.

Poli G, Biswas P & Fauci AS (1994a) Interferons in the pathogenesis and treatment of human immunodeficiency virus infection. *Antiviral Research* **24**:221–233.

Poli G, Kinter A & Fauci A (1994b) Interleukin 1 induces expression of the human immunodeficiency virus alone and in synergy with interleukin 6 in chronically infected U1 cells: inhibition of inductive effects by the interleukin 1 receptor antagonist. *Proceedings of the National Academy of Science, USA* **91**:108–112.

Pomerantz RJ, Feinberg MB, Trono D & Baltimore D (1990) Lipopolysaccharide is a potent monocyte/macrophage-specific stimulator of human immunodeficiency virus type 1 expression. *Journal of Experimental Medicine* **172**:253–261.

Radrizzani M, Accorsero P, Amidei A, Delia D, Kurrle R & Colombo MP (1995) IL-12 inhibits apoptosis induced in a human Th1 clone by gp120/CD4 cross-linking and CD3/TCR activation or by IL-2 deprivation. *Cellular Immunology* **161**:14–21.

Re M, Zauli G, Furlini G, Ranieri S & La Placa M (1992) Progressive and selective impairment of IL-3 and IL-4 production by peripheral blood CD4+ T-lymphocytes during the course of HIV-1 infection. *Viral Immunology* **5**:185–194.

Rho M, Wesselingh S, Glass J, McArthur J, Choi S, Griffin J & Tyor W (1995) A potential role for interferon-alpha in the pathogenesis of HIV-associated dementia. *Brain, Behavior and Immunity* **9**: 366–377.

Rincon M, Anguita J, Nakamura T, Fikrig E & Flavell R (1997) Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. *Journal of Experimental Medicine* **185**:461–469.

Rizzardi G, Marriott J, Cookson S, Lazzarin A, Dalgleish A & Barcellini W (1998) Tumour necrosis factor (TNF) and TNF-related molecules in HIV-1+ individuals: relationship with *in vitro* Th1/Th2-type response. *Clinical and Experimental Immunology* **114**:61–65.

Rollins B (1997) Chemokines. *Blood* **90**:909–928.

Sadeghi H, Weiss L, Kazatchkine M & Haeffner-Cavaillon N (1995) Antiretroviral therapy suppresses the constitutive production of interleukin-1 associated with human immunodeficiency virus infection. *Journal of Infectious Diseases* **172**:547–550.

Schmidtmayerova H, Nottet H, Nuovo G, Raabe T, Flanagan C, Dubrovsky L, Gendelman H, Cerami A, Bukrinsky M & Sherry B (1996) Human immunodeficiency virus type 1 infection alters chemokine beta peptide expression in human monocytes: implications for recruitment of leukocytes into brain and lymph nodes. *Proceedings of the National Academy of Science, USA* **93**:700–704.

Schols D & De Clercq E (1996) Human immunodeficiency virus type 1 gp120 induces anergy in human peripheral blood lymphocytes by inducing interleukin-10 production. *Journal of Virology* **70**:4953–4960.

Schols D, Este JA, Henson G & De Clercq E (1997) Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4. *Antiviral Research* **35**:147–156

Schuitemaker H, Kootstra NA, van Oers MH, van Lambalgen R, Tersmette M & Miedema F (1990) Induction of monocyte proliferation and HIV expression by IL-3 does not interfere with antiviral activity of zidovudine. *Blood* **76**:1490–1493.

Schuitemaker H, Kootstra N, Koppelman M, Bruisten S, Huisman H, Tersmette M & Miedema F (1992) Proliferation-dependent HIV-1 infection of monocytes occurs during differentiation into

macrophages. *Journal of Clinical Investigation* **89**:1154–1160.

Shapiro L, Puren AJ, Barton HA, Novick D, Peskind RL, Shenkar R, Gu Y, Su MS & Dinarello CA (1998) Interleukin 18 stimulates HIV type 1 in monocytic cells. *Proceedings of the National Academy of Science, USA* **95**:12550–12555.

Shirazi Y & Pitha PM (1992) Alpha interferon inhibits early stages of the human immunodeficiency virus type 1 replication cycle. *Journal of Virology* **66**:1321–1328.

Skowron G, Stein D, Drusano G, Melbourne K, Bilello J, Mikolich D, Rana K, Agosti JM, Mongillo A, Whitmore J & Gilbert MJ (1999) The safety and efficacy of GM-CSF (Sargramostim) added to indinavir-or ritonavir-based antiretroviral therapy: a randomized double blind, placebo-controlled trial. *Journal of Infectious Diseases* **180**:1064–1071.

Smith K (1988) Interleukin-2: inception, impact, and implications. *Science* **240**:1169–1176.

Smith R & Baglioni C (1987) The active form of tumor necrosis factor is a trimer. *Journal of Biological Chemistry* **262**:6951–6954.

Smithgall MD, Wong JG, Critchett KE & Haffar OK (1996) IL-7 up-regulates HIV-1 replication in naturally infected peripheral blood mononuclear cells. *Journal of Immunology* **156**:2324–2330.

Sonza S, Maerz A, Deacon N, Meanger J, Mills J. & Crowe S (1996) Human immunodeficiency virus type 1 replication is blocked prior to reverse transcription and integration in freshly isolated peripheral blood monocytes. *Journal of Virology* **70**:3863–3869.

Swingler S, Mann A, Jacque J, Brichacek B, Sasseville V, Williams K, Lackner A, Janoff E, Wang R, Fisher D & Stevenson M (1999) HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. *Nature Medicine* **9**:997–1003.

Takeshita S, Breen E, Ivashchenko M, Nishanian P, Kishimoto T, Vredevoe D & Martinez-Maza O (1995) Induction of IL-6 and IL-10 production by recombinant HIV-1 envelope glycoprotein 41 (gp41) in the THP-1 human monocytic cell line. *Cellular Immunology* **165**:234–242.

Tambussi G, Ghezzi S, Nozza S, Vallanti G, Magenta L, Guffanti M, Brambilla A, Vicenzi E, Carrera P, Racca S, Soldini L, Gianotti N, Murone M, Veglia F, Poli G & Lazzarin A (2001) Efficacy of low-dose intermittent subcutaneous interleukin (IL)-2 in antiviral drug-experienced human immunodeficiency virus-infected persons with detectable virus load: a controlled study of 3 IL-2 regimens with antiviral drug therapy. *Journal of Infectious Diseases* **183**:1476–1484.

Taoufik Y, Lantz O, Wallon C, Charles A, Dussaix E & Delfraissy JF (1997) Human immunodeficiency virus gp120 inhibits interleukin-12 secretion by human monocytes: an indirect interleukin-10-mediated effect. *Blood* **89**:2842–2848.

Trinchieri G & Scott P (1999) Interleukin-12: basic principles and clinical applications. *Current Topics in Microbiology and Immunology* **238**:57–78.

Truong MJ, Darcissac EC, Hermann E, Dewulf J, Capron A & Bahr GM (1999) Interleukin-16 inhibits human immunodeficiency virus type 1 entry and replication in macrophages and in dendritic cells. *Journal of Virology* **73**:7008–7013.

Tuosto L, Piazza C, Moretti S, Modesti A, Greenlaw R, Lechler R, Lombardi G & Piccolella E (1995) Ligation of either CD2 or CD28 rescues CD4+ T cells from HIV-gp120-induced apoptosis. *European Journal of Immunology* **25**:2917–2922.

Vazquez JA, Gupta S & Villanueva A (1998) Potential utility of recombinant human GM-CSF as adjunctive treatment of refractory oropharyngeal candidiasis in AIDS patients. *European Journal of Clinical Microbiology and Infectious Diseases* **17**:781–783.

Vicenzi E, Bagnarelli P, Santagostino E, Ghezzi S, Alfano M, Sinnone M, Fabio G, Turchetto L, Moretti G, Lazzarin A, Mantovani A, Mannucci P, Clementi M, Gringeri A & Poli G (1997) Hemophilia and nonprogressing human immunodeficiency virus type 1 infection. *Blood* **90**:2846–2848.

Vicenzi E, Alfano M, Ghezzi S, Gatti A, Veglia F, Lazzarin A, Sozzani S, Mantovani A & Poli G (2000) Divergent regulation of HIV-1 replication in PBMC of infected individuals by CC chemokines: suppression by RANTES, MIP-1alpha, and MCP-3, and enhancement by MCP-1. *Journal of Leukocyte Biology* **68**:405–412.

Vieillard V, Cremer J, Lauret E, Rozenbaum W, Debre P, Autran B & De Maeyer E (1997) Interferon beta transduction of peripheral blood lymphocytes from HIV- infected donors increases Th1-type cytokine production and improves the proliferative response to recall antigens. *Proceedings of the National Academy of Science, USA* **94**:11595–11600.

Vyakarnam A, McKeating J, Meager A & Beverley P (1990) Tumour necrosis factors (alpha, beta) induced by HIV-1 in peripheral blood mononuclear cells potentiate virus replication. *AIDS* **4**:21–27.

Wang J, Roderiquez G, Oravecz T & Norcross MA (1998) Cytokine regulation of human immunodeficiency virus type 1 entry and replication in human monocytes/macrophages through modulation of CCR5 expression. *Journal of Virology* **72**:7642–7647.

Weissman D, Poli G & Fauci AS (1994) Interleukin 10 blocks HIV replication in macrophages by inhibiting the autocrine loop of tumor necrosis factor alpha and interleukin 6 induction of virus. *AIDS Research and Human Retroviruses* **10**:1199–1206.

Weissman D, Poli G & Fauci AS (1995) IL-10 synergizes with multiple cytokines in enhancing HIV production in cells of monocytic lineage. *Journal of Acquired Immune Deficiency Syndromes and Human Retroviruses* **9**:442–449.

Weissman D, Daucher J, Barker T, Adelsberger J, Baseler M & Fauci A (1996) Cytokine regulation of HIV replication induced by dendritic cell-CD4-positive T cell interactions. *AIDS Research and Human Retroviruses* **12**:759–767.

Xin KQ, Hamajima K, Sasaki S, Tsuji T, Watabe S, Okada E & Okuda K (1999) IL-15 expression plasmid enhances cell-mediated immunity induced by an HIV-1 DNA vaccine. *Vaccine* **17**:858–866.

Yang OO, Swanberg SL, Lu Z, Dziejman M, McCoy J, Luster AD, Walker BD & Herrmann SH (1999) Enhanced inhibition of human immunodeficiency virus type 1 by met-stromal-derived factor 1β correlates with down-modulation of CXCR4. *Journal of Virology* **73**:4582–4589.

Yarchoan R, Pluda JM, Perno CF, Mitsuya H, Thomas RV, Wyvill KM & Broder S (1990) Initial clinical experience with dideoxynucleosides as single agents and in combination therapy. *Annals of the New York Academy of Sciences* **616**:328–343.

Zack J, Arrigo S, Weitsman S, Go A, Haislip A & Chen I (1990) HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell* **61**:213–222.

Zanussi S & De Paoli P (2000) The effects of interleukin-2 therapy on the viral reservoir in HIV+ patients. *Biomedicine and Pharmacotherapy* **54**:316–320.

Zou W, Dufloust A, Fior R, Durand-Gasselin I, Boue F, Galanaud P & Emilie D (1997) Increased T-helper-type 2 cytokine production in chronic HIV infection is due to interleukin (IL)-13 rather than IL-4. *AIDS* **11**:533–534.

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FcγR-mediated phagocytosis by human macrophages involves Hck, Syk, and Pyk2 and is augmented by GM-CSF

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Abstract: The receptors for the constant region of immunoglobulin G (FcγR) are widely expressed on cells of hemopoietic lineage and plays an important role in host defense. We investigated the signaling pathways during FcγR-mediated phagocytosis in human monocyte-derived macrophages (MDMs) and examined the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) on these events. FcγR-mediated phagocytosis resulted in enhanced tyrosine phosphorylation of a wide range of cellular proteins and activation of tyrosine kinases Hck, Syk, and Pyk2, as well as the multidomain adapter protein paxillin. Stimulation of MDMs with GM-CSF augmented FcγR-mediated phagocytosis and increased the levels of tyrosine phosphorylation in phagocytosing MDM cultures, indicating tyrosine kinase-mediated activation. GM-CSF treatment of MDMs without a phagocytic stimulus did not activate Syk, suggesting that GM-CSF may act either distally to Syk in the FcγR-mediated signaling cascade or on a parallel pathway activated by the FcγR. This study shows that early signaling events during FcγR-mediated phagocytosis in human MDMs involve activation of Syk, Hck, and paxillin. It also provides the first evidence for Pyk2 activation during phagocytosis and a baseline for further studies on the effect of GM-CSF on FcγR-mediated phagocytosis. *J. Leukoc. Biol.* 70: 322–328; 2001.

Key Words: paxillin · monocyte-derived macrophage · tyrosine kinase · signaling

INTRODUCTION

The receptors for the constant region of immunoglobulin (Ig) G (FcγRI, FcγRII, and FcγRIIIA) are the major means by which cells of the monocyte/macrophage lineage recognize IgG-opsonized particles and promote host defenses, including phagocytosis, antibody-dependent cellular cytotoxicity, and cytokine and chemokine secretion. The major Fcγ receptors expressed on monocytes are the high-affinity FcγRI (CD64) and a low-affinity FcγRII (CD32), whereas macrophages also express FcγRIIIA (CD16) [reviewed in reference 1]. Most studies delineating the specific signaling events during FcγR-mediated phagocytosis have been performed using murine macrophages [2–7] or cell lines transfected with FcγR [8–10], with only two studies done in human macrophages [11] or monocytes [12]. Several signal transduction pathways utilized by macrophages activated during FcγR-mediated phagocytosis have been described [reviewed in 13], including requirements for isoforms of protein kinase C [12], phosphatidylinositol 3-kinase [14, 15], and the Rho family of GTPases [16, 17]. These signaling events are initiated after clustering of FcγR via activation of tyrosine kinases of the Src family [18, 19]. Src kinase activation results in the rapid and transient phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on either the ligand-binding subunit in the cytoplasmic domain of Fcγ RII or the associated γ chain of FcγRI and RIIIA [10, 20, 21]. Phosphorylated ITAM motifs may represent docking sites for Syk that allow its subsequent activation [22, 23]. A requirement for Syk in FcγR-mediated phagocytosis was firstly demonstrated in human monocytes by using antisense oligodeoxynucleotides to eliminate Syk mRNA [24]. Gene knockout studies subsequently confirmed an absolute and specific requirement for Syk in FcγR-mediated phagocytosis by murine macrophages [3]. Macrophages derived from the fetal livers of Syk-deficient mice had defective FcγR-mediated phagocytosis and actin assembly but unimpaired phagocytosis of *Escherichia coli*, yeasts, or latex particles [3].

Activated Syk is thought to promote phosphorylation and localized accumulation of a number of cytoskeletal substrates [reviewed in 25], including the actin-binding proteins paxillin, vinculin, talin, and α-actinin [6, 26, 27]. Paxillin, a multidomain adapter protein, is thought to interact with a variety of proteins, such as focal adhesion kinase (FAK), Pyk2, and Vav, and in this way it organizes focal adhesion complexes and cytoskeletal rearrangement [reviewed in 28]. Human monocytes and macrophages do not express FAK [29], but they activate and phosphorylate another member of the FAK family,

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Pyk2. This kinase is 45% identical in amino acid sequence to FAK [30] and is shown to be involved in cytoskeletal engagement upon adherence and subsequent calcium or PKC co-stimulatory activation in human monocytes [29]. A role for Pyk2 in phagocytosis has not yet been reported.

Here we demonstrate that FcγR-mediated internalization of IgG-opsonized targets by human monocyte-derived macrophages (MDMs) was dependent on protein tyrosine phosphorylation, with transient activation of the protein tyrosine kinases Hck, Syk and Pyk2, and redistribution of the multidomain adaptor protein paxillin between Triton-soluble and Triton-insoluble cell fractions. In addition, acute stimulation of MDMs with granulocyte-macrophage colony-stimulating factor (GM-CSF) augmented phagocytosis of IgG-opsonized targets and concomitantly tyrosine phosphorylation of cellular proteins in an additive manner but did not activate Syk. These observations suggest that GM-CSF might either stimulate phagocytic pathways activated by the FcγR downstream of Syk kinase or might do so independently of Syk activation.

MATERIALS AND METHODS

Isolation and culture of monocytes

Human monocytes were isolated from buffy coats of HIV-, hepatitis B virus-, and human T-cell-lymphotropic virus-seronegative blood donors (supplied by the Red Cross Blood Bank, Melbourne) by Ficoll-Paque density gradient centrifugation and plastic adherence as previously described [31]. Immediately after isolation, cell viability was greater than 95% as assessed by trypan blue exclusion, and the purity of monocytes was greater than 95% as determined by immunofluorescent staining with anti-CD14 monoclonal antibody (mAb) (Becton Dickinson, Mountain View, CA) and analysis by flow cytometry (FACStar^{Plus}; Becton Dickinson). Monocytes were cultured in supplemented Iscove's medium [Iscove's modified Dulbecco medium (Cytosystem, Castle Hill, Australia) supplemented with 10% heat-inactivated human AB$^+$ serum, 2 mM L-glutamine, and 24 μg/mL of gentamicin] in suspension in polytetrafluorethylene (Teflon) pots (Savillex, Minnetonka, MN) at an initial concentration of 10^6 cells/mL. Monocytes were cultured for 5 to 7 days prior to phagocytosis assay. All the reagents and culture supernatants were tested for lipopolysaccharide levels using the *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, MD).

Opsonization of target particles

Target particles were opsonized immediately prior to the phagocytosis assay. Sheep red blood cells (E; ICN-Cappel, Aurora, OH) were washed three times in phosphate-buffered saline (PBS; Trace Biosciences, Castle Hill, Australia), and opsonized with a subagglutinating titer (1:300) of rabbit anti-E antibody (ICN-Cappel, Aurora, OH) for 30 min at room temperature. Cells were washed five times in cold PBS and resuspended at a concentration of 1×10^8/mL in Iscove's medium. Latex beads (3 μm in diameter; Sigma, St Louis, MO) were coated with bovine serum albumin (BSA) by incubation in 1 mL of PBS containing 10 mg of BSA (Sigma) overnight at 4°C, followed by five washes with cold PBS. Beads were opsonized with 10% rabbit anti-BSA antiserum (ICN-Cappel, Aurora, OH) for 30 min at room temperature, washed three times in PBS, and resuspended in PBS at a concentration of 4×10^8 cells/mL.

Phagocytosis assay using IgG-opsonized E

On days 5–7 after isolation, MDMs were plated onto 96-well plates (Costar, Cambridge, MA) at 5×10^4 cells per well in 100 μL of supplemented Iscove's medium and allowed to adhere for 2 h at 37°C in a 5% CO_2 humidified incubator. IgG-opsonized or unopsonized E was added to adhered MDMs at an E/MDM ratio of 10:1. The plate was centrifuged at 100 *g* for 5 min at 4°C and then placed at 37°C for phagocytosis to proceed. Phagocytosis was terminated after 30 min by placing the plates on ice and washing cells with ice-cold PBS. Phagocytosis of erythrocytes was quantified by a colorimetric assay [32]. Briefly, after unbound E were removed by washing with PBS, non-phagocytosed erythrocytes attached to MDMs were lysed with 0.2% NaCl for 3 min, followed by three washes with warm Iscove's medium. Phagocytosed erythrocytes were measured by reaction of hemoglobin with 2,7-diaminoflurene (Sigma) after lysis of MDMs in 0.2 M Tris-HCl buffer containing 6 M urea. Absorbance was determined at 620 nm in a plate reader (Labsystems Multiskan, Helsinki, Finland) and compared to a standard curve generated using known numbers of E (ranging from 4×10^5 to 5×10^5). In selected experiments MDMs were incubated with the protein tyrosine kinase inhibitor Genistein (Calbiochem, Croydon, Australia) at concentrations ranging from 0.1 to 30 μM at 37°C for 30 min prior to phagocytosis assay.

Phagocytosis assays using IgG-opsonized beads

MDMs (2×10^6 cells) were dispensed into 4 mL polypropylene tubes (Becton Dickinson, Paramus, NJ), washed twice in calcium- and magnesium-free PBS (PBS-CMF; 500 *g* for 5 min), and cooled on ice for 20 min in 100 μL of PBS-CMF. MDMs were incubated with or without IgG-opsonized beads at 37°C in a shaking-water bath with a target/MDM ratio of 10:1. At specified time points, phagocytosis was arrested by plunging the tubes into ice and washing the MDMs in ice-cold PBS-CMF, followed by centrifugation at 20,000 *g* for 30 s. For immunoblotting and immunoprecipitation experiments, washed cells were lysed in 100 μL of Triton lysis buffer containing 25 mM Tris-HCl (pH 7.5), 0.14 M NaCl, 1 mM EDTA, 1% Triton X-100, supplemented with phosphatase inhibitors [50 mM NaF, 1 mM sodium orthovanadate (Sigma), 40 mM β-glycerophosphate (Sigma)], and the following protease inhibitors: 1 mM pefabloc, 1 μM pepstatin, and 1 μM leupeptin (Boehringer-Mannheim, Mannheim, Germany).

GM-CSF stimulation

MDMs were stimulated with GM-CSF at 100 ng/mL (kindly provided by A. Lopez, Hanson Centre, Adelaide, Australia), immediately prior to addition of phagocytic targets. These phagocytic assays always commenced within 2 min of adding GM-CSF. Lysates were analyzed either for phagocytosis by a colorimetric assay, for tyrosine-phosphorylated proteins by immunoblotting, or for Syk phosphorylation by immunoprecipitation.

Immunoblotting and immunoprecipitation

Cell extracts containing equal amounts of proteins as determined by DC protein assay (Bio-Rad Laboratories, Hercules, CA) were boiled in sodium dodecyl sulfate (SDS) sample buffer [10 mM Tris (pH 8.0), 2 mM EDTA, 1% SDS, 5% β-mercaptoethanol, 5% glycerol], resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blocked for 2 h in either 3% BSA or 5% nonfat milk. The blots were probed with a recombinant antibody directed against phosphotyrosine (RC20; Transduction Laboratories, Lexington, KY) or paxillin (Transduction Laboratories) overnight at 4°C, followed by secondary antibody conjugated with horseradish peroxidase (Amersham Pharmacia, Buckinghamshire, England), and developed for enhanced chemiluminescence according to manufacturer's instructions (Amersham Pharmacia). Alternatively, to determine the involvement of various proteins in FcγR-mediated phagocytosis, cell lysates were immunoprecipitated with either a rabbit antibody against Hck (gift from H. C. Cheng, School of Biochemistry, Melbourne University), anti-Syk mAb (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-paxillin mAb and then probed with anti-phosphotyrosine antibody (PY20; Transduction Laboratories) or conversely, immunoprecipitated with anti-phosphotyrosine mAb and then probed with anti-Pyk2 (Transduction Laboratories) or anti-Syk mAb. Proteins were immunoprecipitated from extracts with antibodies overnight at 4°C, collected with 15 μL of protein G-Sepharose beads (1 h of incubation at 4°C) (Pharmacia Biotech, Uppsala, Sweden), washed five times in Triton lysis buffer, boiled in SDS sample buffer, resolved by 10% SDS-polyacrylamide gel electrophoresis, and analyzed by immunoblotting as described above. To verify the equal protein, input blots were reprobed with relevant antibodies.

Immunofluorescence microscopy

Cells fixed with 1 mL of 3% Ultrapure formaldehyde (Polysciences, Warrington, PA) for 20 min were washed twice with cold 0.1 M glycine in PBS-CMF and permeabilized with 0.1% Triton X-100 (Merck, Kilsyth, Aus-

tralia) for 1 min. After two washes with 1% fetal bovine serum in PBS-CMF, cells were stained for intracellular proteins with mouse anti-phosphotyrosine mAb (PY20) or isotype-matched control IgG1 (MOPC 21; Bionetics, Charleston, SC) for 30 min on ice. After two washes with cold PBS-CMF, cells were incubated with biotin-conjugated anti-mouse Ig (Silenus, Melbourne, Australia) for 30 min on ice, followed by two further washes with cold PBS-CMF. Subsequently, MDMs were incubated with Texas Red-conjugated streptavidin (Amersham Pharmacia) for 30 min on ice, washed once with cold PBS-CMF, fixed with 200 μL of 1% formaldehyde, and cytocentrifuged onto glass slides. Protein tyrosine phosphorylation in both resting and phagocytosing MDMs was analyzed by confocal laser microscopy (Bio-Rad MRC500). For some samples, cells were also stained with phalloidin conjugated to Alexa 488 (Molecular Probes, Eugene, OR) to determine colocalization of F-actin and phosphotyrosine.

RESULTS

Enhanced protein tyrosine phosphorylation during FcγR-mediated phagocytosis by MDMs

Two minutes after the addition of IgG-opsonized latex beads, increased tyrosine phosphorylation of a number of human MDM cytoplasmic proteins was evident by immunoblot analysis, with phosphorylation reaching a peak between 5 and 15 min (**Fig. 1a**). Phagocytosis of IgG-opsonized E was inhibited by the protein tyrosine kinase inhibitor genistein in a dose-dependent manner (Fig. 1b), suggesting that tyrosine kinase activity was required for FcγR-mediated phagocytosis. Phagocytosis of unopsonized E by MDM cultured in the presence or absence of genistein was below the detection level of the assay.

The majority of antiphosphotyrosine immunoreactivity in MDMs was localized around the internalized IgG-opsonized latex beads (**Fig. 2b**) colocalized with sites of actin polymerization (Fig. 2d). This finding is consistent with binding of tyrosine-phosphorylated proteins to a cytoskeleton which has been rearranged around the phagosome. Within 2 min of initiation of phagocytosis, paxillin, a multidomain adapter protein involved in organizing focal adhesion complexes, was also found to be preferentially localized around the phagosome (Fig. 2c). Control experiments verified a lack of nonspecific staining with the isotype-matched antibody (Fig. 2a).

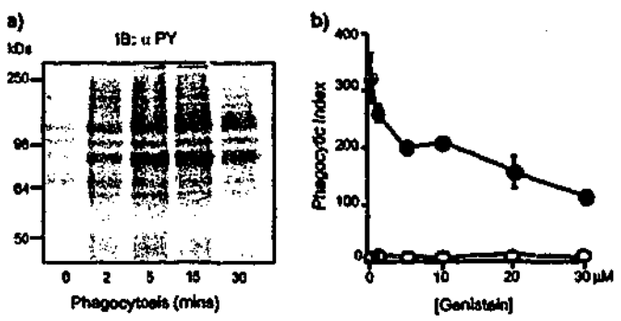

Fig. 1. FcγR-mediated phagocytosis and protein tyrosine phosphorylation. (a) MDMs incubated with IgG-opsonized latex beads (2×10^7) for the indicated times were lysed in Triton X-100 buffer, and samples of lysate containing 50 μg of protein were resolved by SDS-polyacrylamide gel electrophoresis, then probed with antiphosphotyrosine conjugated to horseradish peroxidase (RC20). Results shown are representative of five experiments using MDMs prepared from different donors. (b) Inhibition of phagocytosis by genistein. Phagocytosis of IgG-opsonized E (●) or E (○) was measured in the presence of the indicated concentrations of genistein via colorimetric assay as described in Materials and Methods. Data represent means (±SD) of triplicate determinations.

Activation of Hck, Syk, and Pyk2 tyrosine kinases

Having demonstrated a requirement for tyrosine kinase activity during FcγR-mediated phagocytosis by MDMs, we sought to identify the proteins which were tyrosine phosphorylated in the experiments depicted in Fig. 1. Based on estimated size, we investigated several tyrosine kinases that could potentially be activated during this process. Enhanced tyrosine phosphorylation of Hck, Syk, and Pyk2 was observed within 2–5 min of FcγR-mediated phagocytosis (**Fig. 3**). The input of Syk, Hck, and Pyk2 proteins was standardized according to Lowry's determinations. Protein levels are shown by reprobing the blots with relevant antibodies [Fig. 3a and b (lower panels)].

Redistribution of paxillin

Within 2 to 5 min of FcγR-mediated phagocytosis, there was an increase in paxillin phosphorylation which had declined by 30 min (**Fig. 4a**). Immunoblot analysis of Triton-soluble lysates probed with anti-paxillin antibody showed that the total level of extracted paxillin levels changed in a corresponding fashion (Fig. 4b). Total extracted paxillin levels did not vary during phagocytosis when MDMs were lysed in SDS sample buffer (total cell extract) (Fig. 4c). Taken together, these results suggest that in different phases of FcγR-mediated phagocytosis, paxillin is distributed predominantly in either the cytoskeletal (detergent-insoluble) fraction or cytoplasmic (detergent-soluble) fraction.

Stimulation of FcγR-mediated phagocytosis by GM-CSF

Stimulation of MDMs with GM-CSF (100 ng/mL) augmented the level of FcγR-mediated phagocytosis by 20–30% (**Fig. 5a**). GM-CSF stimulated tyrosine phosphorylation of MDM cellular proteins, and this effect was further augmented during phagocytosis of IgG-opsonized latex beads (Fig. 5b). We next determined whether GM-CSF directly activated Syk, because in murine macrophages this kinase is a well-established critical intermediate in Fcγ receptor signaling [3]. Incubation of MDM with GM-CSF for 2 to 5 min did not alter the extent of tyrosine phosphorylation of Syk above the levels observed during FcγR-mediated phagocytosis (Fig. 5c). In addition, control MDMs which had not been exposed to target particles did not display detectable Syk phosphorylation in either GM-CSF-treated or untreated samples. The level of Syk protein was equal for all the samples (Fig. 5c, lower panel). Syk coimmunoprecipitated from Triton lysates with several other phosphoproteins in a reproducible pattern. This pattern may represent a stable complex of Syk with Fcγ receptors and/or other signaling molecules located around the phagosome because a similar pattern was observed in experiments when MDM lysates were immunoprecipitated with anti γ-chain antibody (data not shown). Taken together, these data suggest that GM-CSF augments the process of FcγR-mediated phagocytosis by triggering tyrosine phosphorylation of a number of cellular proteins and most probably by acting downstream of Syk (and,

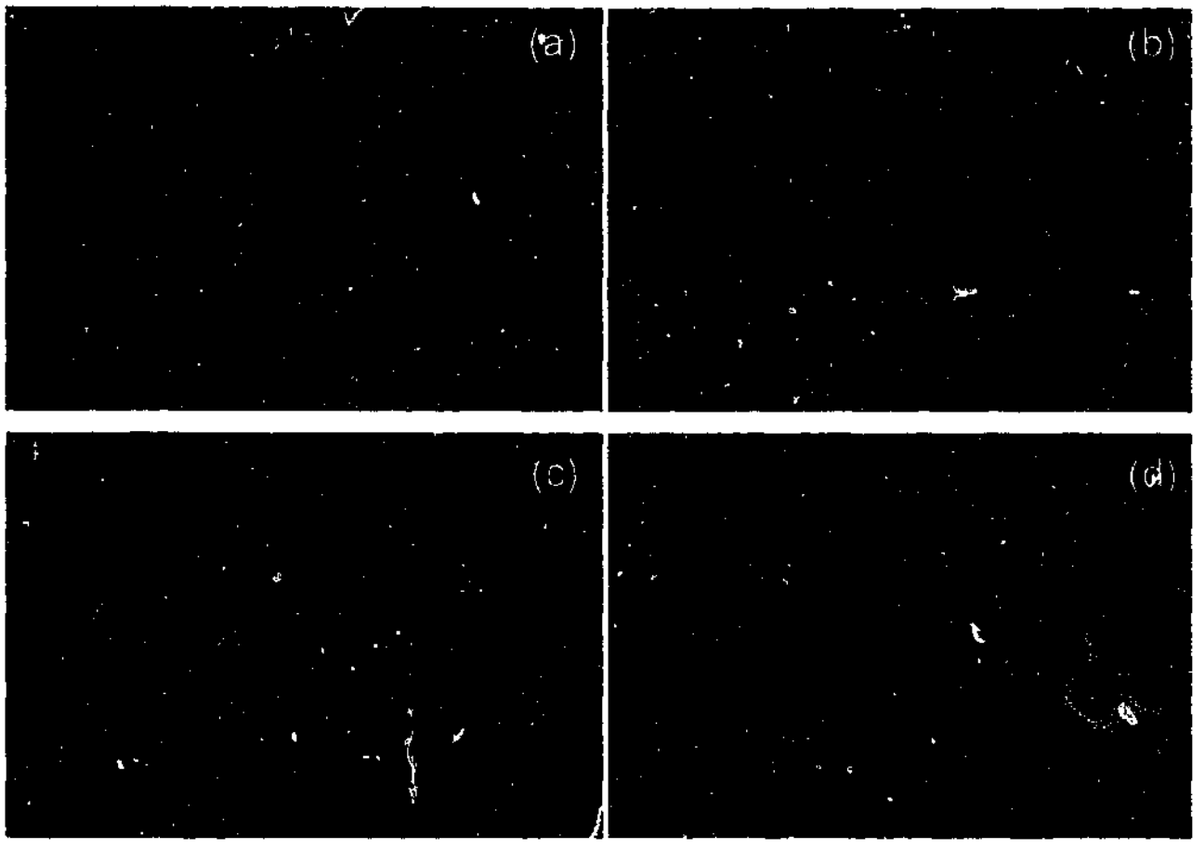

Fig. 2. Colocalization of tyrosine-phosphorylated proteins and polymerized actin around phagocytic cups in MDMs ingesting IgG-opsonized latex beads. MDMs were incubated with IgG-opsonized latex beads (3 μm diameter) for 2 min, fixed with 3% formaldehyde, permeabilized with 0.1% Triton X-100, and stained with (a) an isotype-matched control (MOPC 21); (b, d) mouse anti-phosphotyrosine mAb; or (c) mouse anti-paxillin mAb, followed by biotinylated anti-mouse Ig and Texas Red streptavidin conjugate; (d) MDMs were double stained for phosphotyrosine first as above and then with Alexa 488-labeled phalloidin. Areas of the cell staining with both antibodies appear yellow. MDMs were examined by confocal laser microscopy. Bar = 3 μm.

by extension, downstream of Src) in the FcγR-mediated cascade or via a pathway parallel to these events.

DISCUSSION

Our data show that FcγR-mediated phagocytosis by human macrophages was mediated by tyrosine phosphorylation of a number of cellular proteins which localized around the phagocytic cup and that phagocytosis was blocked by a protein tyrosine kinase inhibitor, genistein. Both Hck (a macrophage-specific Src family kinase) and Syk were phosphorylated after engagement of human MDM Fcγ receptors. Here, we also provide the first evidence for activation of Pyk2 and redistribution of the phosphorylated form of paxillin between the cytoplasm and cytoskeleton during FcγR-mediated phagocyto-

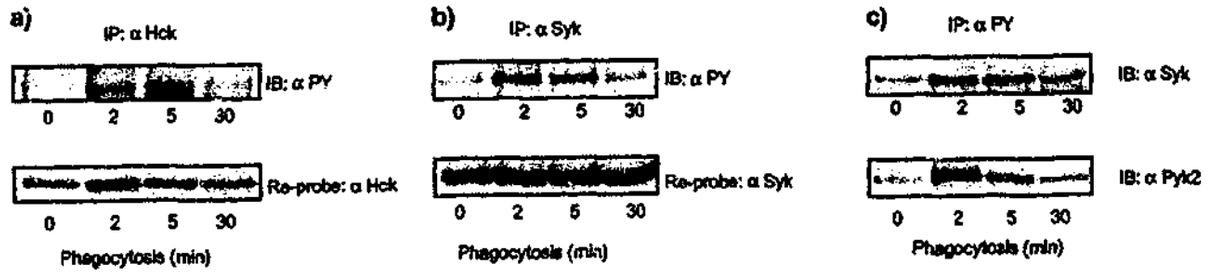

Fig. 3. Activation of Hck, Syk, and Pyk2 tyrosine kinases during FcγR-mediated phagocytosis in human MDMs. Cells (2×10^6) were incubated with IgG-opsonized latex beads (2×10^7) in polypropylene tubes in a shaking water bath for the indicated times (0 to 30 min) at 37°C and then lysed in Triton X-100 buffer. Samples of lysate containing 100 μg of protein were immunoprecipitated with (a) anti-Hck or (b) anti-Syk; resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-PY-horseradish peroxidase; or immunoprecipitated with (c) anti-PY mAb, resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-Syk or anti-Pyk2 mAb. The data are representative of three experiments using MDMs from different donors. Protein levels are shown by re-probing the blots with (a) anti-Hck mAb (lower panel) or (b) anti-Syk mAb (lower panel).

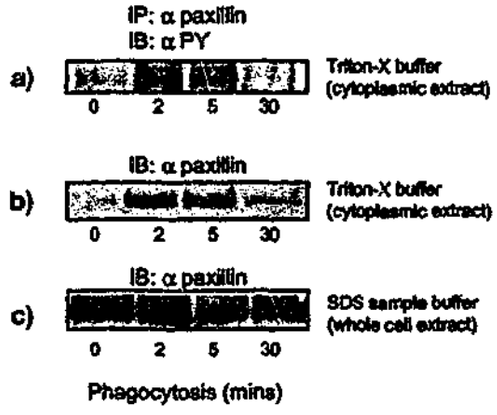

Fig. 4. Redistribution of paxillin between cytoplasm and cytoskeleton during FcγR-mediated phagocytosis in MDMs. Cells were incubated with IgG-opsonized latex beads (2×10^7) in a shaking water bath for the indicated times (0 to 30 min) at 37°C and lysed in either Triton X-100 or SDS sample buffer. (a) Triton X-100 lysates containing 100 μg of protein were immunoprecipitated with anti-paxillin mAb, resolved by SDS-polyacrylamide gel electrophoresis, and probed with anti-PY mAb. (b) Triton X-100 lysates (cytoplasmic fraction) or (c) SDS lysates (whole-cell extracts) containing 50 μg of protein were resolved by SDS-polyacrylamide gel electrophoresis and probed with anti-paxillin mAb. The data shown are representative of three experiments.

sis. In addition, we show augmentation of FcγR-mediated phagocytosis by GM-CSF, concomitant with increased tyrosine phosphorylation of a number of cellular proteins, which did not however include Syk.

A role for Hck in FcγR signaling has been suggested, based on data showing that macrophages prepared from mice deficient in three Src family tyrosine kinases, Hck, Fgr, and Fyn, exhibit poor signaling downstream of FcγR (i.e., Syk activation) as well as reduced levels of FcγR-induced functional responses such as phagocytosis, actin cup formation, and respiratory burst [3, 33]. A specific requirement for Hck in phagocytosis has also been demonstrated using Hck knock-out mice, which fail to internalize IgG-opsonized E, while other hemopoietic functions remain intact [34]. This study also suggested that some functions of Hck are subsumed through compensatory increases in the activity of another Src kinase, Lyn, although Lyn could not substitute for Hck in phagocytosis. Both Hck and Lyn have been shown also to coimmunoprecipitate with FcγR after cross-linking of receptors on THP-1 monocytic cell lines [35] and human monocytes [36]. In the present work we demonstrate activation of Hck during FcγR-mediated phagocytosis in human macrophages.

Phosphorylation of ITAM creates docking sites for Syk, a kinase shown by a number of studies to play a critical role in coupling phagocytosis-promoting FcγRs to the actin-based cytoskeleton [reviewed in 25]. A study using stem-loop Syk antisense oligonucleotides in human monocytes [24], as well as transfection studies using COS and DT40 cell lines and gene knockout studies, has demonstrated that Syk activation is absolutely required for FcγR-mediated phagocytosis, actin assembly, and FcγR-mediated transport to lysosomes [2, 3, 8, 22]. Furthermore, Syk has also been found to be a part of the activated FcγR complex after cross-linking of receptors in U937 and THP-1 cell lines [37, 38]. Our data confirm that Syk is phosphorylated in human MDMs after FcγR-mediated phagocytosis.

Activation of Syk is thought to result in the phosphorylation of various substrates located in the submembranous region beneath phagocytosed particles, which are required for actin polymerization and cytoskeletal rearrangement [8]. Paxillin is a potential downstream effector of Syk, because this protein has been shown to interact with a variety of proteins involved in growth control and cytoskeletal reorganization [reviewed in 28]. The abundance of binding motifs in paxillin for structural and regulatory proteins has led to a suggestion that it is important in recruiting signaling molecules at sites of actin

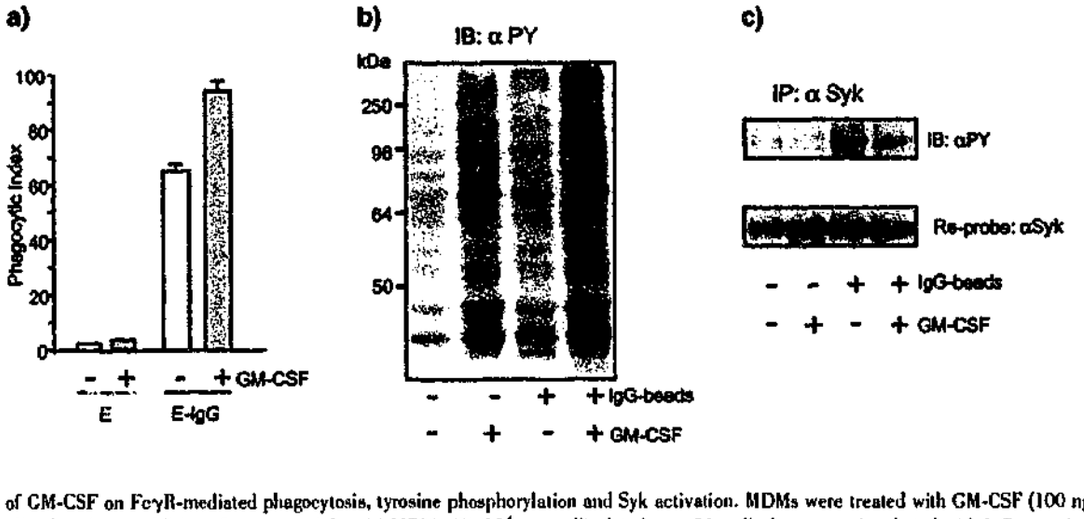

Fig. 5. Effect of GM-CSF on FcγR-mediated phagocytosis, tyrosine phosphorylation and Syk activation. MDMs were treated with GM-CSF (100 ng/mL), where indicated, immediately prior to addition of target particles. (a) MDMs (5×10^4 per well) plated onto 96-well plates were incubated with IgG-opsonized E or E for 20 min, and phagocytosis was determined as described in Materials and Methods. Data are representative of 3 donors and are means ± SD. MDMs (2×10^6) were incubated with IgG-opsonized latex beads (2×10^7) for the indicated times, and Triton X-100 buffer lysates were analyzed for (b) tyrosine-phosphorylated proteins by probing with horseradish peroxidase-conjugated anti-PY antibody (representative of five experiments using MDMs prepared from different donors); or (c) Syk tyrosine phosphorylation by immunoprecipitation of Syk and immunoblotting with horseradish peroxidase-conjugated anti-PY antibody (representative of five MDM donors). Input level of Syk was determined by reprobing the blots with Syk mAb.

rearrangement and in facilitating their interaction during phagocytosis [39]. Paxillin has been previously shown to be tyrosine phosphorylated in murine peritoneal macrophages during FcγR-mediated phagocytosis [6] and to colocalize with phagosomes [6, 26]. Here, we confirm and extend those observations by showing that the phosphorylated form of paxillin is redistributed between cytoplasm and cytoskeleton during phagocytosis in human macrophages.

Paxillin phosphorylation after cell adhesion has been postulated to involve the kinase p125 FAK, shown previously to bind to paxillin LD motifs [28]. The involvement of FAK in FcγR-mediated phagocytosis remains controversial, with one study showing no enhancement of tyrosine phosphorylation of FAK during phagocytosis in murine macrophages [6] and another report demonstrating the expression and phosphorylation of FAK as a result of FcγR cross-linking in human monocytes [40]. However, it has been reported that human monocytes/macrophages isolated under stringent conditions and free of platelet contamination do not express FAK [29; A. Jaworowski, unpublished results). We therefore determined whether Pyk2, a kinase with 45% sequence identity to FAK [41], known to be expressed in human monocytes/macrophages [29] and to bind to paxillin [42], is activated during FcγR-mediated phagocytosis. Our results demonstrated that tyrosine phosphorylation of Pyk2 was increased during FcγR-mediated phagocytosis. This is the first report of stimulation of Pyk2 tyrosine phosphorylation in response to phagocytosis, although this kinase has been implicated in other processes involving reorganization of the cytoskeleton, such as locomotion and adhesion [29, 43, 44].

FcγR phagocytosis by both human [45] and murine [46] macrophages has been found to be up-regulated by GM-CSF, a cytokine known to augment a number of macrophage effector functions [reviewed in 47, 48]. Since GM-CSF receptor does not possess an intrinsic tyrosine kinase catalytic domain, the activation of protein tyrosine kinases associated with the β subunit of the receptor (Janus kinase, signal-transducing activator of transcription, and Src families) mediates GM-CSF-stimulated proliferation, differentiation, and gene expression [49, 50]. Stimulation of human monocytes with GM-CSF for 48 h resulted in increased expression of FcγR II and increased binding of IgG-opsonized particles [51]. In our experiments, short-term pretreatment of MDMs with GM-CSF (2–5 min) was unlikely to increase FcγR expression and de novo receptor synthesis. Our observations and those of Rossman et al. suggest that GM-CSF may augment FcγR-mediated phagocytosis by several mechanisms, i.e., by stimulating the level of Fcγ receptor expression as well as the phosphorylation of intracellular tyrosine kinases. Because GM-CSF treatment of MDMs did not enhance phosphorylation of Syk, we postulate that GM-CSF exerts its stimulatory effect on FcγR-phagocytosis by acting either distally to Syk kinase in the FcγR-mediated signaling cascade or in a parallel pathway mediated via Fcγ receptor.

Our study shows that the early signaling events during FcγR-mediated phagocytosis in human MDMs were similar to those previously characterized in murine macrophages. In both species, key protein tyrosine kinases were activated during this process. In addition, we have demonstrated a novel role for the FAK-related kinase, Pyk2, in phagocytosis and have provided a baseline for further studies on the stimulatory role of GM-CSF on macrophage function. Because recent clinical studies have demonstrated the successful outcome of adjunctive GM-CSF treatment for opportunistic infections in immunosuppressed patients [52, 53], an understanding of the mechanism underlying macrophage stimulation by GM-CSF is potentially of therapeutic benefit.

ACKNOWLEDGMENTS

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Anthony Jaworowski and Suzanne M. Crowe contributed equally to this work.

REFERENCES

1. Daeron, M. (1997) Fc receptor biology. Annu. Rev. Immunol. 15, 203–234.
2. Kiefer, F., Brumell, J., Al-Alawi, N., Latour, S., Cheng, A., Veillette, A., Grinstein, S., Pawson, T. (1998) The Syk protein tyrosine kinase is essential for Fcγ receptor signaling in macrophages and neutrophils. Mol. Cell. Biol. 18, 4209–4220.
3. Crowley, M. T., Costello, P. S., Fitzer-Attas, C. J., Turner, M., Meng, F., Lowell, C., Tybulewicz, V. L., DeFranco, A. L. (1997) A critical role for Syk in signal transduction and phagocytosis mediated by Fcgamma receptors on macrophages. J. Exp. Med. 186, 1027–1039.
4. Strzelecka, A., Pyrzynska, B., Kwiatkowska, K., Sobota, A. (1997) Syk kinase, tyrosine-phosphorylated proteins and actin filaments accumulate at forming phagosomes during Fcgamma receptor-mediated phagocytosis. Cell. Motil. Cytoskeleton 38, 287–296.
5. Aderem, A. (1995) The MARCKS family of protein kinase-C substrates. Biochem. Soc. Trans. 23, 587–591.
6. Greenberg, S., Chang, P., Silverstein, S. C. (1994) Tyrosine phosphorylation of the gamma subunit of Fc gamma receptors, p72syk, and paxillin during Fc receptor-mediated phagocytosis in macrophages. J. Biol. Chem. 269, 3897–3902.
7. Greenberg, S., Chang, P., Silverstein, S. C. (1993) Tyrosine phosphorylation is required for Fc receptor-mediated phagocytosis in mouse macrophages. J. Exp. Med. 177, 529–534.
8. Cox, D., Chang, P., Kurosaki, T., Greenberg, S. (1996) Syk tyrosine kinase is required for immunoreceptor tyrosine activation motif-dependent actin assembly. J. Biol. Chem. 271, 16597–16602.
9. Greenberg, S., Chang, P., Wang, D. C., Xavier, R., Seed, B. (1996) Clustered Syk tyrosine kinase domains trigger phagocytosis. Proc. Natl. Acad. Sci. USA 93, 1103–1107.
10. Indik, Z. K., Hunter, S., Huang, M. M., Pan, X. Q., Chien, P., Kelly, C., Levinson, A. I., Kimberly, R. P., Schreiber, A. D. (1994) The high affinity Fc gamma receptor (CD64) induces phagocytosis in the absence of its cytoplasmic domain: the gamma subunit of Fc gamma RIIIA imparts phagocytic function to Fc gamma RI. Exp. Hematol. 22, 599–606.
11. Kusner, D. J., Hall, C. F., Jackson, S. (1999) Fc gamma receptor-mediated activation of phospholipase D regulates macrophage phagocytosis of IgG-opsonized particles. J. Immunol. 162, 2266–2274.
12. Zheleznyak, A., Brown, E. J. (1992) Immunoglobulin-mediated phagocytosis by human monocytes requires protein kinase C activation: evidence for protein kinase C translocation to phagosomes. J. Biol. Chem. 267, 12042–12048.
13. Aderem, A., Underhill, D. (1999) Mechanisms of phagocytosis in macrophages. Annu. Rev. Immunol. 17, 593–623.
14. Kanakaraj, P., Duckworth, B., Azzoni, L., Kamoun, M., Cantley, L. C., Perussia, B. (1994) Phosphatidylinositol-3 kinase activation induced upon Fc gamma RIIIA-ligand interaction. J. Exp. Med. 179, 551–558.

15. Ninomiya, N., Hazeki, K., Fukui, Y., Seya, T., Okada, T., Hazeki, O., Ui, M. (1994) Involvement of phosphatidylinositol 3-kinase in Fc gamma receptor signaling. J. Biol. Chem. 269, 22732–22737.
16. Hackam, D. J., Rotstein, O. D., Schreiber, A., Zhang, W., Grinstein, S. (1997) Rho is required for the initiation of calcium signaling and phagocytosis by Fcgamma receptors in macrophages. J. Exp. Med. 186, 955–966.
17. Cox, D., Chang, P., Zhang, Q., Reddy, P. G., Bokoch, G. M., Greenberg, S. (1997) Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. J. Exp. Med. 186, 1487–1494.
18. Jouvin, M. H., Adamczewski, M., Numerof, R., Letourneur, O., Valle, A., Kinet, J. P. (1994) Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. J. Biol. Chem. 269, 5918–5925.
19. Sarmay, G., Pecht, I., Gergely, J. (1994) Protein-tyrosine kinase activity tightly associated with human type II Fc gamma receptors. Proc. Natl. Acad. Sci. USA 91, 4140–4144.
20. Indik, Z. K., Park, J. G., Hunter, S., Mantaring, M., Schreiber, A. D. (1995) Molecular dissection of Fc gamma receptor-mediated phagocytosis. Immunol. Lett. 44, 133–138.
21. Park, J. G., Murray, R. K., Chien, P., Darby, C., Schreiber, A. D. (1993) Conserved cytoplasmic tyrosine residues of the gamma subunit are required for a phagocytic signal mediated by Fc gamma RIIIA. J. Clin. Invest. 92, 2073–2079.
22. Bonnerot, C., Briken, V., Brachet, V., Lankar, D., Cassard, S., Jabri, B., Amigorena, S. (1998) Syk protein tyrosine kinase regulates Fc receptor gamma-chain-mediated transport to lysosomes. EMBO J. 17, 4606–4616.
23. Indik, Z. K., Park, J. G., Pan, X. Q., Schreiber, A. D. (1995) Induction of phagocytosis by a protein tyrosine kinase. Blood 85, 1175–1180.
24. Matsuda, M., Park, J. G., Wang, D. C., Hunter, S., Chien, P., Schreiber, A. D. (1996) Abrogation of the Fc gamma receptor IIA-mediated phagocytosis signal by stem-loop Syk antisense oligonucleotides. Mol. Biol. Cell. 7, 1095–1106.
25. Greenberg, S. (1995) Signal transduction of phagocytosis. Trends Cell. Biol. 5, 93–99.
26. Allen, L. A., Aderem, A. (1996) Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. J. Exp. Med. 184, 627–637.
27. Greenberg, S., Burridge, K., Silverstein, S. C. (1990) Colocalization of F-actin and talin during Fc receptor-mediated phagocytosis in mouse macrophages. J. Exp. Med. 172, 1853–1856.
28. Turner, C. E. (1998) Paxillin. Int. J. Biochem. Cell. Biol. 30, 955–959.
29. Li, X., Hunter, D., Morris, J., Haskill, J. S., Earp, H. S. (1998) A calcium-dependent tyrosine kinase splice variant in human monocytes: activation by a two-stage process involving adherence and a subsequent intracellular signal. J. Biol. Chem. 273, 9361–9364.
30. Avraham, S., London, R., Fu, Y., Ota, S., Hiregowdara, D., Li, J., Jiang, S., Pasztor, L. M., White, R. A., Groopman, J. E., Avraham, H. (1995) Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. J. Biol. Chem. 270, 27742–27751.
31. Crowe, S. M., Mills, J., McGrath, M. S. (1987) Quantitative immunocytofluorographic analysis of CD4 surface antigen expression and HIV infection of human peripheral blood monocyte/macrophages. AIDS Res. Hum. Retroviruses 3, 135–145.
32. Gebran, S. J., Romano, E. L., Pons, H. A., Cariani, L., Soyano, A. N. (1992) A modified colorimetric method for the measurement of phagocytosis and antibody-dependent cell cytotoxicity using 2,7-diaminofluorene. J. Immunol. Methods 151, 255–260.
33. Fitzer-Attas, C. J., Lowry, M., Crowley, M. T., Finn, A. J., Meng, F., DeFranco, A. L., Lowell, C. A. (2000) Fcgamma receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn. J. Exp. Med. 191, 669–682.
34. Lowell, C. A., Soriano, P., Varmus, H. E. (1994) Functional overlap in the src gene family: inactivation of hck and fgr impairs natural immunity. Genes Dev. 8, 387–398.
35. Ghazizadeh, S., Bolen, J., Fleit, H. (1994) Physical and functional association of Src-related protein tyrosine kinases with Fc gamma RII in monocytic THP-1 cells. J. Biol. Chem. 269, 8878–8884.
36. Wang, A., Scholl, P., Geha, R. (1994) Physical and functional association of the high affinity immunoglobulin G receptor (Fc gamma RI) with the kinases Hck and Lyn. J. Exp. Med. 180, 1165–1170.
37. Darby, C., Geahlen, R., Schreiber, A. (1994) Stimulation of macrophage Fc gamma RIIIA activates the receptor-associated protein tyrosine kinase Syk and induces phosphorylation of multiple proteins including p95Vav and p62/GAP-associated protein. J. Immunol. 152, 5429–5437.
38. Ghazizadeh, S., Bolen, J., Fleit, H. (1995) Tyrosine phosphorylation and association of Syk with Fc gamma RII in monocytic THP-1 cells. Biochem. J. 305, 669–674.
39. Turner, C., Brown, M., Perrotta, J., Riedy, M., Nikolopoulos, S., McDonald, A., Bagrodia, S., Thomas, S., Leventhal, P. (1999) Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: a role in cytoskeletal remodeling. J. Cell Biol. 145, 851–863.
40. Pan, X. Q., Darby, C., Indik, Z. K., Schreiber, A. D. (1999) Activation of three classes of nonreceptor tyrosine kinases following Fc gamma receptor crosslinking in human monocytes. Clin. Immunol. 90, 55–64.
41. Sasaki, H., Nagura, K., Ishino, M., Tobioka, H., Kotani, K., Sasaki, T. (1995) Cloning and characterization of cell adhesion kinase beta, a novel protein-tyrosine kinase of the focal adhesion kinase subfamily. J. Biol. Chem. 270, 21206–21219.
42. Hiregowdara, D., Avraham, H., Fu, Y., London, R., Avraham, S. (1997) Tyrosine phosphorylation of the related adhesion focal tyrosine kinase in megakaryocytes upon stem cell factor and phorbol myristate acetate stimulation and its association with paxillin. J. Biol. Chem. 272, 10804–10810.
43. Duong, L. T., Lakkakorpi, P. T., Nakamura, I., Machwate, M., Nagy, R. M., Rodan, G. A. (1998) Pyk2 in osteoclasts is an adhesion kinase, localized in the sealing zone, activated by ligation of alpha/beta integrin, and phosphorylated by Src kinase. J. Clin. Invest. 102, 881–892.
44. Astier, A., Avraham, H., Manie, S. N., Groopman, J., Canty, T., Avraham, S., Freedman, A. S. (1997) The related adhesion focal tyrosine kinase is tyrosine-phosphorylated after beta1-integrin stimulation in B cells and binds to p130cas. J. Biol. Chem. 272, 228–232.
45. Capsoni, F., Minonzio, F., Ongari, A. M., Rizzardi, P. G., Lazzarin, A., Zanussi, C. (1992) Monocyte-derived macrophage function in HIV-infected subjects: in vitro modulation by rIFN-gamma and rGM-CSF. Clin. Immunol. Immunopathol. 62, 176–182.
46. Coleman, D. L., Chodakewitz, J. A., Bartiss, A. H., Mellors, J. W. (1988) Granulocyte-macrophage colony-stimulating factor enhances selective effector functions of tissue-derived macrophages. Blood 72, 573–578.
47. Metcalf, D. (1985) The granulocyte-macrophage colony stimulating factors. Science 229, 16–21.
48. Armitage, J. (1998) Emerging applications of recombinant human granulocyte-macrophage colony stimulating factor. Blood 92, 4491–4508.
49. Corey, S. J., Anderson, S. M. (1999) Src-related protein tyrosine kinases in hematopoiesis. Blood 93, 1–14.
50. Mui, A.-F., Wakao, H., O'Farrel, A.-M., Harada, N., Miyajima, A. (1995) Interleukin-1, granulocyte-macrophage colony stimulating factor and interleukin -5 transduce signal through two STAT5 homologs. EMBO J. 14, 1166–1175.
51. Rossman, M. D., Ruiz, P., Comber, P., Gomez, F., Rottem, M., Schreiber, A. D. (1993) Modulation of macrophage FC gamma receptors by rGM-CSF. Exp. Hematol. 21, 177–183.
52. Kedzierska, K., Mak, J., Mijch, A., Cooke, I., Rainbird, M., Roberts, S., Paukovics, G., Jolley, D., Lopez, A., Crowe, S. M. (2000) Granulocyte-macrophage colony-stimulating factor augments phagocytosis of Mycobacterium avium complex by human immunodeficiency virus type 1-infected monocytes/macrophages in vitro and in vivo. J. Infect. Dis. 181, 390–394.
53. Vazquez, J. A., Gupta, S., Villanueva, A. (1998) Potential utility of recombinant human GM-CSF as adjunctive treatment of refractory oropharyngeal candidiasis in AIDS patients. Eur. J. Clin. Microbiol. Infect. Dis. 17, 781–783.

nef-deleted HIV-1 inhibits phagocytosis by monocyte-derived macrophages *in vitro* but not by peripheral blood monocytes *in vivo*

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Objective: HIV-1 infection impairs a number of macrophage effector functions, but the mechanism is unknown. We studied the role of HIV-1 Nef in modulating phagocytosis by human monocytes and monocyte-derived macrophages (MDM).

Design and methods: Using a flow cytometric assay, phagocytosis of *Mycobacterium avium* complex (MAC) by monocytes in whole blood of Sydney Blood Bank Cohort (SBBC) members infected with a *nef*-deleted (Δ*nef*) strain of HIV-1 was compared with that of monocytes from uninfected or wild-type (WT) HIV-infected subjects. The specific impact of Nef on phagocytosis by MDM was determined by either infecting cells *in vitro* with Δ*nef* strains of HIV-1 or electroporating Nef into uninfected MDM.

Results: MAC phagocytic capacity of monocytes from SBBC members was equivalent to that of cells from uninfected individuals ($P = 0.81$); it was greater than that of cells from individuals infected with WT HIV-1 ($P < 0.0001$); irrespective of CD4 counts and HIV viral load. In contrast, *in vitro* infection of MDM with either Δ*nef* or WT strains of HIV-1 resulted in similar levels of HIV replication and equivalent impairment of phagocytosis via Fcγ and complement receptors. Electroporation of Nef into MDM did not alter phagocytic capacity.

Conclusions: This study provides evidence demonstrating the complex indirect effect of Nef on phagocytosis by peripheral blood monocytes (infrequently infected with HIV-1) *in vivo*. Conversely, the fact that MDM infected with either Δ*nef* or WT HIV-1 *in vitro* (high multiplicity of infection) show comparably impaired phagocytosis, indicates that HIV-1 infection of macrophages can directly impair function, independent of Nef.

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Keywords: Phagocytosis, monocyte/macrophages, HIV-1, Nef

Introduction

Cells of macrophage lineage including peripheral blood monocytes and tissue macrophages are important effector cells against a number of intracellular pathogens including *Mycobacterium avium* complex (MAC), *Toxoplasma gondii*, *Candida albicans* and *Pneumocystis carinii*. These cells provide critical functions in the cell-

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mediated response to these opportunistic pathogens such as chemotaxis, phagocytosis and intracellular killing. We and others have reported that a number of these functions are impaired following HIV-1 infection [1–3]. These defects contribute to the pathogenesis of AIDS by allowing reactivation or infection with otherwise uncommon opportunistic pathogens (reviewed in [4]), resulting in significant morbidity and mortality.

The exact mechanism by which HIV-1 impairs monocyte/macrophage function and promotes disease progression remains unclear. However, the importance of Nef (a 25 to 30 kDa myristylated accessory protein) for HIV-1 pathogenesis has been demonstrated in prospective studies of the Sydney Blood Bank Cohort (SBBC), a blood donor and eight transfusion recipients who were all infected with a strain of HIV-1 containing deletions within the *nef* gene and deletions and duplications within the overlapping long terminal repeat overlap [5–7]. Although there is evidence of recent clinical progression in two of the cohort members after 16 years of infection, the virus is clearly attenuated in comparison with wild-type (WT) HIV-1 strains, supporting the role for a functional Nef in HIV-1 pathogenesis in humans [5–7]. An intact *nef* gene has also been shown to be important for disease progression in simian immunodeficiency virus (SIV)-infected adult rhesus macaques [8] as well as in HIV-infected severe combined immunodeficient (SCID)-hu mice [9]. However, viral load may play an important role, as infant macaques infected with *nef*-deleted SIV at high multiplicity of infection developed disease [10].

Nef is thought to contribute to HIV-1 pathogenesis through a variety of mechanisms such as down-regulating CD4 and MHC class I expression [11–15], enhancing virion infectivity [16–18] and modulating signalling pathways via interactions with host cell proteins (reviewed in [19]). As these latter include interactions of Nef with cellular proteins and kinases which are also involved in the process of phagocytosis (such as Src kinases, Hck and Lyn [20–23], p21-activated kinase [24,25], guanine-nucleotide exchange factor Vav [26] and small GTPases Rac1 and Cdc42 [27,28]), it is possible that Nef modulation of signalling pathways involving these proteins inhibits phagocytosis. The role of Nef on phagocytosis by human monocytes and macrophages following HIV-1 infection has not yet been elucidated.

In this study we have investigated the effects of Nef on phagocytosis of MAC, by comparing monocytes from members of SBBC with monocytes from subjects who are either uninfected or infected with WT HIV-1. We have examined the indirect (e.g. cytokine or chemokine) effects of HIV-1 infection on phagocytosis by monocytes (infrequently infected with HIV-1) in whole blood from HIV-infected individuals including SBBC members and patients with WT HIV-1 infection. To examine the direct effects of HIV-1 replication within monocyte-derived macrophages (MDM) we have infected MDM with either *nef*-deleted (Δ*nef*) or WT strains of HIV-1 *in vitro*, at relatively high multiplicity of infection, and assessed phagocytosis of both IgG- and complement (C')-opsonized targets. To examine the effects of Nef on phagocytosis directly we have electroporated Nef protein into uninfected MDM. The results of this study demonstrate that the impact of HIV-1 Nef protein on phagocytosis differs markedly in MDM infected *in vitro* and in monocytes studied *ex vivo*, reflecting the potentially different mechanisms underlying inhibition of phagocytosis in these two situations.

Methods

Sources of monocytes

Blood (2 ml) was collected from six members of the Sydney Blood Bank Cohort in lithium heparin anticoagulant, with their informed consent, and dispensed into polypropylene tubes. Similarly, blood was collected from HIV-1-infected patients and from uninfected controls. Peripheral blood was assessed for plasma HIV RNA by bDNA assay (Chiron Corporation, Emeryville, USA) or reverse transcriptase (RT)-polymerase chain reaction (Roche Diagnostics, Nutley, New Jersey, USA) according to manufacturers' instructions.

Whole blood phagocytosis assay

Fluorescein isothiocyanate (FITC)-labelled MAC (5×10^6 and 1.5×10^7) was added to 100 μl of blood dispensed in polypropylene tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA) and placed into a shaking waterbath at 37°C for phagocytosis to proceed, as previously described by this laboratory [3,29]. Phagocytosis was terminated after 10 min by plunging the tubes into ice. The fluorescence of MAC-FITC adherent to the monocyte surface was quenched using quenching agent (Orpegen, Heidelberg, Germany). Monocytes were identified by staining with anti-CD14 conjugated to phycoerythrin (Becton Dickinson). Erythrocytes were lysed with FACS lysing solution (Becton Dickinson) and the remaining cells were fixed with 1% formaldehyde (Polysciences, Warrington, Pennsylvania, USA) for flow cytometric analysis (FACStar^{PLUS}). The proportion of monocytes that had ingested MAC-FITC were plotted against the ratio of MAC-FITC : monocyte which was calculated from the total monocyte count as previously described [3].

F-actin content of peripheral blood monocytes during phagocytosis

Blood (100 μl) was dispensed in polypropylene tubes and cooled on ice for 20 min. To perform phagocyto-

sis, blood samples were incubated with or without IgG-opsonized latex beads (3 μm in diameter; Sigma, St Louis, Missouri, USA) at a concentration of 5×10^7 beads/ml, at 37°C. Phagocytosis was terminated at various times (0 to 10 min) by plunging the tubes into ice and fixing the cells with 1 ml of 3% formaldehyde (20 min, 4°C). Following two washes with cold 0.1 mol/l glycine in phosphate-buffered saline (PBS)-calcium magnesium free (CMF), the monocytes within blood were stained with anti-CD14 Mab conjugated to phycoerythrin for 30 mins at 4°C. After a wash in cold (4°C) PBS-CMF, the erythrocytes were lysed with FACS' lysing solution at 4°C. The remaining white blood cells were permeabilized with 0.1% Triton-X 100 (Merck, Kilsyth, Australia) for 1 min, washed twice with 1% FCS/PBS-CMF and stained for F-actin levels with phalloidin-Alexa 488 (Molecular Probes, Eugene, Oregon, USA) for 30 min at 4°C. The cells were washed with cold PBS-CMF, fixed with 1% formaldehyde and analysed by flow cytometry.

HIV-1 infection of MDM *in vitro*

A subproviral Δ*nef* construct, obtained from NIH AIDS Reagent Program (contributed by Ronald Desrosiers), was used to generate the full length NL4.3Δ*nef* proviral DNA. The DNA constructs pNL(AD8) and pNL(AD8Δ*nef*) were prepared by substituting the respective envelope coding DNA sequences from NL4.3 and NL4.3Δ*nef* with monocytotropic AD8 envelope coding sequences, converting a T-tropic virus to M-tropic. A laboratory-adapted M-tropic strain ($HIV\text{-}1_{Ba\text{-}L}$) of HIV-1 was also used for MDM infections as described previously [30]. The *nef*-deleted primary isolates from D36 and C18 (two of the SBBC members from which virus could be successfully isolated) were prepared by co-culturing peripheral blood mononuclear cells (PBMC) from D36 or C18 with HIV-1 seronegative CD8-depleted PBMC that were pre-stimulated with phytohaemagglutinin (10 μg/ml; Murex Diagnostics, Dartford, UK) and macrophage colony-stimulating factor (750U/ml, Genzyme, Cambridge, Massachusetts, USA) for 3 days prior to co-culture. Cells were then co-cultured in the presence of recombinant human interleukin (IL)-2 (10 U/ml; Boehringer Mannheim, Mannheim, Germany). Purified MDMs from HIV-1 seronegative buffy coats were infected with $HIV\text{-}1_{NL(AD8)}$ chimera, $HIV\text{-}1_{NL(AD8\Delta nef)}$ chimera, $HIV\text{-}1_{Ba\text{-}L}$, and primary isolates $HIV\text{-}1_{D36}$ or $HIV\text{-}1_{C18}$ from SBBC members at the same multiplicity of infection. Control cells were mock-infected and cultured under identical conditions. HIV-1 infections were all performed using MDM 5 days after their isolation, cultured in suspension in polytetrafluorethylene (Teflon) jars (Savillex, Minnetonka, Minnesota, USA) at a concentration of 1×10^6 cells/ml. HIV-1 replication in MDM was quantified by flow cytometry by measuring intracellular p24 antigen using a MAb directed against p24 (2μg/ml; IgG1, Olympus, Lake Success, New York, USA), followed by goat anti-mouse IgG conjugated to FITC (FITC-GAM; Tago, Burlingame, California, USA) or by monitoring RT activity using a micro RT assay as previously described [30].

Phagocytosis by MDM infected with HIV-1 *in vitro*

Phagocytosis of MAC-FITC

The phagocytic capacity of MDM for MAC-FITC was assessed 7 days after HIV-1 infection. Cells (2×10^5 cells in 100 μl of PBS) were dispensed in polypropylene tubes (Becton Dickinson) and cooled on ice for 20 min. MDM were incubated at 37°C (or on ice as a control) for 2 h with MAC-FITC, at various MAC-FITC : MDM ratios (ranged from 1 : 10 to 1 : 75), in duplicate. After washing MDM with PBS and quenching adherent MAC-FITC, the cells were fixed and the proportion of MDM that had ingested MAC-FITC was quantified by flow cytometry as previously described [3].

Fc-gamma receptor (FcγR)-mediated phagocytosis

On day 7–10 following HIV-1 infection, the MDM were plated onto 96-well plates (Costar, Cambridge, Massachusetts, USA) at 5×10^4 cells per well in 100 μl of supplemented Iscove's medium (Cytosystem, Castle Hill, Australia), and allowed to adhere for 2 h in a 37°C, 5%CO_2 humidified incubator. Sheep red blood cells (sRBC; ICN-Cappel, Aurora, Ohio, USA) were opsonized immediately prior to the phagocytosis assay with a subagglutinating titre of rabbit anti-sRBC antibody (1 : 300; ICN-Cappel) for 30 min at room temperature. IgG-opsonized or unopsonized sRBC were added to adhered MDM at a sRBC : MDM ratio of 10 : 1. The plate was centrifuged at $100 \times g$ for 5 min at 4°C and then placed at 37°C, 5%CO_2 for phagocytosis to proceed. Phagocytosis was terminated after 10 min by placing the plates on ice and washing the cells with ice-cold PBS. The level of phagocytosed sRBC was determined by colorimetric assay [31]. Briefly, after unbound sRBC were removed by washing with PBS, the bound non-phagocytosed sRBC were lysed with 0.2% NaCl for 3 min. Phagocytosed sRBC were assessed after total cell lysis in 0.2 mol/l Tris-HCl buffer containing 6 mol/l urea by reaction of haemoglobin with 2,7-diaminoflurene (Sigma). Absorbance was determined at 620 nm in a plate reader (Labsystems, Multiskan, Helsinki, Finland), and compared with a standard curve generated using known amounts of sRBCs (ranging from 2×10^3 to 5×10^5).

C'R-mediated phagocytosis

sRBC were opsonized with pre-warmed 2% AB-negative human serum as a source of human complement components for 30 min at room temperature (Chan H-T, unpublished). As controls, sRBC were opsonized with the same source of AB-negative serum that had

been heat-inactivated at 56°C for 45 min (HI-sRBC). Prior to the phagocytosis assay, MDM that had adhered to 96-well plates were activated by treatment with phorbol-12-myristate-13-acetate (PMA) at a final concentration of 200 nmol/l per well for 10 min at 37°C. Subsequently those cells were exposed to C′-sRBC or HI-sRBC at an sRBC : MDM ratio of 20 : 1. C′R-mediated phagocytosis was performed at 37°C for 60 min and assessed by colorimetric assay as described above for FcγR-mediated phagocytosis.

Phagocytosis by Nef-electroporated MDM

To assess the direct effect of Nef on phagocytosis *in vitro*, purified recombinant Nef protein [32] was introduced by electroporation into MDM on day 5 post-isolation. Cells that were electroporated with purified recombinant glutathione-s-transferase (GST) or mock-electroporated (no protein) were used as controls. Proteins (300 nmol/l per 1×10^6 cells) were introduced into MDM using a square-wave electroporator (Bio-Rad, Hercules, California, USA; amplitude, 5 kV; pulse frequency, 28; burst time, 0.8 s; cycle number, 10). Electroporation of Nef into MDM was confirmed by immunofluorescence staining using specific anti-Nef MAb (AE6; AIDS Research and Reference Reagent Program, NIAID, NIH: HIV-1 Nef monoclonal antibody from Dr James Hoxie) immediately after electroporation and at later times as previously described [32]. Electroporated MDM were returned to cultures and allowed to recover for 24 h at 37°C in a humidified incubator and subsequently used for phagocytosis assays. The impact of Nef on macrophage function was investigated by assessing its role on MAC-FITC phagocytosis (as described above) as well as on the level of tyrosine phosphorylation during FcγR-mediated phagocytosis. The latter was performed by incubating MDM with IgG-opsonized latex beads at a ratio of 1 : 10 respectively at 37°C. At indicated time points phagocytosis was arrested by plunging the tubes into ice and washing MDM in ice-cold PBS, followed by centrifugation at 14 000 rpm for 45 s. Cells were lysed in 100 µl of Triton-X lysis buffer containing 25 mmol/l Tris-HCl (pH 7.5), 0.14 mol/l NaCl, 1 mmol/l EDTA, 1% Triton-X-100, supplemented with the following phosphatase inhibitors: 50 mmol/l NaF, 1 mmol/l sodium orthovanadate (Sigma), 40mmol/l β-glycerophosphate (Sigma), and the following protease inhibitors: 1 mmol/l pefabloc, 1 µmol/l pepstatin, 1 µmol/l leupeptin (Boehringer-Mannheim). After 30 min lysis at 4°C, MDM extracts were centrifuged at 14 000 rpm for 10 min and assessed by immunoblot analysis.

Immunoblotting

Cell extracts containing equal amounts of proteins, as determined by DC protein assay (Bio-Rad Laboratories), were boiled in sodium dodecyl sulphate (SDS) sample buffer (10 mmol/l Tris pH 8.0, 2 mmol/l EDTA, 1% SDS, 5% β-mercaptoethanol, 5% glycerol) resolved by 10% SDS-polyacrylamide gel (SDS-PAGE), transferred to Hybond-C nitrocellulose (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK) and blocked for 2 h in 3% bovine serum albumin (Sigma). The blots were probed with a recombinant antibody directed against phosphotyrosine conjugated with horseradish peroxidase (RC20; Transduction Laboratories, Lexington, Kentucky, USA) overnight at 4°C, washed five times in 1 × Tris-buffered saline containing 0.3% Tween-20 (Astral, Gymea, Australia) and subsequently developed for enhanced chemiluminescence (ECL) according to manufacturer's instructions (Amersham Pharmacia).

Statistical analyses

Multiple regression was used to model the association between the percentage of phagocytosis and MAC-FITC:monocyte ratio, adjusting for HIV-1 infection status (HIV-1 positive, HIV-1 negative, and *nef*-deleted SBBC HIV-1 positive). Robust standard errors were used to account for repeat tests on individuals. To account for the highly skewed data, the MAC-FITC : monocyte ratio was transformed using natural logarithms. Interactions between MAC-FITC : monocyte ratio and HIV infection status were investigated by fitting interaction terms in the model. The analyses were carried out using Stata statistical software analysis package (Stata, Collage Station, Texas, USA).

The significance of F-actin contents of monocytes from HIV-positive and -negative individuals, *in vitro* infections with WT HIV-1(HIV-1Ba-L or HIV-1NL (AD8)) and *nef*-deleted strains of HIV-1 (primary isolates HIV-1D36 and HIV-1C18 or HIV-1NL (AD8)(nef chimera) as well as the impact of Nef electroporation on phagocytosis was assessed using the Student's t test (paired, two-tailed).

Results

Subjects

Blood was collected from a total of six Sydney Blood Bank Cohort members (median CD4, 805 × 10^6 cells/µl; range, 306 to 2331 × 10^6 cells/µl; median plasma HIV RNA < 400 copies/ml; range, < 400 to 4000 copies/ml), 16 persons with WT HIV-1 infection (median CD4, 138 cells × 10^6 cells/µl; range, 20 to 713 × 10^6 cells/µl for 11 subjects tested at the time of assay; median plasma HIV RNA 900 copies/ml; range, < 500 to 385 500 copies/ml for 13 subjects tested) and three uninfected persons. Blood samples from the same HIV-uninfected donors were used as a source of control monocytes in every experiment (Table 1).

Table 1. Phagocytosis of *Mycobacterium avium* complex by peripheral blood monocytes in *nef*-deleted Sydney Blood Bank Cohort (SBBC) and wild-type (WT) HIV-infected subjects *ex vivo*. The phagocytosis of *Mycobacterium avium* complex (MAC)-fluorescein isothiocyanate (FITC) by monocytes of SBBC and WT HIV-seropositive subjects was compared with that of uninfected controls and is reported as a percentage of the phagocytosis by seronegative controls for the same MAC : monocyte ratio (% phagocytosis). The values obtained at each MAC : monocyte ratio for the seronegative controls were standardized as 100% of phagocytic efficiency, as previously described [3]. Each phagocytosis experiment was performed in duplicate.

		nef-deleted SBBC		WT HIV-infected controls			
Subject	Expt.	Phagocytosis (% control)	Viral load[a] (copies/ml)	CD4 count (cells × 10^6/l)	Phagocytosis (% control)	Viral load[b] (copies/ml)	CD4 count (cells × 10^6/l)
D36	(i)	124	3100	361	40	< 500	713
	(ii)	101	2600	414	67	< 500	50
	(iii)	80	4000	540	32	< 500	100
C49	(i)	141	< 400	918	23	N/T	N/T
C54	(i)	123	1500	1260	20	< 500	20
	(ii)	116	1700	1419	35	< 500	363
	(iii)	96	1800	1120	65	600	N/T
	(iv)	96	1600	1152	92	N/T	N/T
C64	(i)	137	< 400	805	32	385500	N/T
	(ii)	123	< 400	805	83	N/T	N/T
	(iii)	101	< 400	1026	22	95600	40
	(iv)	100	< 400	2331	44	119800	120
C98	(i)	110	< 400	612	57	10700	400
	(ii)	115	< 400	306	13	62400	247
C135	(i)	105	< 400	392	55	26900	138
	(ii)	220	< 400	495	130	900	480
P-value		0.808			< 0.0001		

Viral load assessed by [a]reverse-transcriptase-polymerase chain reaction, [b]bDNA assay; N/T, not tested at time of assay.

Phagocytosis by monocytes in whole blood from individuals infected with WT and *nef*-deleted HIV-1

As SBBC members have been infected with an attenuated strain of HIV-1 for periods in excess of 16 years prior to the onset of any HIV-related symptoms or infections [7], it was of interest to determine whether they had unimpaired phagocytic function, consistent with slow or absent disease progression. Monocytes present in whole blood from members of SBBC phagocytozed MAC-FITC (ingested predominantly via C'R [33]) with an efficiency similar to that of monocytes from HIV-1-uninfected individuals ($P = 0.808$), but significantly better than monocytes from individuals infected with WT HIV-1 ($P < 0.0001$) (Table 1, Fig. 1). In addition, monocytes from members of SBBC phagocytosed *Toxoplasma gondii*–FITC (mediated predominantly via FcγRII [34]) substantially better than monocytes from WT HIV-infected individuals (n = 4; data not shown). These data suggest that both C'R- and FcγR-mediated phagocytosis by monocytes from WT HIV-infected individuals is impaired, whereas those from subjects infected with Δ*nef* HIV-1 are not. No correlation was found between the level of inhibition of phagocytosis and either viral load ($R = -0.27$; $P = 0.38$) or CD4 counts ($R = 0.35$; $P = 0.29$).

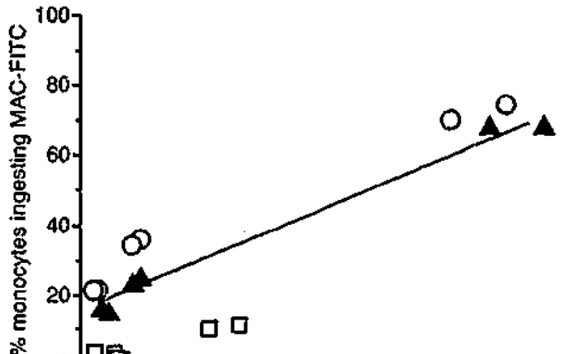

Fig. 1. Quantitation of *Mycobacterium avium* complex (MAC)-fluorescein isothiocyanate (FITC) phagocytosis by peripheral blood monocytes assayed *ex vivo*. A representative phagocytic analysis for one Sydney Blood Bank Cohort (SBBC) member, C54 (open circles), wild-type (WT) HIV-infected individual (open squares) and HIV-seronegative controls (solid triangles). Phagocytosis of MAC-FITC by monocytes obtained from C54 and WT HIV-infected individuals was compared with regression line fitted to the data points obtained from HIV-uninfected controls. Results are corrected for background fluorescence.

Mechanism underlying inhibition of phagocytosis

The surface expression of the complement receptor (CD11c) and Fcγ receptors (CD16, CD32) was not modulated in monocytes from individuals infected with WT or *nef*-deleted HIV-1 when compared with uninfected subjects (data not shown), suggesting a post-receptor mediated mechanism of phagocytosis inhibition in individuals infected with WT HIV-1. As

reorganization of the actin-based cytoskeleton is essential for the formation of the phagocytic cup and the engulfment of phagocytosed particles, the mechanism of defective phagocytosis in HIV-infected individuals was further investigated at the actin polymerization level. A flow cytometric assay was developed to measure the level of polymerized actin (filamentous actin; F-actin) in blood monocytes during FcγR-mediated phagocytosis (Kedzierska, unpublished). Monocytes in blood of HIV-infected individuals showed significantly increased basal levels of polymerized actin in comparison with F-actin level in monocytes from uninfected controls (Fig. 2a; $P < 0.05$; $n = 5$). During phagocytosis there was a significant net increase of F-actin from basal levels in monocytes from uninfected controls at between 2 and 10 min of phagocytosis, but not in monocytes from HIV-infected individuals (Fig. 2b; $n = 3$). These data suggest defective actin rearrangement (and thus abnormal phagocytic cup formation) in blood monocytes from HIV-infected subjects.

Phagocytosis by purified MDM infected with HIV-1 *in vitro*

As phagocytosis by monocytes from SBBC members was not impaired, the direct effect of Nef on phagocytosis was assessed, initially by using Δ*nef* strains to infect MDM *in vitro* in comparison with WT HIV-1. *In vitro* infection of MDM by Δ*nef* strains HIV-1_{D36} (primary isolate from SBBC member D36) or HIV-$1_{NL(AD8)D\,nef}$ chimera impaired phagocytosis of MAC-FITC to the same degree as infection with WT HIV-1 [Ba-L or NL(AD8)]. All infected MDM cultures showed impaired phagocytosis compared with uninfected MDM ($P < 0.001$; Fig. 3).

MDM infected with either WT HIV-1 (Ba-L or NL(AD8)) or Δ*nef* HIV-1_{C18} (primary isolate from SBBC cohort member C18) or HIV-$1_{NL(AD8)\Delta nef}$ chimera had impaired phagocytosis mediated via either Fcγ and C′ receptors using specifically opsonized targets (IgG- and C′-opsonized sRBC) in comparison with uninfected MDM ($P < 0.001$; Fig. 4). Infection of MDM with *nef*-deleted strains of HIV-1 resulted in similar levels of inhibition of phagocytosis as MDM infected with WT HIV-1 ($P = 0.08$ and $P = 0.1$, for FcγR- and C′R-mediated phagocytosis respectively). The presence of a *nef*-deletion had no significant effect on HIV-1 replication *in vitro* as measured by RT activity in the culture supernatant (mean RT values of 204 500 cpm/1 × 10^6 MDM and 125 000 cpm/1 × 10^6 MDM for HIV-$1_{NL(AD8)}$ and HIV-$1_{NL(AD8)\Delta nef}$ chimera respectively; $n = 5$; $P = 0.48$) or by flow cytometric analysis of intracellular p24 antigen (mean of 59 and 74% p24-positive MDM for HIV-$1_{NL(AD8)}$ and HIV-$1_{NL(AD8)\Delta nef}$ chimera respectively; $n = 2$).

Phagocytosis by MDM electroporated with Nef protein

To address directly the effects of Nef on phagocytosis by MDM, cells were electroporated with Nef. Electroporation of Nef protein into MDM did not affect phagocytosis of MAC-FITC compared with mock- or GST-electroporated controls ($n = 4$; $P = 0.16$ and

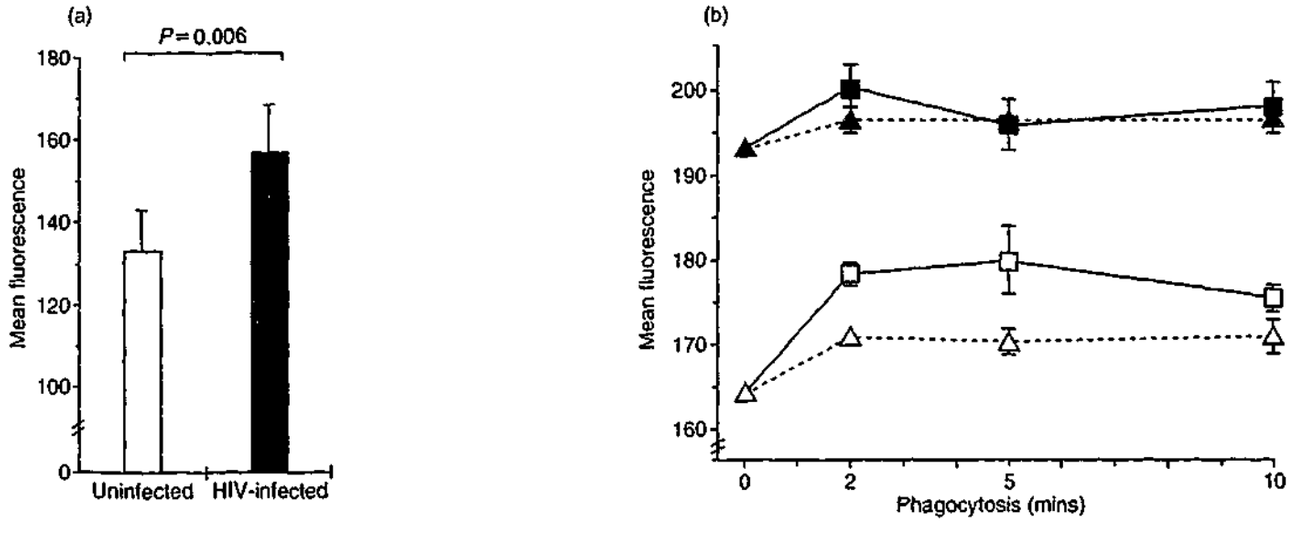

Fig. 2. F-actin content in peripheral blood monocytes during phagocytosis. Blood samples from HIV-positive individuals (solid symbols) or uninfected controls (open symbols) were stained for F-actin levels. Cells were (a) unstimulated; or (b) incubated with IgG-opsonised latex beads (squares, solid lines) or without beads (triangles, dotted lines) at 37°C for indicated times. Following fixation with 3% formaldehyde, monocytes were stained with CD14-PE monoclonal antibody (Mab). Cells were permeabilized with 0.01% Triton-X, stained for F-actin levels with phalloidin-Alexa488 as described in 'Methods' section and analysed by flow cytometry. Data shown represent (a) means ± SEM from five different experiments (b) representative of three experiments using blood samples from HIV-negative ($n = 4$) and HIV-positive ($n = 3$) donors.

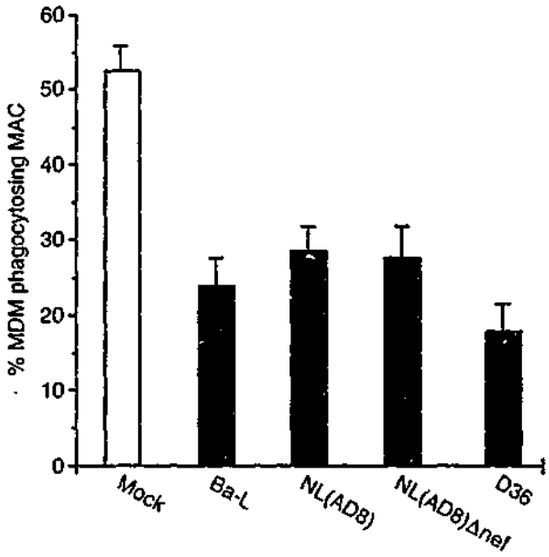

Fig. 3. Effect of wild-type (WT) HIV-1 and *nef*-deleted HIV-1 infection on phagocytosis of *Mycobacterium avium* complex (MAC)-fluorescein isothiocyanate (FITC) by monocyte-derived macrophages (MDM). Phagocytosis of MAC-FITC by MDM infected *in vitro* with mock (open bar), WT HIV-1 (Ba-L or NL(AD8); black bars) or Δ*nef* HIV-1 (NL(AD8)Δ*nef* chimera or primary isolate from SBBC member D36; grey bars) was assessed by a flow cytometric assay on day 7–10 post-infection. The proportion of MDM phagocytosing MAC-labelled with FITC (mean ± SEM) is shown using MDM : MAC-FITC ratio of 1 : 75. These data are the means compiled from results from 10 different MDM donors.

0.54 respectively) (Fig. 5a). As Nef interacts with cellular proteins and kinases which are also phosphorylated during FcγR-mediated phagocytosis (eg Hck), we assessed the impact of Nef on the level of tyrosine phosphorylation during phagocytosis of IgG-opsonized targets. Stimulation of mock-electroporated MDM with IgG-opsonized beads triggered an increase in tyrosine phosphorylation of a wide range of cellular proteins after 2 min of phagocytosis. Nef- and GST-electroporated MDM displayed similar levels and patterns of phosphorylation during FcγR-mediated phagocytosis to that induced by mock electroporation (Fig. 5b). These data suggest that HIV-1 Nef does not inhibit phagocytosis via FcγR or C'R in MDM infected *in vitro*.

Discussion

Our data show that phagocytosis of MAC by blood monocytes from individuals infected with *nef*-deleted strains of HIV-1 was normal, whereas in agreement with our previous data [3] phagocytosis by blood monocytes from WT HIV-1 infected subjects was significantly impaired, in comparison with monocytes from uninfected controls. However, when MDM from HIV-1 seronegative individuals were infected *in vitro* at the same multiplicity of infection with WT or Δ*nef* HIV-1 strains, the phagocytic capacity of these MDM

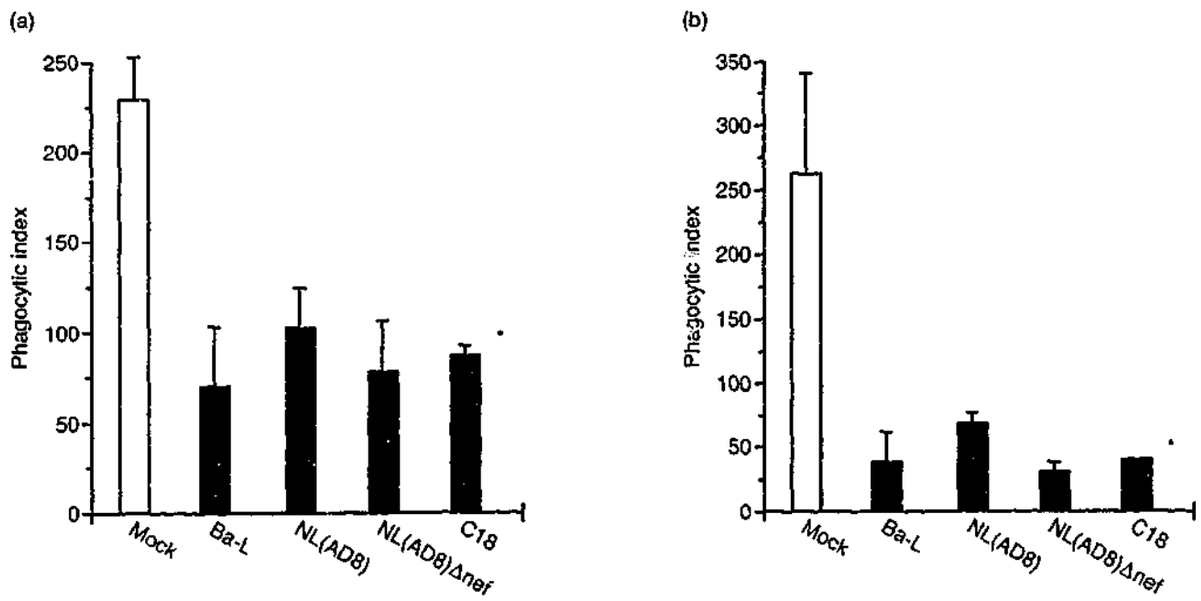

Fig. 4. Effect of *nef*-deleted HIV-1 on FcγR- and C'R-mediated phagocytosis. Phagocytosis of specific targets (a) Fc-opsonized and (b) $C^{\times}$-opsonized sRBC was assessed by colorimetric assay. Monocyte-derived macrophages (MDM) on day 5 post-isolation were infected with either WT HIV-$1_{Ba\text{-}L}$ and HIV-$1_{NL(AD8)}$ (black bars) or Δ*nef* strains (HIV-$1_{NL(AD8)\Delta nef}$ or HIV-1_{C18}, *nef*-deleted primary isolate; grey bars). Phagocytosis was performed on day 7–10 post-infection. These data represent means ± SEM from (a) five different MDM donors; (b) three different donors. *Shown results from a single experiment.

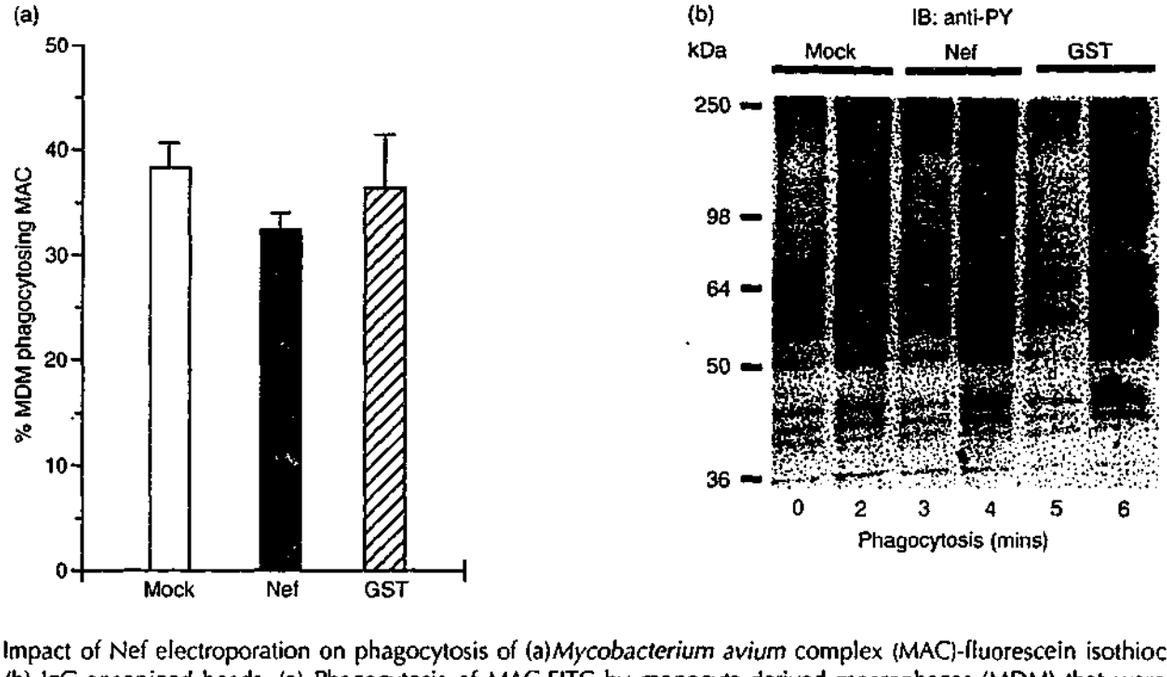

Fig. 5. Impact of Nef electroporation on phagocytosis of (a)*Mycobacterium avium* complex (MAC)-fluorescein isothiocyanate (FITC); (b) IgG-opsonized beads. (a) Phagocytosis of MAC-FITC by monocyte-derived macrophages (MDM) that were either electroporated with mock (open bar), Nef (solid bar) or glutathione-S-transferase (GST) (hatched bar) were compared. Shown are mean ± SEM of four experiments. Student's t-test was performed to measure the significance of these results. (b) MDM (2×10^6) were incubated with IgG-opsonized latex beads (2×10^7) for the indicated times, lysed in Triton-X 100 buffer and samples of lysate containing 50 μg protein were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, then probed with anti-phosphotyrosine conjugated to horseradish peroxidase (RC20). Data shown are representative of two experiments using MDM prepared from different donors.

were equally reduced. These results suggest that if HIV-1 Nef protein has an effect on monocyte and macrophage phagocytic function, it is mediated through a complex indirect effect only evident *in vivo*.

A number of investigators have reported impairment of phagocytosis following WT HIV-1 infection, by monocytes and alveolar macrophages *in vivo* as well as by macrophages infected with WT HIV-1 *in vitro* [35–42], although others have shown normal monocyte and macrophage function following HIV-1 infection [43,44]. These discordant results may be at least partially explained by the differing methods used by investigators, in particular whether cells have been isolated from HIV-infected individuals (where the proportion of infected cells is very low, in the range of 0.001 to 1%) or from seronegative donors whose purified MDM were then infected *in vitro* (with infection rates often in the range of 30 to 70%). As previously demonstrated by our group, monocytes and macrophages are distinct cell populations that differ in their susceptibility to HIV-1 infection (monocytes being highly refractory, whereas macrophages are fully permissive to HIV-1) [45], the expression level of surface receptors (including CD4, CCR5 and phagocytic receptors) [30,46] as well as in their cytokine/chemokine production profile (reviewed in [47]). This study, however, demonstrates defective phagocytosis by either monocytes or macrophages following HIV-1 infection *in vivo* and *in vitro*.

Phagocytosis of opportunistic pathogens such as MAC (utilizing predominantly C'R for phagocytosis; [33]) and *Toxoplasma gondii* (utilizing FcγRII; [34]) was impaired by peripheral blood monocytes from WT HIV-infected individuals but not from SBBC members infected with a Δ*nef* strain of HIV-1. Impaired phagocytosis following HIV-1 infection is likely to directly contribute to HIV pathogenesis, by allowing reactivation of opportunistic pathogens normally controlled by macrophages. Our data demonstrating normal phagocytic efficiency of monocytes in the blood of SBBC members is therefore consistent with their long-term non/slow progression and the absence of opportunistic infections [7]. As individuals infected with *nef*-deleted HIV-1 displayed normal phagocytic efficiency, we considered that Nef might be responsible for the observed impairment of phagocytosis following HIV-1 infection. However, as the attenuated virus from this group also has deletions and rearrangements within the long terminal repeat [6], a tight correlation of phagocytic efficiency and *nef*-deletion is not possible.

As only a small proportion of blood monocytes (< 1%) is infected with HIV-1, impaired phagocytosis may be predominantly an indirect consequence of HIV-1

infection and might reflect dysregulation of cytokine/chemokine production by monocytes and/or other cells present in blood. In support of this proposal, defective phagocytosis by neutrophils (not targets for HIV-1 infection) from HIV-positive individuals has also been reported [41]. Nef may alter phagocytosis through its influence on the cellular environment and subsequently on cytokine and chemokine production. Nef induces the production of two CC-chemokines, MIP1-α and MIP1-β by macrophages [48], induces synthesis of IL-15 by MDM [49], IL-10 [50], tumour necrosis factor-α, IL-6 and IL-1b (Greenway, unpublished) by PBMC or alters IL-2 production by T lymphocytes [51,52]. As monocyte activation and their response to phagocytic stimuli are dependent on cytokine concentration (including IL-2 [53]) Nef may impair phagocytosis via dysregulated cytokine/chemokine production.

The *nef* gene of primate lentiviruses is necessary for high level virus replication *in vivo* [8]. It has been also demonstrated by the work of Ruprecht *et al.* that SIV with *nef* deletion is generally attenuated in adult macaques, but causes disease in infant macaques, probably as a result of a 'threshold' for pathogenicity relating to levels of viral replication and viral load [10,54]. Although it is possible that Nef may influence phagocytic capacity of monocytes *in vivo* by affecting viral load and CD4 counts, our data suggest that the reduction of phagocytosis was not due to viral load or CD4 counts. SBBC members who contributed to this study had low viral load and normal CD4 counts at the time of phagocytosis measurements, whereas individuals infected with WT HIV-1 had a wide range of viral loads and CD4 counts. However, we found no correlation between the observed inhibition of phagocytosis and either viral load (undetectable or low in approximately 50% of subjects) or CD4 counts in individuals infected with WT HIV-1, suggesting that the difference in phagocytosis between *nef*-deleted and WT HIV-infected individuals is not due to either of those factors. Those results are in the agreement with studies showing decreased phagocytosis in patients with both undetectable (< 400 copies/ml) and high viral loads (> 10 000 copies/ml) [42] as well as over a wide range of CD4 counts [39].

The mechanism of defective phagocytosis by peripheral blood monocytes from WT HIV-infected individuals remains unclear. Previous reports [37,38,55], confirmed by our observations, suggest that HIV-1 infection *in vivo* results in either elevated or unchanged expression of complement and Fcγ receptors on monocytes, indicating that inhibition of phagocytosis occurs at a post-receptor level. Recently, Elbim *et al.* [56] showed increased basal levels of F-actin in monocytes from HIV-infected individuals, at different stages of disease, when compared with uninfected subjects. We have confirmed these findings and have further demonstrated that monocytes from HIV-infected individuals display either minimal or no elevation of F-actin above their basal level during phagocytosis. This is in contrast to significant net increases from the basal F-actin level in uninfected controls. As actin polymerization plays a critical role in the formation of phagocytic cup and ingestion of phagocytosed particles, defective actin rearrangement is a potential mechanism underlying impaired phagocytic function in HIV-infected individuals. F-actin content in monocytes from individuals infected with *nef*-deleted HIV-1 remains to be elucidated.

There are other potential mechanisms by which Nef could directly influence phagocytosis. Nef associates with several cellular kinases, including Src family tyrosine kinases, Lck (resulting in impairment of Lck-mediated signalling events) [57] and Hck (resulting in its activation) [23]. As the same signalling pathways are involved in FcγR-mediated phagocytosis by MDM, (reviewed in [58]), Nef may influence phagocytosis by binding to Hck or other relevant kinases or cytoskeletal proteins. To assess this hypothesis we investigated the direct effects of infection and replication of HIV-1 on macrophage function as well as the effect of Nef in the absence of other HIV-1 proteins. Phagocytosis of MAC was defective in MDM infected *in vitro* with either WT or *nef*-deleted strain of HIV-1. Experiments to specifically address both FcγR- and C'R-mediated phagocytosis (occurring via different signalling mechanisms [59]) by HIV-infected MDM showed similar levels of impairment. In our studies deletions within *nef* did not significantly alter the replicative capacity of HIV-1 in MDM (in agreement with previous reports [60,61], in contrast to other studies [62]). Therefore, our results suggest that decreased phagocytosis *in vitro* is predominantly a direct consequence of HIV-1 replication in MDM, and that Nef does not contribute to this impairment of MDM function. Unaltered levels and pattern of tyrosine phosphorylation of key proteins during FcγR-mediated phagocytosis by Nef-electroporated MDM confirmed that Nef on its own is not sufficient to inhibit the process of phagocytosis.

In conclusion, there is no impairment in phagocytosis by monocytes in whole blood from individuals infected with Δ*nef* strains of HIV-1 in contrast to subjects infected with WT HIV-1, possibly reflecting an indirect effect of infection with HIV-1 resulting in altered actin polymerization in these cells. However, *in vitro* infection of MDM with *nef*-deleted strains and WT HIV-1 at relatively high multiplicity of infection both equally impair phagocytosis, indicating a direct effect of HIV-1 infection and replication on macrophage function, and suggesting that Nef does not directly impact on macrophage phagocytic activity.

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References

1. Crowe SM, Vardaxis NJ, Kent SJ, *et al.* **HIV infection of monocyte-derived macrophages *in vitro* reduces phagocytosis of *Candida albicans*.** *J Leukoc Biol* 1994, 56:318–327.
2. Biggs BA, Hewish M, Kent S, Hayes K, Crowe SM. **HIV-1 infection of human macrophages impairs phagocytosis and killing of *Toxoplasma gondii*.** *J Immunol* 1995, 154:6132–6139.
3. Kedzierska K, Mak J, Mijch A, *et al.* **Granulocyte-macrophage colony-stimulating factor augments phagocytosis of *Mycobacterium avium* complex by human immunodeficiency virus type 1-infected monocytes/macrophages *in vitro* and *in vivo*.** *J Infect Dis* 2000, 181:390–394.
4. Crowe SM. **Role of macrophages in the pathogenesis of human immunodeficiency virus (HIV) infection.** *Aust N Z J Med* 1995, 25:777–783.
5. Learmont J, Tindall B, Evans L, *et al.* **Long term symptomless HIV-1 infection in recipients of blood products from a single donor.** *Lancet* 1992, 340:863–867.
6. Deacon NJ, Tsykin A, Solomon A, *et al.* **Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients.** *Science* 1995, 270:988–991.
7. Learmont J, Geczy A, Mills J, *et al.* **Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1. A report from the Sydney Blood Bank Cohort.** *N Engl J Med* 1999, 340:1715–1722.
8. Kestler HW, Ringler DJ, Mori K, *et al.* **Importance of the nef gene for maintenance of high virus loads and for development of AIDS.** *Cell* 1991, 65:651–662.
9. Jamieson BD, Aldrovandi GM, Planelles V, *et al.* **Requirement of human immunodeficiency virus type 1 nef for in vivo replication and pathogenicity.** *J Virol* 1994, 68:3478–3485.
10. Ruprecht R, Baba T, Liska V, Bronson R, Penninck D, Greene M. **"Attenuated" simian immunodeficiency virus in macaque neonates.** *AIDS Res Hum Retroviruses* 1996, 12:159–460.
11. Garcia JV, Alfano J, Miller AD. **The negative effect of human immunodeficiency virus type 1 Nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4.** *J Virol* 1993, 67:1511–1516.
12. Anderson SJ, Lenburg M, Landau NR, Garcia JV. **The cytoplasmic domain of CD4 is sufficient for its down-regulation from the cell surface by human immunodeficiency virus type 1 Nef.** *J Virol* 1994, 68:3092–3101.
13. Rhee SS, Marsh JW. **Human immunodeficiency virus type 1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4.** *J Virol* 1994, 68:5156–5163.
14. Schwartz O, Marechal V, Le Gall S, Lemonnier F, Heard JM. **Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein.** *Nat Med* 1996, 2:338–342.
15. Le Gall S, Prevost MC, Heard JM, Schwartz O. **Human immunodeficiency virus type 1 Nef independently affects virion incorporation of major histocompatibility complex class I molecules and virus infectivity.** *Virology* 1997, 229:295–301.
16. Aiken C, Trono D. **Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis.** *J Virol* 1995, 69:5048–5056.
17. Chowers MY, Pandori MW, Spina CA, Richman DD, Guatelli JC. **The growth advantage conferred by HIV-1 nef is determined at the level of viral DNA formation and is independent of CD4 downregulation.** *Virology* 1995, 212:451–457.
18. Schwartz O, Marechal V, Danos O, Heard JM. **Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell.** *J Virol* 1995, 69:4053–4059.
19. Herna Remkema G, Saksela K. **Interactions of HIV-1 NEF with cellular signal transducing proteins.** *Front Biosci* 2000, 5:D268–283.
20. Saksela K, Cheng G, Baltimore D. **Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4.** *EMBO J* 1995, 14:484–491.
21. Lee CH, Saksela K, Mirza UA, Chait BT, Kuriyan J. **Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain.** *Cell* 1996, 85:931–942.
22. Greenway A, Azad A, Mills J, McPhee DA. **Human immunodeficiency virus type 1 nef binds directly to Lck and mitogen-activated protein kinase, inhibiting kinase activity.** *J Virol* 1996, 70:6701–6708.
23. Greenway A, Dutartre H, Allen K, McPhee D, Olive D, Collette Y. **Simian immunodeficiency virus and human immunodeficiency virus type 1 nef proteins show distinct patterns and mechanisms of Src kinase activation.** *J Virol* 1999, 73:6152–6158.
24. Nunn MF, Marsh JW. **Human immunodeficiency virus type 1 Nef associates with a member of the p21-activated kinase family.** *J Virol* 1996, 70:6157–6161.
25. Fackler OT, Lu X, Frost JA, *et al.* **p21-activated kinase 1 plays a critical role in cellular activation by Nef.** *Mol Cell Biol* 2000, 20:2619–2627.
26. Fackler OT, Luo W, Geyer M, Alberts AS, Peterlin BM. **Activation of Vav by Nef induces cytoskeletal rearrangements and downstream effector functions.** *Mol Cell* 1999, 3:729–739.
27. Lu X, Wu X, Plemenitas A, *et al.* **CDC42 and Rac1 are implicated in the activation of the Nef-associated kinase and replication of HIV-1.** *Curr Biol* 1996, 6:1677–1684.
28. Crespo P, Schuebel KE, Ostrom AA, Gutkind JS, Bustelo XR. **Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product.** *Nature* 1997, 385:169–172.
29. Hewish MJ, Meikle AM, Hunter SD, Crowe SM. **Quantifying phagocytosis of Mycobacterium avium complex by human monocytes in whole blood.** *Immunol Cell Biol* 1996, 74:306–312.
30. Kedzierska K, Maerz A, Warby T, *et al.* **Granulocyte-macrophage colony stimulating factor inhibits HIV-1 replication in monocyte-derived macrophages.** *AIDS* 2000, 14:1739–1748.
31. Gebran SJ, Romano EL, Pons HA, Cariani L, Soyano AN. **A modified colorimetric method for the measurement of phagocytosis and antibody-dependent cell cytotoxicity using 2,7-diaminofluorene.** *J Immunol Methods* 1992, 151:255–260.
32. Greenway A, McPhee DA, Grgacic E, *et al.* **Nef 27, but not the Nef 25 isoform of human immunodeficiency virus type 1 pNL4-3 down regulates surface CD4 and IL-2 R expression in peripheral blood mononuclear cells and transformed T cells.** *Virology* 1994, 198:245–256.
33. Schlesinger LS, Horwitz MA. **Phagocytosis of *Mycobacterium leprae* by human monocyte-derived macrophages is mediated by complement receptors CR1 (CD35), CR3 (CD11b/CD18) and CR4(CD11c/CD18) and IFN-gamma activation inhibits complement receptor function and phagocytosis of the bacterium.** *J Immunol* 1991, 147:1983–1993.
34. Joiner KA, Fuhrman SA, Miettinen HM, Kasper LH, Mellman I. ***Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts.** *Science* 1990, 249:641–646.
35. Musher DM, Watson DA, Nickeson D, Gyorkey F, Lahart C, Rossen RD. **The effect of HIV infection on phagocytosis and killing of Staphylococcus aureus by human pulmonary alveolar macrophages.** *Am J Med Sci* 1990, 299:158–163.
36. Bravo-Cuellar A, Nowacki W, Vuillier F, de Saint-Martin J, Orbach-Arbouys S. **The bactericidal capacity of peripheral blood monocytes from HIV positive patients may collapse very soon after the infection.** *Immunol Lett* 1992, 31:297–299.

37. Capsoni F, Minonzio F, Ongari AM, P RG, Lazzarin A, Zanussi C. **Monocyte-derived macrophage function in HIV-infected subjects: in vitro modulation by rIFN-gamma and rGM-CSF.** *Clin Immunol Immunopathol* 1992, 62:176–182.
38. Capsoni F, Minonzio F, Ongari AM, *et al.* **Fc receptors expression and function in mononuclear phagocytes from AIDS patients; modulation by IFN-gamma.** *Scand J Immunol* 1994, 39: 45–50.
39. Roilides E, Holmes A, Blake C, Pizzo PA, Walsh TJ. **Defective antifungal activity of monocyte derived macrophages from human immunodeficiency virus infected children against *Aspergillus fumigatus*.** *J Infect Dis* 1993, 168:1562–1565.
40. Wehle K, Schirmer M, Dunnebacke-Hinz J, Kupper T, Pfitzer P. **Quantitative differences in phagocytosis and degradation of Pneumocystis carinii by alveolar macrophages in AIDS and non-HIV patients *in vivo*.** Cytopathology 1993,4:231-236.
41. Dobmeyer TS, Raffel B, Dobmeyer JM, *et al.* **Decreased function of monocytes and granulocytes during HIV-1 infection correlates with CD4 cell counts.** *Eur J Med Res* 1995, 1:9–15.
42. Baqui AA, Meiller TF, Zhang M, Falkler WAJ. **The effects of HIV viral load on the phagocytic activity of monocytes activated with lipopolysaccharide from oral microorganisms.** *Immunopharmacol Immunotoxicol* 1999, 21:421–438.
43. Nottet HSLM, de Graaf L, de Vos NM, *et al.* **Phagocytic function of monocyte-derived macrophages is not affected by human immunodeficiency virus type 1 infection.** *J Infect Dis* 1993, 168:84–91.
44. Eversole LR, Fleischmann J, Baldwin GC, Sapp JP. **The effects of human immunodeficiency virus infection on macrophage phagocytosis of *Candida*.** *Oral Microbiol Immunol* 1994, 9:55–59.
45. Sonza S, Maerz A, Deacon N, Meanger J, Mills J, Crowe S. **Human immunodeficiency virus type 1 replication is blocked prior to reverse transcription and integration in freshly isolated peripheral blood monocytes.** *J Virol* 1996, 70:3863–3869.
46. Sonza S, Maerz A, Uren S, *et al.* **Susceptibility of human monocytes to HIV type 1 infection in vitro is not dependent on their level of CD4 expression.** *AIDS Res Hum Retroviruses* 1995, 11:769–776.
47. Fantuzzi L, Conti L, Gauzzi MC, *et al.* **Regulation of chemokine/cytokine network during in vitro differentiation and HIV-1 infection of human monocytes: possible importance in the pathogenesis of AIDS.** *J Leuk Biol* 2000, 68:391–399.
48. Swingler S, Mann A, Jacque J, *et al.* **HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages.** *Nat Med* 1999, 9:997–1003.
49. Quaranta M, Camponeschi B, Straface E, Malorni W, Viora M. **Induction of interleukin-15 production by HIV-1 nef protein: a role in the proliferation of uninfected cells.** *Exp Cell Res* 1999, 250:112–121.
50. Brigino E, Haraguchi S, Koutsonikolis A, *et al.* **Interleukin 10 is induced by recombinant HIV-1 Nef protein involving the calcium/calmodulin-dependent phosphodiesterase signal transduction pathway.** *Proc Natl Acad Sci USA* 1997, 94:3178–3182.
51. Collette Y, Chang HL, Cerdan C, *et al.* **Specific Th1 cytokine down-regulation associated with primary clinically derived human immunodeficiency virus type 1 Nef gene-induced expression.** *J Immunol* 1996, 156:360–370.
52. Schibeci SD, Clegg AO, Biti RA, Sagawa K, Stewart GJ, Williamson P. **HIV-Nef enhances interleukin-2 production and phosphatidylinositol 3-kinase activity in a human T cell line.** *AIDS* 2000, 14:1701–1707.
53. Ennen J, Ernst M, Flad HD. **The effect of interleukin 2 on Fc-gamma receptor function of human monocytes requires specific intercellular interaction.** *Immunobiology* 1989, 179:17–32.
54. Montefiori D, Baba T, Li A, Bilska M, Ruprecht R. **Neutralizing and infection-enhancing antibody responses do not correlate with the differential pathogenicity of SIVmac239delta3 in adult and infant rhesus monkeys.** *J Immunol* 1996, 157:5528–5535.
55. Dunne J, Feighery C, Whelan A. **Beta-2-microglobulin, neopterin and monocyte Fc gamma receptors in opportunistic infections of HIV-positive patients.** *Br J Biomed Sci* 1996, 53:263–269.
56. Elbim C, Pillet S, Prevost MH, *et al.* **Redox and activation status of monocytes from human immunodeficiency virus-infected patients: relationship with viral load.** *J Virol* 1999, 73:4561–4566.
57. Collette Y, Dutartre H, Benziane A, *et al.* **Physical and functional interaction of Nef with Lck. HIV-1 Nef-induced T-cell signaling defects.** *J Biol Chem* 1996, 271:6333–6341.
58. Greenberg S. **Signal transduction of phagocytosis.** *Trends Cell Biol* 1995, 5:93–99.
59. Allen LA, Aderem A. **Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages.** *J Exp Med* 1996, 184:627–637.
60. Hattori N, Michaels F, Fargnoli K, Marcon L, Gallo RC, Franchini G. **The human immunodeficiency virus type 2 vpr gene is essential for productive infection of human macrophages.** *Proc Natl Acad Sci USA* 1990, 87:8080–8084.
61. Meylan PR, Baumgartner M, Ciuffi A, Munoz M, Sahli R. **The nef gene controls syncytium formation in primary human lymphocytes and macrophages infected by HIV type 1.** *AIDS Res Hum Retroviruses* 1998, 14:1531–1542.
62. Miller MD, Warmerdam MT, Gaston I, Greene WC, Feinberg MB. **The human immunodeficiency virus 1 nef gene product, a positive factor for viral infection and replication in primary lymphocytes and macrophages.** *J Exp Med* 1994, 179:101–113.

CONCISE COMMUNICATION

Granulocyte-Macrophage Colony-Stimulating Factor Augments Phagocytosis of *Mycobacterium avium* Complex by Human Immunodeficiency Virus Type 1–Infected Monocytes/Macrophages In Vitro and In Vivo

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The role of human immunodeficiency virus type 1 (HIV-1) infection on the ability of human monocytes/macrophages to phagocytose *Mycobacterium avium* complex (MAC) in vivo and in vitro and the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) on this function were investigated. By use of a flow cytometric assay to quantify phagocytosis, HIV-1 infection was found to impair the ability of monocyte-derived macrophages to phagocytose MAC in vitro, whereas GM-CSF significantly improved this defect. Phagocytosis was not altered by exposure to a mutant form of GM-CSF (E21R) binding only to the α chain of the GM-CSF receptor, suggesting that signaling by GM-CSF that leads to augmentation of phagocytosis is via the β chain of the receptor. In a patient with AIDS and disseminated multidrug-resistant MAC infection, GM-CSF treatment improved phagocytosis of MAC by peripheral blood monocytes and reduced bacteremia. These results imply that GM-CSF therapy may be useful in restoring antimycobacterial function by human monocytes/macrophages.

Infection of monocytes/macrophages with human immunodeficiency virus (HIV) has been reported to impair a number of effector functions carried out by these cells, resulting in defective phagocytosis of *Candida albicans, Aspergillus fumigatus,* and *Toxoplasma gondii;* impaired intracellular killing of *C. pseudotropicalis, T. gondii,* and *Mycobacterium avium* complex (MAC); and dysregulation of cytokine production [1–3]. These alterations in macrophage function are thought to contribute to the pathogenesis of AIDS by allowing the reactivation of opportunistic pathogens (reviewed in [4]).

Disseminated MAC infection is one of the most common opportunistic infections, diagnosed in up to 40% of patients with advanced HIV infection and causing significant morbidity and mortality in them. Although combinations of antimycobacterial drugs are usually effective in suppressing symptoms, drug resistance leading to persistent clinical symptoms is increasingly being recognized. Granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine regulating proliferation, differentiation, and function of granulocytes and macrophages, has been previously shown to up-regulate phagocytosis and intracellular killing of MAC by murine and uninfected human monocyte-derived macrophages (MDM) and alveolar macrophages in vitro (reviewed in [5]). A recent report has demonstrated that GM-CSF therapy can also enhance mycobactericidal activity in monocytes from AIDS patients with MAC bacteremia [6].

We have investigated the ability of human monocytes and MDM to phagocytose MAC after their having been infected with HIV-1 in vitro and have studied the effects of GM-CSF on this function. In addition, we have examined the effect of a course of adjunctive GM-CSF treatment of a patient with advanced HIV-1 infection and disseminated infection with multidrug-resistant MAC.

Materials and Methods

Isolation and Culture of Monocytes

Human monocytes were isolated from HIV-seronegative buffy coats (supplied by the Red Cross Blood Bank, Melbourne) and cultured in suspension in polytetrafluoroethylene pots as described elsewhere [1]. Freshly isolated monocytes were either treated with recombinant human GM-CSF (Genzyme, Cambridge, MA) at a concentration of 100 ng/mL or left unexposed to GM-CSF as controls. Experiments were also done with a mutant form of GM-CSF

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(E21R; 1 μg/mL; BresaGen, Adelaide, Australia) with a site mutation within the first α helix, resulting in a substitution of arginine for glutamic acid [7]. This mutant GM-CSF binds with low affinity only to the α chain of the GM-CSF receptor.

HIV Infection of MDM

MDM in suspension culture were infected 4–6 days after isolation with the macrophage-tropic strain HIV-$1_{Ba\text{-}L}$, as described elsewhere [2]. After 4 h of exposure to HIV, cells were washed with PBS and resuspended in supplemented Iscove's medium (Cytosystem, Castle Hill, Australia) in the presence or absence of GM-CSF or E21R mutant GM-CSF. HIV-1 infection was quantified by measuring p24 antigen (HIV-1 p24 assay; Abbott Laboratories, Abbott Park, IL) in culture supernatants obtained 7 days after infection.

GM-CSF Treatment of the HIV-1–Infected Patient

A 35-year-old man was hospitalized with disseminated MAC infection resistant to ethambutol, clarithromycin, and amikacin. He had a history of extensive prior antiretroviral therapy. Two weeks before commencing GM-CSF treatment, his antiretroviral regimen was changed by the addition of 2 new drugs (nelfinavir and abacavir). On day 1 of therapy, he received recombinant GM-CSF (Schering-Plough, New South Wales, Australia) by subcutaneous injection at an initial dosage of 400 μg/day. GM-CSF treatment was suspended on day 7 of therapy because of neutrophilia and recommenced at a dose of 200 μg/day on day 14, when his neutrophil count declined to 10.2×10^3/mL. On day 38, he developed central catheter–related sepsis and multiorgan failure. He died on day 40 from central catheter–associated complications. Further clinical details of this patient are to be provided elsewhere (authors' unpublished data).

The patient's peripheral blood was collected twice before beginning GM-CSF treatment and 2 or 3 times per week during GM-CSF therapy to assess phagocytosis of MAC by monocytes and to measure plasma HIV RNA (branched DNA assay; Chiron, Emeryville, CA). Blood and duodenal biopsy specimens were cultured for MAC by inoculation into BACTEC 13A media (Becton Dickinson Diagnostic Systems, Cockeysville, MD) by standard procedures and cultured at 35°C for up to 8 weeks. A growth index of >20 determined by use of the BACTEC 460 system indicated a positive culture. Ziehl-Neelsen and auramine-rhodamine staining was done on all positive cultures, and identification was confirmed by the State Reference Laboratory by means of a DNA probe (Accuprobe; Gen-Probe, San Diego) and biochemical tests.

Phagocytosis Assays

With cultured MDM in vitro. On day 7 after HIV infection, MDM (2×10^5 cells in 100 μL of PBS) were dispensed in polypropylene tubes (Becton Dickinson) and cooled on ice for 20 min. MDM were incubated for 2 h at 37°C or 0°C with MAC (*M. avium* and *M. intracellulare*, a mixture of 4 serovars) labeled with fluorescein isothiocyanate (FITC), as described by our group [8], at different ratios of MAC-FITC to MDM, in duplicate. Phagocytosis was arrested by plunging the tubes into ice. The fluorescence of adherent MAC-FITC was quenched by use of quenching agent (Orpegen, Heidelberg, Germany). Cells were washed twice with cold PBS and fixed with 1% formaldehyde (Polysciences, Warrington, PA). The percentage of MDM that phagocytosed MAC-FITC was quantified by flow cytometry (FACStar Plus; Becton Dickinson). The significance of different treatments on phagocytosis was assessed by use of Student's *t* test (paired, two-tailed).

With monocytes within blood. Analysis of phagocytosis of MAC-FITC by monocytes was done by means of a whole blood assay as described elsewhere [8]. Briefly, blood from the HIV-1–infected patient treated with GM-CSF was collected in lithium heparin anticoagulant, along with samples from a minimum of 4 HIV-seronegative and -seropositive untreated controls. Blood samples from the same HIV-seronegative controls were used for each experiment. MAC-FITC (5×10^6 and 1.5×10^7) was added to 100 μL of blood dispensed into polypropylene tubes and incubated in a shaking water bath at 37°C for 10 min. After phagocytosis was arrested and the fluorescence of adherent MAC-FITC was quenched, monocytes were stained for 30 min on ice with anti-CD14 antibody conjugated to phycoerythrin (Becton Dickinson). Erythrocytes were lysed with FACS lysing solution (Becton Dickinson), and white blood cells were fixed with 1% formaldehyde. The percentage of $CD14^+$ monocytes phagocytosing MAC-FITC was calculated as the percentage of FITC-labeled cells in the phycoerythrin-positive cell population.

Statistics

Multiple linear regression analysis was used to determine the effects of HIV infection and GM-CSF therapy on the phagocytic ability of monocytes. A regression equation was constructed to estimate percentage of phagocytosis by monocytes as a function of $\log_{10}$ (MAC-FITC to monocytes) and dummy variables introduced to account for elapsed time, HIV infection status, and GM-CSF treatment status. The analysis was done by use of the STATA (Stata, College Station, TX) statistical analysis package.

Results

Effect of HIV-1 and GM-CSF on phagocytosis of MAC by MDM in vitro. The effect of HIV-$1_{Ba\text{-}L}$ infection of MDM in vitro on phagocytosis of MAC-FITC was assessed by flow cytometry with cells from 10 different donors at a range of ratios of MDM to MAC-FITC (1 : 12 to 1 : 75). By 7 days after HIV-1 infection, we found consistent impairment of phagocytosis compared with that in uninfected controls (significant at ratios of 1 : 50 [$P < .05$] and 1 : 75 [$P < .01$]; table 1). HIV-1 infection of MDM was confirmed in all experiments by demonstrating a rise in p24 antigen in culture supernatants between day 1 and day 7 after infection (data not shown).

GM-CSF treatment of MDM augmented phagocytosis of MAC-FITC by HIV-1–infected MDM (significant at ratios of MDM to MAC of 1 : 25, 1 : 50, and 1 : 75 [$P < .05$, $< .01$, and $< .01$, respectively]). At the highest ratio, HIV-infected GM-CSF–treated MDM phagocytosed a mean of 55.9% MAC-FITC, compared with 34.4% of infected MDM cultured with-

Table 1. Effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) and human immunodeficiency virus (HIV) on phagocytosis of *Mycobacterium avium* complex (MAC) by monocyte-derived macrophages (MDM) in vitro.

No. of experiments	Mock-infected			HIV-infected		
	Control	GM-CSF	E21R GM-CSF	Control	GM-CSF	E21R GM-CSF
10	56.1 ± 4.5	63.6 ± 3.1	—	34.4 ± 4.8	55.9 ± 4.8	—
5	64.1 ± 6.2	69.7 ± 3.5	41.6 ± 11.0	30.9 ± 5.9	54.7 ± 4.0	28.5 ± 7.0

NOTE. Data are % of macrophages containing fluorescein isothiocyanate-labeled MAC. Experiments were done in duplicate with MDM from different donors at a ratio of MDM to MAC of 1 : 75 (mean ± SD).

out GM-CSF (table 1). Furthermore, phagocytosis by HIV-1–infected GM-CSF–treated MDM was restored to levels comparable with those found in uninfected MDM (at a ratio of 1 : 75, 55.9% vs. 56.1%; $P = .487$). The apparent increase in phagocytosis by GM-CSF–treated uninfected MDM was of borderline significance (at a ratio of 1 : 75, $P = .057$).

A mutant form of GM-CSF (E21R), binding only to the α chain of the GM-CSF receptor, did not augment phagocytosis of MAC by either HIV-1–infected ($P = .68$) or paired uninfected MDM ($P = .54$; table 1).

Effect of GM-CSF treatment on phagocytosis of MAC by monocytes from an HIV-1–infected patient. Before GM-CSF therapy, the phagocytic activity of this patient's monocytes was significantly lower than that of HIV-uninfected control subjects ($P < .001$). GM-CSF therapy improved the ability of the patient's monocytes to phagocytose MAC to levels comparable with those of HIV-uninfected persons, starting from day 5 after commencement of treatment ($P = .45$; figure 1*A*). The phagocytic activity of monocytes from HIV-infected patients not undergoing GM-CSF therapy was significantly lower than that of HIV-uninfected controls and the GM-CSF–treated patient ($P < .001$).

The patient was monitored for MAC bacteremia for >1 year before GM-CSF therapy. Over this period, blood samples took a mean of 11.1 days to yield cultures positive for MAC ($n = 10$). One week before GM-CSF treatment, MAC was cultured from blood and duodenal biopsy within 6 and 4 days, respectively, whereas after 28–34 days of GM-CSF, the time was 17–24 days (mean, 20.0 days; $n = 4$; figure 1*B*). The patient experienced only minor fluctuations in plasma HIV RNA levels during therapy, with a possibly significant (defined as >0.5 $\log_{10}$ copies/mL) increase of 0.7 $\log_{10}$ copies/mL occurring at only one time point (day 28 of therapy; figure 1*C*).

Discussion

This study shows that phagocytosis of MAC by human MDM is defective after HIV-1 infection in vitro and that GM-CSF treatment can largely restore this antimycobacterial function. These data are supported by results from an HIV-infected patient with drug-resistant MAC infection whose ex vivo phagocytic capacity was significantly improved during adjunctive therapy with GM-CSF and was accompanied by a reduction in MAC bacteremia as measured by the length of time required for blood cultures to become positive.

The effector cells used in the phagocytosis assay in our study were MDM infected with HIV-1 after 5 days in culture. We observed a marked degree of donor variation in the ability of human MDM to phagocytose MAC under conditions tested (mean, 56%; range, 35%–82%). This may reflect the variability in surface expression of CR3 receptors facilitating phagocytosis of MAC or in complement levels (required for opsonization) present within human serum used in the experiments. There was also variable suppression of phagocytosis resulting from HIV-1 infection of MDM, which may reflect variation in the proportion of MDM infected with HIV-1, resulting from differing levels of surface chemokine coreceptor expression.

Our findings are consistent with data showing that HIV-1 infection of MDM in vitro impairs immunologic defenses against other opportunistic pathogens (reviewed in [4]). Similarly, monocytes from HIV-1–infected persons have been found to be less efficient in terms of phagocytosis and killing [9], although there are also reports of normal macrophage function following HIV infection in vivo and in vitro [10]. There are also inconsistent data regarding the effect of HIV infection on the ability of monocytes/macrophages to control MAC infection, with the data generally indicating reduced antimycobacterial function [3, 11]. A number of variables may be responsible for the inconsistencies in the literature, including the strain of HIV-1 used in vitro, donor variability of MDM regarding susceptibility to HIV-1 infection, the stage of maturation of monocytes/macrophages at the time of functional assessment, the type of intracellular pathogen, and the nature of exogenous cytokines.

Recombinant GM-CSF has been found by most investigators to augment the antimycobacterial function (including intracellular killing) of normal human and murine monocytes (reviewed in [5]). Earlier studies that used impure preparations of GM-CSF reported no improvement in macrophage function after GM-CSF treatment [12]. In our study, there was clear evidence of improvement in phagocytosis of MAC by MDM infected with HIV-1, as well as in paired uninfected MDM from the same donors. A mutant form of GM-CSF, E21R, binding only to the α chain of the receptor, failed to stimulate phagocytosis of MAC, suggesting that the principal signals controlling phagocytosis occur via the receptor β chain.

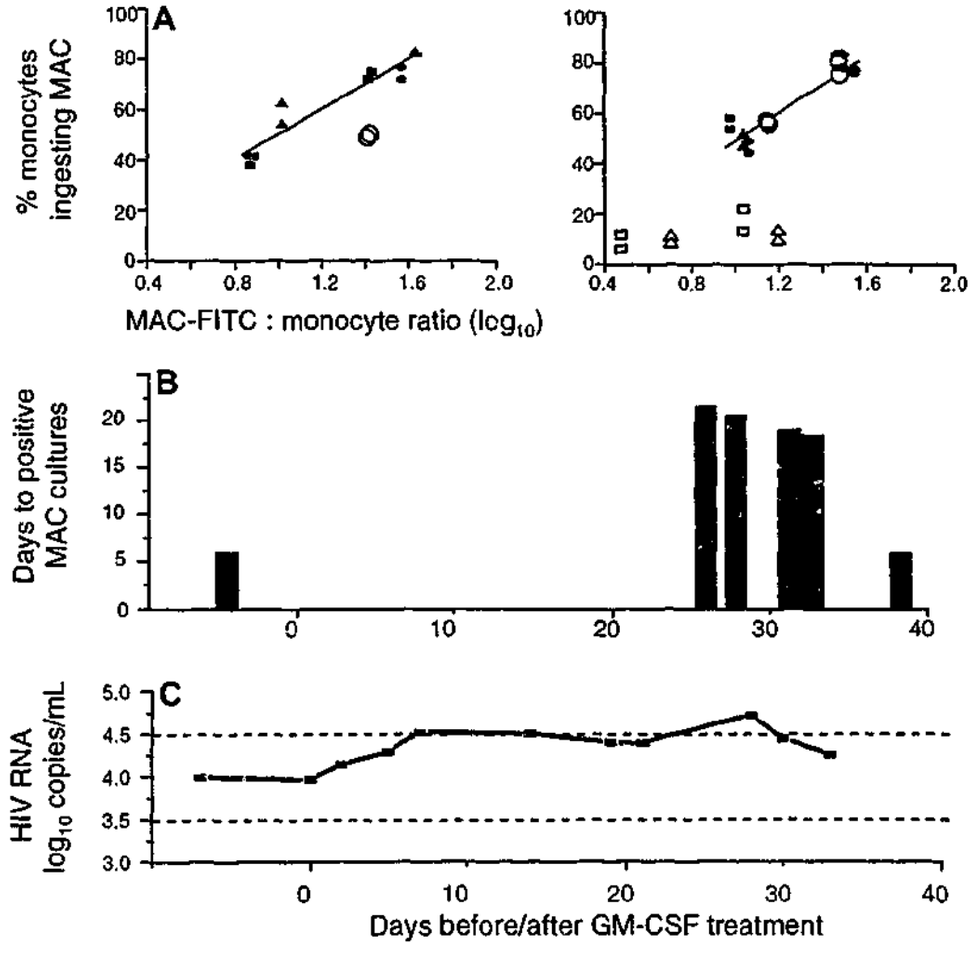

Figure 1. *A*, Effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) therapy on phagocytosis of *Mycobacterium avium* complex (MAC) by monocytes in ex vivo assay. Phagocytosis of fluorescein isothiocyanate (FITC)–labeled MAC by monocytes obtained from human immunodeficiency virus (HIV)–infected patient undergoing GM-CSF therapy (○) was compared with phagocytosis by monocytes obtained from HIV-uninfected controls (▲, ●, ■) and HIV-1–infected patients not receiving GM-CSF (□, △). Shown are representative results from GM-CSF–treated patient obtained before GM-CSF treatment (*left;* $P < .01$) and 30 days after beginning GM-CSF (*right;* $P = .45$). Regression lines were fitted to data points obtained from HIV-uninfected controls. *B*, Time to culture positivity for MAC in samples of patient's blood. *C*, Plasma HIV RNA levels before and during GM-CSF treatment.

The improvement in phagocytosis of MAC and lengthening of time to culture positivity for MAC in blood samples from the single patient with drug-resistant MAC infection adjunctively treated with GM-CSF support our in vitro findings. Similarly, a recent report by Kemper et al. [6] showed enhanced activation and mycobactericidal activity of blood monocytes of 4 AIDS patients with newly diagnosed MAC infection receiving azithromycin combined with GM-CSF, compared with that of monocytes from patients receiving azithromycin alone. However, those authors found no significant difference between groups in the reduction of mycobacteremia, which may reflect the efficacy of azithromycin on previously untreated MAC. We report here not only increased phagocytosis of MAC but also improvement in bacteremia, as measured by the length of time required for blood cultures to become positive, in an HIV-infected patient with multidrug-resistant disseminated MAC infection.

Clinical improvement after GM-CSF therapy has similarly been reported in patients with advanced HIV infection and fluconazole-resistant *C. albicans* infection [13]; augmentation of other aspects of macrophage function has been found in other immunosuppressed patients [14]. Preliminary data from an ongoing study of AIDS patients receiving nucleoside therapy has shown that GM-CSF administrated twice weekly for 6 months not only reduced the frequency of opportunistic infections but also significantly decreased virus load and increased CD4 cell counts of those subjects [15].

Taken together, these data suggest that GM-CSF therapy may be useful to augment macrophage function and to restore

antimycobacterial activity in HIV-1–infected and –uninfected patients.

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References

1. Crowe SM, Vardaxis NJ, Kent SJ, et al. HIV infection of monocyte-derived macrophages in vitro reduces phagocytosis of *Candida albicans*. J Leukoc Biol 1994;56:318–27.
2. Biggs BA, Hewish M, Kent S, Hayes K, Crowe SM. HIV-1 infection of human macrophages impairs phagocytosis and killing of *Toxoplasma gondii*. J Immunol 1995;154:6132–9.
3. Kallenius G, Koivula T, Rydgard KJ, et al. Human immunodeficiency virus type 1 enhances intracellular growth of *Mycobacterium avium* in human macrophages. Infect Immun 1992;60:2453–8.
4. Crowe SM. Role of macrophages in the pathogenesis of human immunodeficiency virus (HIV) infection. Aust N Z J Med 1995;25:777–83.
5. Armitage J. Emerging applications of recombinant human granulocyte-macrophage colony stimulating factor. Blood 1998;92:4491–508.
6. Kemper CA, Bermudez LE, Deresinski SC. Immunomodulatory treatment of *Mycobacterium avium* complex bacteremia in patients with AIDS by use of recombinant granulocyte-macrophage colony-stimulating factor. J Infect Dis 1998;177:914–20.
7. Lopez A, Shannon M, Hercus T, et al. Residue 21 of human granulocyte macrophage colony stimulating factor is critical for biological activity and for high but not low affinity binding. EMBO J 1992;11:909–16.
8. Hewish MJ, Meikle AM, Hunter SD, Crowe SM. Quantifying phagocytosis of *Mycobacterium avium* complex by human monocytes in whole blood. Immunol Cell Biol 1996;74:306–12.
9. Delemarre F, Stevenhagen A, Kroon F, van Eer M, Meenhorst P, Furth R. Reduced toxoplasmastatic activity of monocytes and monocyte-derived macrophages from AIDS patients is mediated via prostaglandin E2. AIDS 1995;9:441–5.
10. Nottet HSLM, de Graaf L, de Vos NM, Bakke LJ, van Strijp JAG, Verhoef J. Phagocytic function of monocyte-derived macrophages is not affected by human immunodeficiency virus type 1 infection. J Infect Dis 1993;168:84–91.
11. Zerlauth G, Maier E, Chehadeh H, Zimmermann K, Eibl M, Mannhalter J. Interaction with human immunodeficiency virus type 1 modulates innate effector functions of human monocytes. J Infect Dis 1995;172:1598–601.
12. Nathan C, Prendergast T, Wiebe M, et al. Activation of human macrophages. Comparison of other cytokines with interferon gamma. J Exp Med 1984;160:600–5.
13. Vazquez JA, Gupta S, Villanueva A. Potential utility of recombinant human GM-CSF as adjunctive treatment of refractory oropharyngeal candidiasis in AIDS patients. Eur J Clin Microbiol Infect Dis 1998;17:781–3.
14. Williams M, Newland A, Kelsey S. Monocyte mediated killing of human leukaemia is enhanced by administration of granulocyte-macrophage colony stimulating factor following chemotherapy. Br J Haematol 1997;98:960–8.
15. Brites C, Badaro R, Pedral-Sampaio D, et al. Granulocyte macrophage colony stimulating factor (GM-CSF) reduces viral load and increases CD4 cell counts in individuals with AIDS receiving AZT [abstract 22494]. In: Conference record of the 12th World AIDS Conference. Geneva, Marathon Multimedia, 1998.

Granulocyte-macrophage colony-stimulating factor inhibits HIV-1 replication in monocyte-derived macrophages

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Background: Previous studies of the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) on HIV-1 replication in macrophages have had inconsistent results, variously reporting no effect, augmentation or inhibition of viral replication.

Objective: To investigate the regulation of HIV-1 in monocyte-derived macrophages (MDM) by GM-CSF *in vitro*.

Methods: The role of GM-CSF on HIV-1 replication was assessed as supernatant and intracellular p24 antigen concentrations and by HIV-1 DNA and mRNA production under different culture conditions. Expression of CD4 and CCR5 receptors was examined. The effect of GM-CSF with an E21R mutation, which binds only to the α-chain of GM-CSF receptor, was used as an additional control.

Results: GM-CSF consistently suppressed HIV-1 replication in human MDM *in vitro*, as assessed by supernatant and intracellular p24 antigen concentrations and HIV-1 *gag* mRNA expression. The inhibitory effect of GM-CSF on HIV-1 replication was observed regardless of HIV-1 strain, source of GM-CSF, stage of MDM maturation or timing of GM-CSF exposure in relation to HIV-1 infection. The effect was dose dependent and reversed by addition of a neutralizing monoclonal antibody (4D4). Flow cytometric analysis of surface expression of CD4 and CCR5 indicates that GM-CSF does not affect HIV-1 entry into MDM. Analysis of intracellular HIV-1 DNA and mRNA suggests that HIV-1 replication is inhibited at or before transcription. E21R GM-CSF had no effect on HIV-1 replication in MDM.

Conclusions: GM-CSF regulates HIV-1 replication in MDM, inhibiting HIV-1 replication through binding to the β-chain of the GM-CSF receptor.

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Introduction

Cells of macrophage lineage can be infected with macrophage-tropic (M-tropic) or CCR5-using strains of HIV-1 *in vitro* and *in vivo* [1–5], via the CD4 molecule and β-chemokine co-receptors [6,7]. Although freshly isolated peripheral blood monocytes (PBMC) are relatively refractory to HIV-1 infection *in*

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vitro, susceptibility to infection is dramatically improved by culturing monocytes prior to infection [8,9]. It is thought that the levels of maturation of monocytes/macrophages affects their susceptibility to HIV-1 infection, as tissue macrophages can be readily infected on the day of their isolation [6]. HIV-1 replication in monocyte-derived macrophages (MDM) infected with M-tropic/CCR5 HIV-1 strains may be influenced by a variety of cytokines and growth factors such as various interleukins, interferons, chemokines, tumour necrosis factor alpha, colony-stimulating factor-1 (CSF-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [10–17].

GM-CSF, produced by a variety of cell types, including activated T cells, monocytes, macrophages and fibroblasts, is required for the survival, proliferation and differentiation of granulocyte–macrophage precursor cells and for the function of their mature progeny (reviewed by Metcalf [18] and Armitage [19]). Early studies investigating the effect of GM-CSF on HIV-1 replication reported that this factor exerted an upregulatory effect on viral production in both MDM [14,20–23] and chronically infected promonocytic lines U937 and U1 [24,25]. However, other investigators have reported inconsistent [13,26] or negative effects of GM-CSF on HIV-1 entry or replication in MDM [27,28] associated with reduced β-chemokine receptor expression.

GM-CSF mediates its activities through binding to a heterodimer receptor comprising a ligand-specific α-chain and a β-chain that is shared with interleukin (IL) 3 and 5 (reviewed by Armitage [19] and Crowe and Lopez [29]). Amino acid 21 within the first α-helix of GM-CSF has been found to be critical for the biological function of GM-CSF and for the interactions between GM-CSF and the β-chain of the GM-CSF receptor that are necessary for high-affinity binding [30]. In order to clarify the effects of GM-CSF on HIV-1 replication in MDM *in vitro*, the activity of this growth factor was examined by several different criteria. A mutant form of GM-CSF (E21R) that binds only to the α-chain of the receptor [30,31] and this form was used to examine the effect of GM-CSF on HIV-1 replication.

Methods

Isolation and culture of monocytes

Human monocytes were isolated from buffy coats of HIV-seronegative blood donors (supplied by the Red Cross Blood Bank, Melbourne, Australia) by Ficoll-Paque density gradient centrifugation and plastic adherence as previously described [2]. Immediately after isolation, cell viability was greater than 95% as assessed by Trypan blue exclusion; the purity of monocytes was greater than 90% as determined by immunofluorescent staining with anti-CD14 monoclonal antibody (MAb) (Becton Dickinson, Mountain View, California, USA) and flow cytometric analysis. Cells were cultured in Iscove's modified Dulbecco medium (Cytosystem, Castle Hill, Australia) supplemented with 10% heat-inactivated human AB+ serum, 2 mmol/l L-glutamine and 24 μg/ml gentamicin (supplemented Iscove's medium). Monocytes were cultured adherent to plastic in 24-well plates (Costar, Cambridge, Massachusetts, USA) or cultured in suspension in polytetrafluorethylene (Teflon) pots (Savillex, Minnetonka, Minnesota, USA) at a concentration of 1×10^6 cells/ml.

Freshly isolated monocytes were treated with recombinant human GM-CSF (Genzyme, Cambridge, Massachusetts, USA or Genetics Institute, Cambridge, Massachusetts, USA) at varying concentrations (1–100 ng/ml). In selected experiments, monocytes were cultured for 5 days prior to the addition of GM-CSF. Control cells from the same donors were cultured in the absence of GM-CSF. MDM cultured in suspension in the presence of GM-CSF (0.01–100 ng/ml) for 21 days showed no evidence of toxicity as assessed by Trypan blue exclusion. To determine the specificity of the effect of GM-CSF on HIV-1 replication in MDM, GM-CSF was incubated with a neutralizing anti-human GM-CSF MAb (0.1–100 μg/ml; 4D4) or a non-neutralizing anti-GM-CSF control MAb (0.1–100 μg/ml; 4A12) for 30 min at 4°C prior to its addition to MDM.

HIV-1 infection of monocyte-derived macrophages

The M-tropic strain of HIV-$1_{Ba\text{-}L}$ (the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, NIH, Bethesda, Maryland, USA) or an M-tropic primary isolate of HIV-1 obtained from an HIV-infected patient, HIV-1_{D36}, was amplified in PBMC. Briefly, PBMC stimulated with phytohaemagglutinin (10 μg/ml, Murex Diagnostics, Dartford, UK) for 3 days were infected with HIV-$1_{Ba\text{-}L}$ or primary isolate and cultured in Iscove's medium containing 10% fetal calf serum (PA Biologicals, New South Wales, Australia) and IL-2 10 U/ml (Boehringer-Mannheim, Mannheim, Germany). The culture supernatants collected 14 days later were clarified using 0.2 μm filters (Schleicher and Schuller, Dassel, Germany), stored at −70°C in small volumes and thawed immediately before use.

Following their isolation, monocytes were cultured under three sets of conditions to determine whether alteration of HIV replication depended upon the timing of addition of GM-CSF with respect to cellular maturation or addition of HIV. Cells were (i) cultured

from time of isolation in the presence or absence of GM-CSF, infected with HIV-1 on day 5 (day 0 GM-CSF, day 5 infection), as described earlier [20,21]; (ii) treated with GM-CSF on the day of isolation and infected the following day (day 0 GM-CSF, day 1 infection) [23]; or (iii) exposed to GM-CSF and HIV-1 on day 5 after isolation (day 5 GM-CSF, day 5 infection) [13,14]. For infections, HIV-$1_{Ba\text{-}L}$ was pre-treated with 10 U RNase-free DNase (Boehringer Mannheim Australia, Castle Hill, New South Wales, Australia) for 20 min at room temperature in the presence of 10 mmol/l $MgCl_2$to remove contaminating viral DNA and used at a concentration of $> 50\,000$ pg/ml HIV antigen (HIV-1 p24 antigen immunoassay, Abbott Laboratories, Abbott Park, Illinois, USA) for 1×10^6 cells for 2 h. Cells were then washed with phosphate-buffered saline (PBS; Trace Biosciences, New South Wales, Australia) and resuspended in fresh Iscove's supplemented medium in the presence or absence of GM-CSF. MDM were then cultured for 10 days following infection either in suspension in Teflon pots or adherent to plastic in 24-well plates. Uninfected MDM from the same donors were used as controls for each experiment. Since endotoxin contamination has been shown to alter HIV-1 replication in MDM, culture supernatants and GM-CSF stocks were tested for LPS levels using the Limulus Amebocyte Lysate Assay (Biowhittaker, Walkersville, Maryland, USA).

Quantification of HIV-1 replication

HIV-1 replication in MDM cultured in 24-well plates was measured as p24 antigen production (HIV-1 p24 antigen immunoassay, Abbott Laboratories, according to manufacturer's instructions) using serial dilutions of culture supernatant obtained 10 days after infection or by monitoring reverse transcriptase (RT) activity using a micro-RT assay. Briefly, 10 µl culture supernatant was added to 10 µl 0.3% NP40 in a 96-well plate. Thereafter, 40 µl RT mixture [50 mmol/l Tris pH 7.8, 7.5 mmol/l KCl, 5 mmol/l $MgCl_2$, 2 mmol/l dithiothreitol (Sigma, St Louis, Montana, USA), distilled H_2O up to 4 ml per plate], 5 µg/ml template-primer p.An.dT12-18 (Pharmacia-Biotec, Buckinghamshire, UK) and 3 µCi ^{33}P-dTTP (Amersham Co., Amersham, UK) was added and the mixture was incubated for 2 h at 37°C. The reaction products were spotted on a DE81 chromatography paper (Whatman, Maidstone, UK) and air-dried. Dry filters were washed six times with 2× SSC buffer (0.3 mol/l sodium chloride and 34 mmol/l sodium citrate) to remove free radioactive dNTP, rinsed once in 95% ethanol and air-dried. Meltilex scintillant (Wallax, Turku, Finland) was spotted onto the filters and bound radioactivity was counted in the LKB micro betacounter (Wallex).

Infection in suspension-cultured macrophages was determined in pre-fixed and permeabilized cells using a previously described method [2], by staining for intracellular p24 antigen using a MAb directed against p24 (2 µg/ml; IgG1, Olympus, Lake Success, New York, USA) or isotype-matched control (MOPC 21; 2 µg/ml; IgG_1, Bionetics, Charlestone, South Carolina, USA) followed by goat anti-mouse IgG conjugated to FITC (FITC-GAM; Tago, Burlingame, California, USA). The proportion of cells containing intracellular p24 antigen was quantified by flow cytometric analysis (FACStar^{Plus}, Becton Dickinson).

Expression of GM-CSF, CCR5 and CD4 receptors

Monocytes from HIV-1 seronegative donors were analysed for expression of the α- and β-chain of the GM-CSF receptor on the day of isolation and at times up to 14 days in culture in Teflon pots. Cells were stained with MAb directed against the α-chain (8G6; 5 µg/ml), β-chain (4F3; 5 µg/ml) [32] or isotype-matched control followed by FITC conjugate. The proportion of monocytes expressing α- or β-chain was quantified by flow cytometric analysis.

To determine regulation of CCR5 and CD4 surface expression by GM-CSF, monocytes were exposed to GM-CSF on the day of isolation and infected with HIV-$1_{Ba\text{-}L}$7 days after isolation. CCR5 and CD4 surface levels were assessed on the day of isolation, the day of HIV-1 infection and 2 and 5 days after infection. CCR5 expression was quantified using MAb against CCR5 (5C7; IgG2a, 0.5 µg/ml, LeukoSite Cambridge, Massachusetts, USA) or isotype-matched control (RPC5; IgG2a) on ice for 30 min. After two washes with cold PBS, cells were incubated with anti-mouse immunoglobulin conjugated with biotin (Silenus, Melbourne, Australia). After two further washes with cold PBS, MDM were incubated with fluorescein-conjugated streptavidin (Calbiochem, La Jolla, California, USA) for 30 min, washed with cold PBS and analysed by flow cytometry. The expression of CD4 receptor was assessed using anti-CD4 MAb conjugated to FITC (Leu-3; 1 µg; Becton Dickinson) on ice for 30 min, followed by a wash with cold PBS and flow cytometric analysis.

The effect of mutant granulocyte-macrophage colony-stimulating factor on HIV-1 replication

In selected experiments, MDM were also exposed to a mutant form of GM-CSF (E21R; BresaGen, Adelaide, South Australia) with a site mutation within the first α-helix, resulting in a substitution of arginine for glutamic acid [30,31]. This mutant GM-CSF only binds with a low affinity to the α-chain of the GM-CSF receptor and was used at a concentration of 100 ng/ml. The significance of the effect of treatment with GM-CSF or the E21R mutant GM-CSF on HIV replication was assessed using the Student's t-test (paired, 2-tailed).

HIV-1 DNA and RNA extraction and amplification

DNA was extracted as previously described [33]. Briefly, 1×10^6 MDM cells infected with HIV-1 were cultured for 7 days in the presence or absence of GM-CSF or mutant GM-CSF (E21R). Cells were lysed in 0.5 ml lysis buffer [50 mmol/l KCl, 10 mmol/l Tris (pH 8.3), 2.5 mmol/l $MgCl_2$, 0.5% Tween 20 (BDH Chemicals, Kilsyth, Australia), 0.5% NP40 (Sigma)]. Following the addition of 5 µl proteinase K (Boehringer Mannheim), cell lysates were heated at 60°C for 1 h and 95°C for 30 min. Sample lysates and lysates from controls (8E5 cells, containing a single HIV-1 provirus per cell) [10] were used in the polymerase chain reaction (PCR) reaction after making serial threefold dilutions in lysis buffer. Amplification of *gag* using SK38 and SK39 primers and hybridization using a ^{32}P-labelled probe SK19 was according to published methods in use in our laboratory [6]. HLA-DQ alpha was amplified in the same reaction using primers GH-26 and -27 to standardize the amount of DNA in the lysates.

Using oligo $(dT)_{25}$ beads (Dynabeads, Dynal, Australia), mRNA was extracted from MDM lysates according to the manufacturer's protocol. Briefly, 1×10^6 MDM that had been cultured in the presence or absence of GM-CSF for 7 days after infection were lysed in lysis/binding buffer [100 mmol/l Tris-HCl (pH 8), 500 mmol/l LiCl, 10 mmol/l ethylenediamine tetraacetic acid (EDTA) (pH 8), 0.1% LiDS (ICN Pharmaceuticals Mesa, California, USA), 5 mmol/l dithiothreitol]. After beads were preconditioned in lysis/binding buffer, MDM lysates were hybridized with beads (1×10^6 MDM/20 µl beads) and incubated at room temperature for 10 min to form a Dynabeads oligo$(dT)_{25}$/mRNA complex. Beads were washed twice with 100 µl washing buffer [10 mmol/l Tris-HCl (pH 8), 0.15 mol/l LiCl, 1 mmol/l EDTA (pH 8)] (BDH Chemicals) with 0.1% LiDS (ICN Pharmaceuticals Mesa, California, USA), and then three times with 100 ml washing buffer. After beads were washed four times with RT buffer [10 mmol/l Tris-HCl (pH 8.3), 75 mmol/l KCl] the Dynabeads oligo$(dT)_{25}$/mRNA complex was resuspended in 25 µl AMV-RT mix (1 mmol/l each of dGTP, dATP, dTTP, dCTP, 4 mmol/l sodium pyrophosphate, 25 U RNasin, 18 U AMV/RT, 5 µl 5× RT-buffer) and incubated for 1 h at 42°C to synthesize cDNA. After removing the RT mix, beads were resuspended in 100 µl elution solution (2 mmol/l EDTA) and heated at 95°C to remove mRNA. Beads were resuspended in 100 µl TE buffer (pH 8) and stored at 4°C in 10-fold dilutions in lysis buffer until used for PCR using *gag*-specific primers and detection by hybridization with a ^{32}P-labelled probe SK19. Levels of cDNA were standardized according to β-actin levels [34] as assessed by laser densitometry. To check for viral DNA contamination, samples prepared without AMV-RT were included within each experiment.

Results

GM-CSF inhibited HIV-$1_{Ba\text{-}L}$ replication in MDM in a dose-dependent manner (Table 1). Results of six experiments using MDM from different donors confirmed that 0.1 ng/ml or greater GM- CSF significantly inhibited HIV-$1_{Ba\text{-}L}$ replication by a mean of $67.7 \pm 4.9\%$ (standard error of the mean, SEM) compared with untreated controls. GM-CSF inhibition was blocked by a neutralizing MAb (4D4) against GM-CSF in a concentration-dependent fashion but not by a non-neutralizing anti-GM-CSF MAb (4A12), suggesting the specificity of the observed effect (Table 2).

MDM from a total of 30 donors were cultured in the absence of GM-CSF for the first 5 days after isolation then infected with HIV-$1_{Ba\text{-}L}$ on day 5 and simultaneously treated with GM-CSF (day 5 onwards). In 26

Table 1. Granulocyte-macrophage colony-stimulating factor (GM-CSF) inhibits HIV-1 replication in monocyte-derived macrophages.

Granulocyte-macrophage colony-stimulating factor (ng/ml)	Inhibition (%)[a]	*P* value
0.01	22.2 ± 21.2	0.25
0.1	67.7 ± 4.9	0.048
1	79.3 ± 5.1	0.042
10	83.2 ± 4.8	0.043
100	86.8 ± 1.9	0.046

[a]Each experiment was performed in duplicate using monocyte-derived macrophages from six different donors by reverse transcriptase or p24 antigen assay. Percentage of inhibition is expressed as mean ± SD.

Table 2. Specificity of the granulocyte-macrophage colony-stimulating factor (GM-CS[F])-induced inhibition of HIV-1 replication in monocyte-derived macrophages.

	Reverse transcriptase activity (Mean cpm ± SD)
No GM-CSF	1560 ± 192
GM-CSF 1 ng/ml	574 ± 209
+4D4 antibody (µg/ml)	
100	1849 ± 68
10	1229 ± 72
1	939 ± 70
0.1	672 ± 100
+4A12 antibody (µg/ml)	
100	398 ± 65
10	327 ± 76
1	701 ± 70
0.1	588 ± 38

Representative results from a single donor. 4D4, anti-GM-CSF blocking monoclonal antibody; 4A12, anti-GM-CSF non-blocking monoclonal antibody.

of 30 experiments, GM-CSF pre-treatment of MDM resulted in a two- to tenfold (mean 54.6 ± 5.5%, n = 30) decrease in the level of HIV p24 antigen in culture supernatants harvested 10 days after infection compared with untreated cells. In the remaining four experiments, there was no or insignificant (< 10%) inhibition (data not shown). Replication of a primary isolate HIV-1_{D36} was similarly inhibited. There was no difference observed in results from experiments using GM-CSF from Genzyme (n = 8) and Genetics Institute (n = 10) with a mean inhibition of 41.0 ± 8.4% (SEM) and 51.0 ± 11.3%, respectively, compared with p24 antigen concentrations in control supernatants (data not shown).

To determine whether the level of maturation of the monocyte at the time of exposure to GM-CSF, and the timing of exposure to GM-CSF prior to infection influenced the effect of GM-CSF on HIV-1 replication, monocytes were cultured under one of three conditions. The addition of GM-CSF resulted in downregulation of HIV-1 replication under all conditions ($P < 0.01$) compared with controls. As anticipated from results of previous studies in this laboratory [9,35], infection on day 1 resulted in lower levels of viral replication than in MDM infected on day 5, regardless of length of time of exposure to GM-CSF prior to infection (data not shown).

Intracellular p24 antigen was quantified in MDM cultured and infected in suspension; GM-CSF dramatically decreased intracellular p24 antigen concentrations as assessed by flow cytometry, whereas the mutant GM-CSF E21R did not significantly alter intracellular

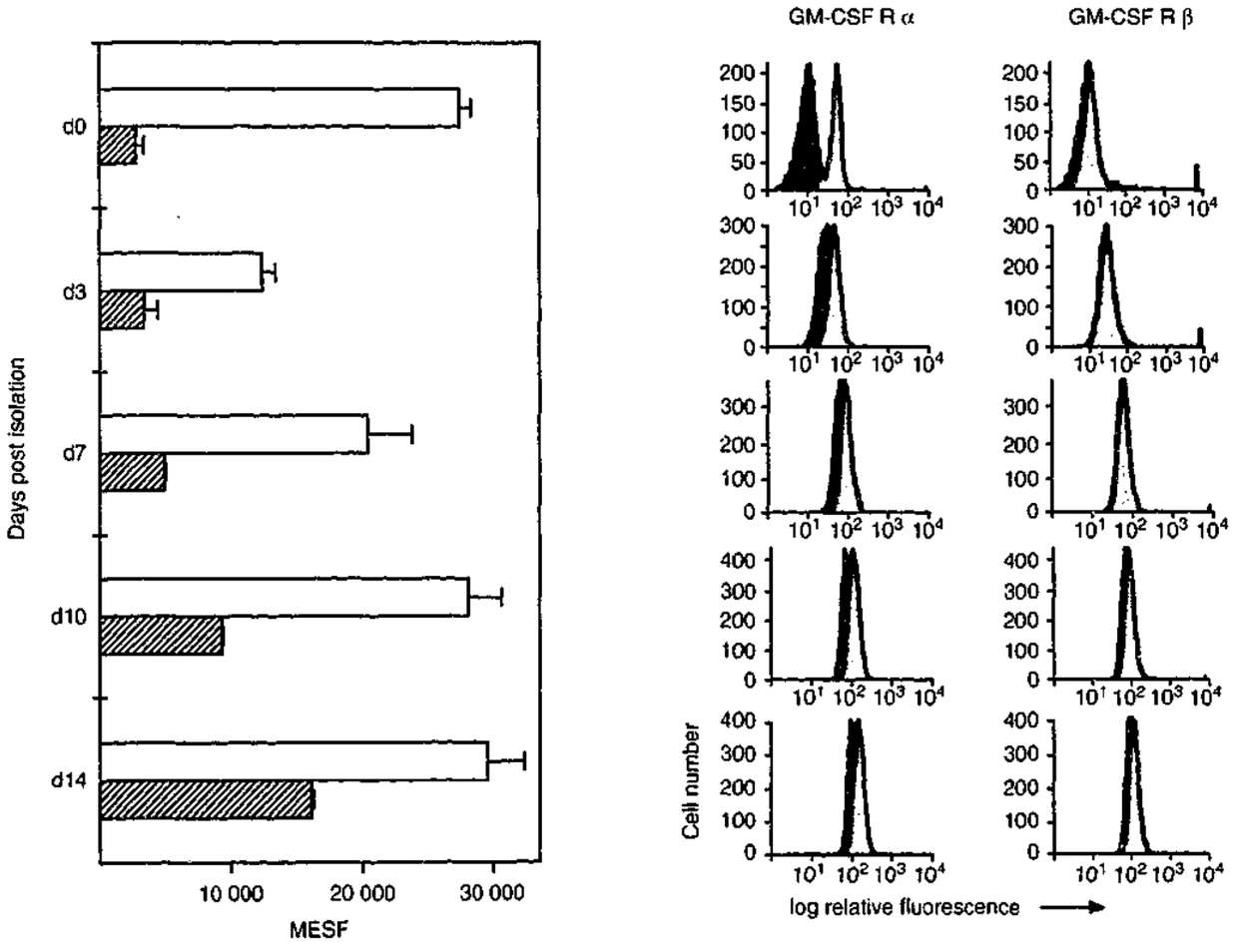

Fig. 1. Flow cytometric analysis of surface expression of α- and β-chains of granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor. The kinetics of expression of α- (clear bar) and β- (hatched bar) receptor subunit expression on monocyte-derived macrophages during 14 days culture in Teflon pots was measured using monoclonal antibodies against the α-chain (8G6; 5 μg/ml), and β-chain (4F3; 5 μg/ml) of the GM-CSF receptor and flow cytometric analysis. Results are expressed as net mean fluorescence values that have been converted to MESF (molecules of equivalent soluble fluorochrome) units using QuickCal program and corrected for background fluorescence. Histograms were unimodal. Results are corrected for background fluorescence and expressed as net MESF values.

p24 antigen levels from those in MDM not exposed to GM-CSF. The MESF (molecules of equivalent soluble fluorochrome) values were 7.2×10^5, 1.8×10^5 and 5.2×10^5 for intracellular p24 fluorescence of MDM cultured in the absence of GM-CSF, in presence of GM-CSF and in presence of mutant E21R GM-CSF, respectively. GM-CSF also inhibited supernatant RT production ($82 \pm 5.2\%$ inhibition; $n = 7$; $P < 0.01$), whereas E21R GM-CSF did not significantly suppress RT ($13.6 \pm 18\%$ inhibition; $n = 7$; $P = 0.5$).

To establish whether the surface expression of α- and β-chains of GM-CSF receptor on freshly isolated monocytes and differentiated MDM could affect their susceptibility to GM-CSF and potentially contribute to the effect of GM-CSF on HIV-1 replication, the level of both chains were assessed during 14 days of culture. The α-chain of the GM-CSF receptor was present in abundance on monocytes on the day of isolation, declined during the first 3 days of culture and then subsequently increased to the same level as on the day of isolation (Fig. 1). Levels of β-chain expression on monocytes were low on the day of isolation and increased during the 14-day culture period (Fig. 1).

To investigate the effect of GM-CSF at the viral entry level, surface expression of both CD4 receptor and CCR5 chemokine co-receptor was assessed by flow cytometry. As previously reported by our group [35], surface CD4 was readily detected on freshly isolated monocytes and increased more than fivefold after 7 days of culture. GM-CSF significantly downregulated surface expression of CD4 by more than 50% on days 7, 9 or 12 after monocyte isolation and GM-CSF treatment (Fig. 2a). GM-CSF also decreased expression of CD4 in HIV-infected MDM on days 2 and 5 after HIV-infection (corresponding to days 9 and 12 post-isolation) (Fig. 2a). Surface expression of CCR5 was undetectable or barely detectable on freshly isolated monocytes and significantly increased with *in vitro* differentiation (as previously demonstrated by a number of studies [28,36,37]). Exposure of fresh monocytes and MDM to GM-CSF resulted in an increase in CCR5 surface expression in both mock- and HIV-infected cultures compared with cells unexposed to GM-CSF (Fig. 2b).

The effect of GM-CSF on HIV-1 DNA and mRNA levels was examined to establish whether the decrease in HIV replication occurs before or after transcription. There was a minimal one- to threefold decrease in the level of HIV *gag* DNA in infected cells cultured in the

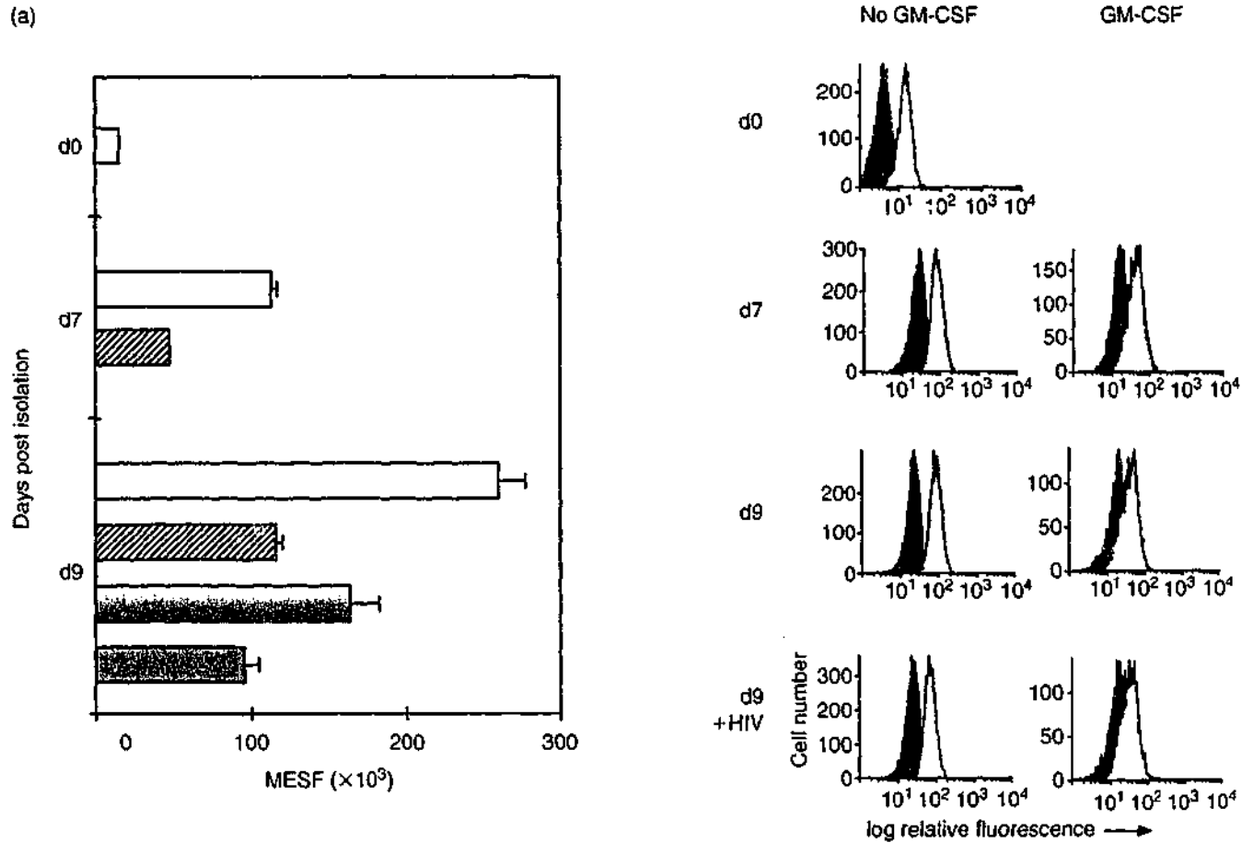

Fig. 2.

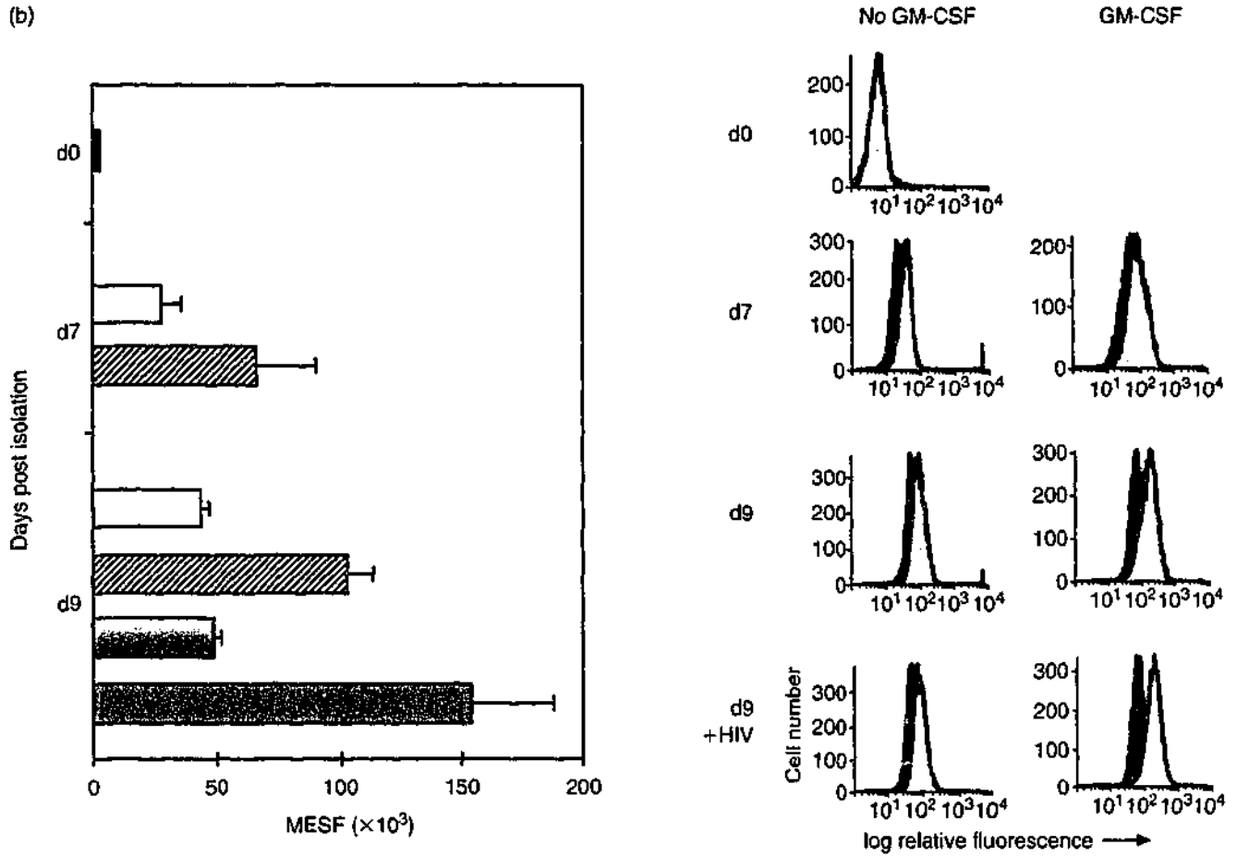

Fig. 2. Flow cytometric analysis of surface expression of CD4 (a) and CCR5 (b). Monocyte-derived macrophages (MDM) were pretreated with granulocyte-macrophage colony-stimulating factor (GM-CSF) (hatched bars) or media alone (open bars) for 7 days in Teflon jars prior to HIV-1 infection. Cells were infected for 4 h with HIV-1_{Ba-L} and then cultured in the presence (shaded bars) or absence of GM-CSF (dotted bars). MDM were stained with monoclonal antibodies on the day of isolation, after 7 days in culture and 2 days after HIV-1 infection. Results are expressed as net mean fluorescence values that have been converted to MESF (molecules of equivalent soluble fluorochrome) units using QuickCal program and corrected for background fluorescence. (a) Antibodies against against CD4 (Leu-3; gray histograms) and isotype-matched control monoclonal antibody (MOPC 21; black histograms). (b) Antibodies against CCR5 (5C7; gray histograms) and isotype-matched control monoclonal antibody (RPC5; black histograms).

presence of GM-CSF (n = 3) compared with cells not exposed to GM-CSF or treated with E21R GM-CSF (Fig. 3a). As assessed by laser densitometry, the ratio of *gag* to *DQ* signal was 0.47, 0.18 and 0.47 (mean of the first three dilutions, results for a representative experiment) for cells cultured in the absence of GM-CSF, presence of GM-CSF and presence of E21R GM-CSF, respectively. Analysis of cell lysates from three donors showed a three- to tenfold decrease in HIV-1 *gag* mRNA expression in MDM infected with HIV-1_{Ba-L} that were exposed to GM-CSF and an approximately twofold inhibition in those exposed to GM-CSF E21R compared with that in untreated MDM, suggesting that HIV-1 replication is inhibited before or at transcription. Densitometry units for *gag* signal standardized to equivalent levels of β-actin levels were 1956, 732 and 957 for MDM cultured in the absence of GM-CSF, presence of GM-CSF and presence of E21R GM-CSF, respectively (Fig. 3b).

Discussion

This study shows that GM-CSF consistently suppresses HIV-1 replication in human MDM in a dose-dependent manner. The inhibitory effect is specific since it is totally reversed by addition of neutralizing MAb 4D4 but not by addition of non-neutralizing anti-GM-CSF control MAb, 4A12. The inhibitory effect of GM-CSF is unrelated to the level of maturation of MDM at the time of GM-CSF stimulation or HIV infection. E21R GM-CSF, binding only to the α-chain of the GM-CSF receptor [30], does not affect HIV-1 replication. We conclude, therefore, that the inhibition of HIV-1 replication by GM-CSF results from signaling through the β-chain of its receptor.

Our report addresses a longstanding controversy in the literature, with most of the early studies reporting augmentation [14,20–25] or no change [13,26] in viral

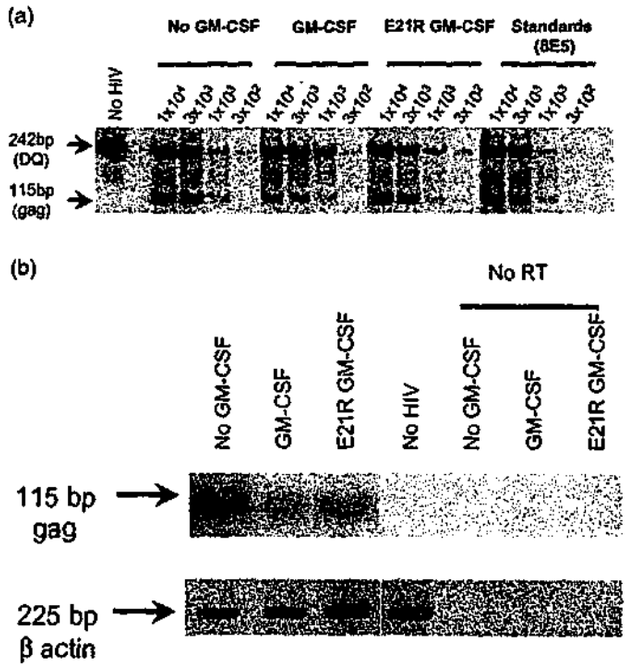

Fig. 3. Effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) on HIV-1 *gag* DNA and mRNA concentrations in HIV-infected monocyte-derived macrophages (MDM). Monocytes were cultured for 5 days in presence or absence of wild-type GM-CSF (Genzyme) or mutant GM-CSF (E21R), infected on day 5, and DNA and mRNA extractions were performed 7 days after infection. (a) HIV-1 *gag* DNA was assessed in threefold dilutions of cell extracts prepared for polymerase chain reaction (PCR) using *DQ* and *gag* primers and detected by hybridization with a ^{32}P-labelled probe GH 26 for HLA-DQ and SK19 for *gag*. Dilutions were similarly prepared from cell lysates of 8E5 cells (containing 1 HIV-1 provirus per cell) and amplified concurrently to serve as standards. (b) HIV-1 mRNA was assessed as cDNA synthesized from mRNA by PCR using *gag*-specific primers and detected by hybridization with a ^{32}P labelled probe SK19. Levels of cDNA were standardized according to β-actin levels. Polymerase chain reaction on samples prepared without reverse transcriptase were negative.

production and two recent studies suggesting inhibition of HIV-1 replication in MDM by GM-CSF [27,28]. A number of laboratory variables could potentially contribute to such variation. We have extensively examined potential confounders to determine why our results differ to some previous studies. We have reproduced the assay conditions used by other investigators, including strain of HIV-1, source/concentration of GM-CSF, timing of incubation with cytokine in relation to cell maturity and HIV-1 infection. Regardless of the experimental conditions, we observed reduced replication of HIV-1.

The mechanism by which GM-CSF alters HIV replication in MDM and in promonocytic cell lines is also controversial. Wang *et al.* [23] reported that the GM-CSF-induced increase in HIV-1 replication in MDM was attributable to upregulation of CCR5 expression. These data are in direct contrast to those of Di Marzio *et al.* [28], who demonstrated that GM-CSF suppressed CCR5 and CD4 expression on MDM and reduced HIV-1 entry into these cells.

Stimulation of monocytes with GM-CSF for 7 days prior to HIV-1 infection resulted in modest downregulation of CD4 surface expression and augmentation of CCR5 levels on MDM, coincident with inhibition of HIV-1 replication in those cells. It is unlikely that these opposing effects on the expression of CD4 and CCR5 will affect viral entry of M-tropic strains of HIV-1. We have previously demonstrated that the susceptibility of human monocytes/macrophages to HIV-1 infection is not dependent on the level of CD4 expression [35]. Recent studies by Fear *et al.* [38] and Kozak *et al.* [39] demonstrate that expression of CD4 is not a rate-determining factor for viral entry in the presence of adequate CCR5 levels, since M-tropic strains of HIV-1 have been able to infect CCR5-expressing cells by utilizing very low densities of CD4.

In support of our findings, other cytokines have also been shown to have opposing effects on the expression of CD4 and CCR5. Interferon-gamma (IFNγ), a cytokine that has bidirectional effects on HIV-1 replication in MDM, significantly upregulated CCR5 surface expression and inhibited CD4 surface levels, coincident with suppression of HIV-1 replication [40]. These results are not surprising since both GM-CSF and IFNγ are known to transduce signals via common pathways, e.g., JAK/STAT. The relationship between JAK2 activation and HIV-1 replication in MDM is currently being investigated.

GM-CSF mediates its activities through binding to its receptor, a heterodimer comprising a ligand-specific α-chain with low-affinity binding [41] and a non-ligand-binding β-chain that increases binding affinity [42]. Although there is high expression of the α-chain and low expression of the β-chain on the surface of monocytes [43], to our knowledge the kinetics of expression over time in culture has not previously been documented. It appears that monocyte differentiation is associated with an increase in α/β dimers available for GM-CSF binding and transduction of the signal to the cell.

Our data suggest minimal inhibition at *gag* DNA level and a three- to tenfold decrease in *gag* mRNA within MDM treated with GM-CSF compared with untreated cells, suggesting that the block to HIV-1 replication occurs at or prior to transcription. The difference in the inhibitory effect for DNA and mRNA levels could be a consequence of the effect of GM-CSF at multiple points, donor variation or the semiquantitative nature of the assays. Our results are in agreement with

Matsuda *et al.* [27], who has also reported inhibition of HIV-1 replication by GM-CSF with a decrease in proviral DNA.

Currently, GM-CSF is used rarely for the treatment of HIV-infected patients because of concerns regarding potential activation of HIV replication. Early studies showed that GM-CSF treatment of HIV-infected patients increased serum p24 antigen and plasma HIV RNA titres [44,45]. However, when used in combination with effective antiretroviral therapy, GM-CSF has been safely administered to patients [46–49]. Data from two studies of HIV-infected patients receiving antiretroviral therapy and GM-CSF have shown that patients experienced a decrease in viral load and an increase in CD4 counts [50,51]. Clinical improvement and augmented macrophage function without an increase in viral load has been also reported in patients with advanced HIV infection and drug-resistant opportunistic infections when treated with GM-CSF [46,52]. Our *in vitro* data, together with that contained in another recent report [28] and the clinical studies mentioned above, suggest that clinical utility of GM-CSF in the setting of HIV infection (especially with M-tropic strains of the virus) may be cautiously revisited.

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References

1. Gartner S, Markovits P, Markovitz DM, Gallo RC, Popovic M. **The role of mononuclear phagocytes in HTLVIII/LAV infection.** *Science* 1986, 233:215–219.
2. Crowe SM, Mills J, McGrath MS. **Quantitative immunocytofluorographic analysis of CD4 surface antigen expression and HIV infection of human peripheral blood monocyte/macrophages.** *AIDS Res Hum Retroviruses* 1987, 3:135–145.
3. Ho DD, Rota TR, Hirsch MS. **Infection of monocyte-macrophages by human T lymphotropic virus type III.** *J Clin Invest* 1986, 77:1712–1715.
4. Collman R, Hassan NF, Walker R *et al.* **Infection of monocyte-derived macrophages with human immunodeficiency virus type 1 (HIV-1). Monocyte tropic and lymphocyte tropic strains of HIV-1 show distinctive patterns of replication in a panel of cell types.** *J Exp Med* 1989, 170:1149–1163.
5. Salahuddin SZ, Rose RM, Groopman JE, Markham PD, Gallo RC. **Human T lymphotropic virus type III infection of human alveolar macrophages.** *Blood* 1986, 68:281–284.
6. Lewin SR, Sonza S, Irving LB, McDonald CF, Mills J, Crowe SM. **Surface CD4 is critical to *in vitro* HIV infection of human alveolar macrophages.** *AIDS Res Hum Retroviruses* 1996, 12:877–883.
7. Alkhatib GC, Combadiere C, Broder C *et al.* **CC CKR5: a RANTES, MIP-1 alpha, MIP-1 beta receptor as a fusion cofactor for macrophage tropic HIV-1.** *Science* 1996, 272:1955–1958.
8. Rich EA, Chen ISY, Zack JA, Leonard ML, O'Brien WA. **Increased susceptibility of differentiated mononuclear phagocytes to productive infection with human immunodeficiency virus-1 (HIV-1).** *J Clin Invest* 1992, 89:176–183.
9. Sonza S, Maerz A, Deacon N, Meanger J, Mills J, Crowe S. **Human immunodeficiency virus type 1 replication is blocked prior to reverse transcription and integration in freshly isolated peripheral blood monocytes.** *J Virol* 1996, 70:3863–3869.
10. Kalter DC, Nakamura M, Turpin JA *et al.* **Enhanced HIV replication in macrophage colony stimulating factor treated monocytes.** *J Immunol* 1991, 146:298–306.
11. Gendelman HE, Orenstein JM, Martin MA *et al.* **Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1 treated monocytes.** *J Exp Med* 1988, 167:1428–1441.
12. Mellors JW, Griffith BP, Ortiz MA, Landry ML, Ryan JL. **Tumor necrosis factor alpha/cachectin enhances human immunodeficiency virus type 1 replication in primary macrophages.** *J Infect Dis* 1991, 163:78–82.
13. Kornbluth RS, Oh PS, Munis JR, Cleveland PH, Richman DD. **Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by human immunodeficiency virus in vitro.** *J Exp Med* 1989, 169:1137–1151.
14. Koyanagi Y, O'Brien WA, Zhao JQ, Golde DW, Gasson JC, Chen ISY. **Cytokines alter production of HIV-1 from primary mononuclear phagocytes.** *Science* 1988, 241:1673–1675.
15. Kedzierska K, Rainbird M, Lopez A, Crowe S. **Effect of GM-CSF on HIV-1 replication in monocytes/macrophages in vivo and in vitro: a review.** *Vet Immunol Immunopathol* 1998, 63:111–121.
16. Verani A, Scarlatti G, Comar M *et al.* **C-C chemokines released by lipopolysaccharide (LPS) stimulated human macrophages suppress HIV-1 infection in both macrophages and T cells.** *J Exp Med* 1997, 185:805–816.
17. Coffey MJ, Woffendin C, Phare SM, Strieter RM, Markovitz DM. **RANTES inhibits HIV-1 replication in human peripheral blood monocytes and alveolar macrophages.** *Am J Physiol* 1997, 272:L1025–L1029.
18. Metcalf D. **Hemopoietic regulators.** *Trends Biochem Sci* 1992, 17:286–289.
19. Armitage J. **Emerging applications of recombinant human grranulocyte-macrophage colony stimulating factor.** *Blood* 1998, 92:4491–4508.
20. Perno CF, Yarchoan R, Cooney DA *et al.* **Replication of human immunodeficiency virus in monocytes. Granulocyte/macrophage colony-stimulating factor (GM-CSF) potentiates viral production yet enhances the antiviral effect mediated by 3×-azido-2×3×-dideoxythymidine (AZT) and other dideoxynucleoside congeners of thymidine.** *J Exp Med* 1989, 169:933–951.
21. Schuitemaker H, Kootstra NA, van Oers MH, van Lambalgen R, Tersmette M, Miedema F. **Induction of monocyte proliferation and HIV expression by IL-3 does not interfere with anti-viral activity of zidovudine.** *Blood* 1990, 76:1490–1493.
22. Perno CF, Cooney DA, Gao WY *et al.* **Effects of bone marrow stimulatory cytokines on human immunodeficiency virus replication and the antiviral activity of dideoxynucleosides in cultures of monocytes/macrophages.** *Blood* 1992, 80:995–1003.
23. Wang J, Roderiquez G, Oravecz T, Norcross A. **Cytokine regulation of human immunodeficiency virus type 1 entry and replication in human monocytes/macropohages through modulation of CCR5 expression.** *J Virol* 1998, 72:7642–7647.
24. Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS. **Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line.** *Science* 1987, 238:800–802.
25. Pomerantz RJ, Feinberg MB, Trono D, Baltimore D. **Lipopolysaccharide is a potent monocyte/macrophage specific stimulator of human immunodeficiency virus type 1 expression.** *J Exp Med* 1990, 172:253–261.
26. Hammer SM, Gillis JM, Pinkston P, Rose RM. **Effect of zidovudine and granulocyte-macrophage colony stimulating factor on human immunodeficiency virus replication in alveolar macrophages.** *Blood* 1990, 75:1215–1219.
27. Matsuda S, Akagawa K, Honda M, Yokota Y, Takebe Y, Takemon T. **Suppression of HIV replication in human monocyte derived macrophages induced by granulocyte macrophage colony**

stimulating factor. *AIDS Res Hum Retroviruses* 1995, 11:1031–1038.
28. Di Marzio P, Tse J, Landau N. **Chemokine receptor regulation and HIV type 1 tropism in monocyte-macrophages.** *AIDS Res Hum Retroviruses* 1998, 14:129–138.
29. Crowe S, Lopez A. **GM-CSF and its effects on replication of HIV-1 in cells of macrophage lineage.** *J Leukoc Biol* 1997, 62:41–48.
30. Lopez A, Shannon M, Hercus T *et al.* **Residue 21 of human granulocyte macrophage colony stimulating factor is critical for biological activity and for high but not low affinity binding.** *EMBO J* 1992, 11:909–916.
31. Hercus T, Cambareri B, Dottore M, *et al.* **Identification of residues in the first and fourth helices of human granulocyte macrophage colony stimulating factor involved in biologic activity and in binding to the alpha and beta chains of its receptor.** *Blood* 1994, 83:3500–3508.
32. Woodcock J, McClure B, Stomski F, Elliott M, Bagley C, Lopez A. **The human granulocyte macrophage colony stimulating factor (GM-CSF) receptor exists as a preformed receptor complex that can be activated by GM-CSF, interleukin-3, or interleukin-5.** *Blood* 1997, 90:3005–3017.
33. Lee T, Sunzeri F, Tobler L, Williams B, Busch M. **Quantitative assessment of HIV-1 DNA load by coamplification of HIV-1 gag and HLA-DQ-alpha genes.** *AIDS* 1991, 5:683–691.
34. Grassi G, Pozzato G, Moretti M, Giacca M. **Quantitative analysis of hepatitis C virus RNA in liver biopsies by competitive reverse transcription and polymerase chain reaction.** *J Hepatol* 1995, 23:403–411.
35. Sonza S, Maerz A, Uren S *et al.* **Susceptibility of human monocytes to HIV-1 infection *in vitro* is not dependent on their level of CD4 expression.** *AIDS Res Hum Retroviruses* 1995, 11:769–776.
36. Tuttle DL, Harrison JK, Anders C, Sleasman JW, Goodenow MM. **Expression of CCR5 increases during monocyte differentiation and directly mediates macrophage susceptibility to infection by human immunodeficiency virus type 1.** *J Virol* 1998, 72:4962–4969.
37. Naif HM, Li S, Mohammed A *et al.* **CCR5 expression correlates with susceptibility of maturing monocytes to human immunodeficiency virus type 1 infection.** *J Virol* 1998, 72:830–836.
38. Fear WR, Kesson AM, Naif H, Lynch GW, Cunningham AL. **Differential tropism and chemokine receptor expression of human immunodeficiency virus type 1 in neonatal monocytes, monocyte-derived macrophages, and placental macrophages.** *J Virol* 1998, 72:1334–1344.
39. Kozak SL, Platt EJ, Madani N, Ferro Jr FE, Peden K, Kabat D. **CD4, CXCR-4, and CCR5 dependencies for infections by primary patient and laboratory-adapted isolates of human immunodeficiency virus type 1.** *J Virol* 1997, 71:873–882.
40. Hariharan D, Douglas SD, Lee B, Lai JP, Campbell DE, Ho WZ. **Interferon-γ upregulates CCR5 expression in cord and adult blood mononuclear phagocytes.** *Blood* 1999, 93:1137–1144.
41. Gearing D, King J, Gough N, Nicola N. **Expression cloning of a receptor for human granulocyte macrophage colony stimulating factor.** *EMBO J* 1989, 8:3667–3676.
42. Bazan J. **Structural design and molecular evolution of a cytokine receptor superfamily.** *Proc Natl Acad Sci USA* 1990, 87:6934–6938.
43. Jubinsky P, Laurie A, Nathan D, Yetz-Aldepe J, Sieff C. **Expression and function of the human granulocyte macrophage colony stimulating factor receptor alpha subunit.** *Blood* 1994, 84:4174–4185.
44. Kaplan LD, Kahn JO, Crowe S *et al.* **Clinical and virological effects of recombinant human granulocyte-macrophage colony-stimulating factor in patients receiving chemotherapy for human immunodeficiency virus-associated non-Hodkin's lymphoma: results of randomized trial.** *J Clin Oncol* 1991, 9:929–940.
45. Lafeuillade A, Poggi C, Tamalet C. **GM-CSF increases HIV-1 load.** *Lancet* 1996, 347:1123–1124.
46. Kedzierska K, Mak J, Mijch A *et al.* **GM-CSF augments phagocytosis of *Mycobacterium avium* complex by HIV-1 infected monocytes /macrophages *in vitro* and *in vivo*.** *J Infect Dis* 2000, 181:390–394.
47. Krown SE, Pardes J, Bundow D, Polsky B, Gold JWM Homenberg N. **Interferon-alpha, zidovudine and granulocyte macrophage colony stimulating factor; a phase I AIDS clinical trials group study in patients with Kaposi's Sarcoma associated with AIDS.** *J Clin Oncol* 1992, 10:1344–1351.
48. Yarchoan R, Pluda JM, Perno CF *et al.* **Initial clinical experience with dideoxynucleosides as single agents and in combination therapy.** *Ann NY Acad Sci* 1990, 616:328–343.
49. Ross SD, DiGeorge A, Conneli JE, Whiting GW, McDonnel N. **Safety of GM-CSF in patients with AIDS: a review of the literature.** *Pharmacotherapy* 1998, 18:1290–1297.
50. Brites C, Badaro R, Pedral-Sampaio D *et al.* **Granulocyte macrophage colony stimulating factor (GM-CSF) reduces viral load and increases CD4 cell counts in individuals with AIDS receiving AZT.** *XII International Conference on AIDS, Geneva,* June 1998 [abstract 22494].
51. Skowron G, Stein D, Drusano G *et al.* **The safety and efficacy of granulocyte-macrophage colony-stimulating factor (Sargramostim) added to indinavir- or ritonavir-based antiretroviral therapy: a randomized double blind, placebo-controlled trial.** *J Infect Dis* 1999, 180:1064–1071.
52. Vazquez JA, Gupta S, Villanueva A. **Potential utility of recombinant human GM-CSF as adjunctive treatment of refractory oropharyngeal candidiasis in AIDS patients.** *Eur J Clin Microbiol Infect Dis* 1998, 17:781–783.

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Effect of GM-CSF on HIV-1 replication in monocytes/macrophages in vivo and in vitro: a review

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Effect of GM-CSF on HIV-1 replication in monocytes/macrophages in vivo and in vitro: a review

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Abstract

Cells of macrophage lineage constitute the main cellular target of Human Immunodeficiency Virus type 1 (HIV-1). Replication of HIV-1 in monocyte/macrophages is generally augmented by factors promoting their differentiation. Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a key regulator of the differentiation of cells of macrophage lineage. The effects of GM-CSF on HIV-1 replication in vitro are still controversial. Most of the published studies suggest that GM-CSF upregulates HIV-1 expression in both primary cultured macrophages and promonocytic cell lines. There have also been reports demonstrating that GM-CSF does not affect HIV-1 replication in cells of macrophage lineage or that GM-CSF can actually suppress HIV-1 expression. In vivo, GM-CSF administrated to HIV-positive patients at any stage of disease, without any antiretroviral therapy, appears to increase HIV-1 activity. The possible mechanism by which GM-CSF might affect HIV-1 replication in macrophages remains unclear.

Keywords: Macrophage; GM-CSF; HIV

1. Introduction

Human Immunodeficiency Virus (HIV) is a member of the lentivirus subfamily of retroviruses. It is the virus responsible for the progressive degeneration of the immune system leading to the development of Acquired Immunodeficiency Syndrome (AIDS)

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(Barre-Sinoussi et al., 1983; Gallo et al., 1984). The main cellular targets of HIV-1 are cells expressing CD4 + surface receptor: the CD4 + subset of T lymphocytes and cells of macrophage lineage. Both blood monocytes and tissue macrophages can be infected with HIV-1 in vitro and in vivo and the HIV-1 infected macrophage is considered a likely viral reservoir in vivo (Gartner et al., 1986; Salahuddin et al., 1986; Crowe et al., 1987; McUrath et al., 1989). Cells of macrophage lineage have many important roles in immunological and inflammatory responses. Their hematopoietic and immunologic functions are regulated through their surface receptors by various cytokines and other growth factors. It has been shown that the susceptibility of monocytes to HIV-1 infection depends on their differentiation (Sonza et al., 1995; Sonza et al., 1996) suggesting that factors promoting differentiation of monocytes/macrophages can directly affect HIV-1 infection and replication. Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a critical regulator of the differentiation of cells of this lineage.

2. The structure and function of GM-CSF and its receptor

GM-CSF is a glycoprotein of 127 amino acids with a molecular weight of 23 000–28 000 produced by a variety of cell types including activated T cells, endothelial cells, macrophages and fibroblasts (Hercus et al., 1994a,b). This cytokine is a multipotential hemopoietic growth factor regulating cells of eosinophilic, neutrophilic, monocytic and megakaryocytic lineages (Fig. 1) [reviewed by Metcalf (1985)]. A number of studies investigating the effects of GM-CSF on monocytes/macrophages have demonstrated that GM-CSF at relatively high concentrations enhances monocyte number by stimulating cell proliferation (Elliott et al., 1989; Bratton et al., 1995), whereas low concentrations of GM-CSF (pM ranges) have been found to enhance monocyte survival (Elliott et al., 1989; Eischen et al., 1991). GM-CSF induces the differentiation of monocytes into macrophages. Circulating monocytes upon migration into the tissues differentiate into specialised macrophages such as alveolar macrophages, Kupffer cells or microglial cells. In vitro, GM-CSF has also been reported to enhance the effector function of mature macrophages including increasing their phagocytic capacity (Smith et al., 1990) as well as cytotoxicity against tumor cells (Parhar et al., 1992) and antiparasitic and antimycobacterial activity (Bermudez, 1994; Laursen et al., 1994). Being a pleiotropic cytokine, GM-CSF also stimulates the effector function of circulating neutrophils and eosinophils (Vadas et al., 1983).

The structure of GM-CSF has been determined by X-ray crystallography revealing the presence of four alpha helices with a novel fold combining a two-stranded antiparallel beta sheet (Diederichs et al., 1991). Although the structure of GM-CSF was extensively studied in vivo and in vitro, little is known about regions of the molecule essential for its biological activity and for receptor recognition. However, it has been shown that elimination of 14 most N-terminal amino acids and six C-terminal amino acids does not have any effect on GM-CSF bioactivity, whereas the comprising amino acid residues 14–28 region in the first alpha helix (Clark-Lewis et al., 1988) and the region between residues 88–96 (Brown et al., 1990) are essential for its biological

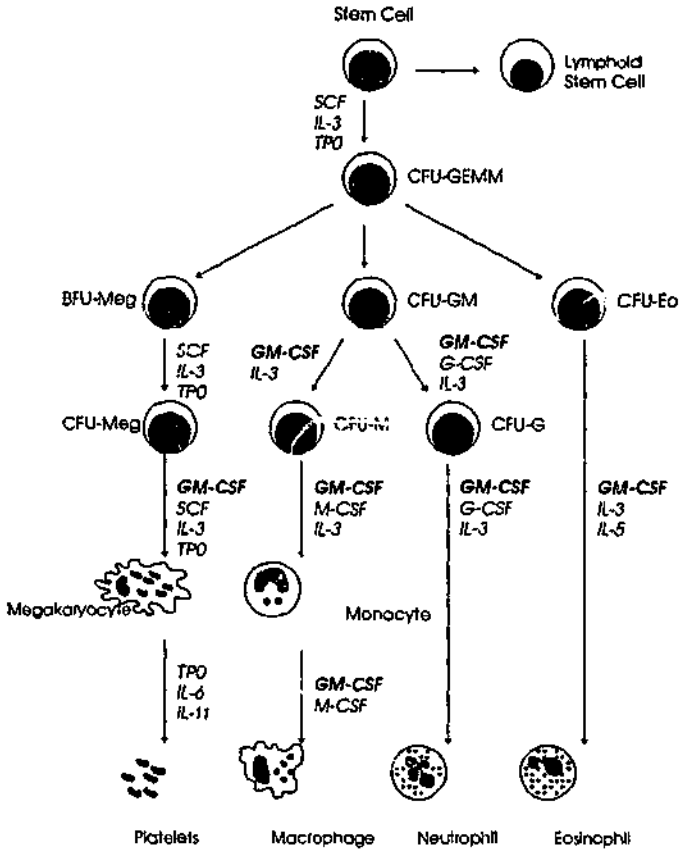

Fig. 1. GM-CSF as a multipotential hemopoietic growth factor that regulates cells of eosinophilic, neutrophilic, monocytic and megakaryocytic lineages (BFU: burst-forming unit; CFU: colony-forming unit; Eo: eosinophil; GEMM: granulocyte, erythrocyte macrophage, megakaryocyte; G: granulocyte; GM: granulocyte, macrophage; M: monocyte; MM: monocyte, macrophage; SCF: stem cell factor; TPO: thrombopoietin). This figure has been modified from the work of Roitt et al., 1993 with permission.

activity. Using site-directed mutagenesis it has been determined that Glu21 in helix A (Lopez et al., 1992) and Asp101 in helix D (Hercus et al., 1994a,b) are critical for the full biological activity of GM-CSF. These residues have been postulated to bind to the separate subunits of the GM-CSF receptor (Hercus et al., 1994a,b).

The human GM-CSF receptor has been cloned and comprises a low affinity α chain (2.6 nM) and a β chain which provides a high affinity binding when co-expressed with α chain, increasing the binding affinity to the pM range (Gearing et al., 1989; Hayashida et al., 1990). The β chain of GM-CSF receptor is shared with IL-3 and IL-5 receptors and does not appear to bind GM-CSF by itself. Recently, it has been demonstrated that E21R, a mutant form of GM-CSF binding selectively to the α chain of GM-CSF receptor and behaving as a competitive GM-CSF antagonist, induces apoptosis of primary hemopoietic cells (Iversen et al., 1996). These results suggest that engaging of the β chain is important for cell survival, which may have important implications for our understanding of cytokine receptor function.

3. The effect of GM-CSF on HIV-1 replication in vivo

Clinical trials in HIV-seronegative patients have indicated that recombinant GM-CSF can shorten the duration of chemotherapy-induced neutropenia in persons receiving chemotherapy for various malignancies (Antman et al., 1988) and can accelerate myeloid recovery after chemotherapy and autologous bone marrow transplantation (Brandt et al., 1988; Nemunaitis et al., 1988). GM-CSF has also been demonstrated to significantly increase the number of circulating neutrophils in patients with HIV-associated neutropenia (Groopman et al., 1987). However, several studies have shown that recombinant GM-CSF administrated subcutaneously to patients with HIV-1 infection at any stage of disease, without any antiretroviral therapy, can increase HIV-1 activity, as assessed by serum p24 antigen levels and plasma HIV RNA titres (Kaplan et al., 1991; Lafeuillade et al., 1996). The observed effect of GM-CSF can be ameliorated by addition of effective antiretroviral therapy (Yarchoan et al., 1990; Krown et al., 1992). Of particular relevance was the observation that although GM-CSF administrated to HIV-seropositive patients without any antiretroviral therapy induced HIV-1 replication (Kaplan et al., 1991), when HIV-1 infected monocytes/macrophages were exposed to GM-CSF it also increased the sensitivity of infected cells to antiretroviral drugs such as zidovudine (Perno et al., 1989).

4. The effect of GM-CSF on HIV-1 replication in vitro

4.1. Increased HIV-1 replication in promonocytic cell lines and primary monocyte cultures

Most of the published studies suggest that GM-CSF exerts an upregulatory effect on HIV-1 infection and replication in both monocyte-derived cell lines and in primary monocyte cultures (Poli and Fauci, 1992). The chronically infected promonocytic cell line, U937 and U937-derived clone, U1, characterised by two integrated copies of proviral HIV-1 and minimal constitutive expression of HIV-1, have often been used as an in vitro model of monocyte infection (Petit et al., 1987). Folks et al. (1987), when studying the expression of HIV-1 induced by various cytokines, found that GM-CSF enhanced HIV-1 replication of the U1 clone by approximately 100%. In this study HIV-1 expression of U1 cells exposed to GM-CSF (10 U per 5×10^5 cells) for 48 h was assessed by reverse transcription (RT) assay and compared to control cells not treated with GM-CSF. Other investigators, using the same clone, found a 27-fold increase in HIV-1 p24 antigen level following 72 h treatment with GM-CSF at concentration 500 U/ml (Pomerantz et al., 1990). Furthermore, they have also shown that GM-CSF enhanced by 200-fold the lipopolysaccharide-induced expression of HIV-1 in U1 cells. The stimulatory effects of GM-CSF on HIV-1 production resulted in increased levels of HIV-1 specific RNA.

Most of the studies reported the effects of various growth factors and cytokines on HIV-1 replication in primary peripheral blood derived monocytes and tissue macrophages. Koyanagi et al. (1988) were one of the first to report that a number of

cytokines including colony stimulating factors, interleukins and interferons can alter HIV-1 replication in macrophages infected with HIV-1. They have shown that GM-CSF treatment of primary peripheral blood monocytes infected with HIV-1 enhanced the level of HIV-1 replication from 4 to 90 times (varying among the different donors) as assessed by quantifying supernatant p24 antigen concentrations in culture supernatants. Neutralising antiserum to GM-CSF could inhibit the induction of HIV-1 replication by GM-CSF suggesting that the observed effect was GM-CSF-specific. Work by a number of other groups, has shown similar results to those obtained by Koyanagi et al. Other investigators have consistently observed augmentation of HIV-1 replication in primary monocyte/macrophage culture stimulated by GM-CSF (Perno et al., 1989; Schuitemaker et al., 1990; Perno et al., 1992). Using different experimental protocols they have reported increases in replication of HIV-1_{Ba-L} in cultured monocytes exposed to GM-CSF (100 U/ml) for 5 days prior to HIV-1 infection. These investigators found a 2- to 30-fold augmentation in HIV-1 replication in cultures pretreated with GM-CSF (or IL-3, which shares a common β receptor with GM-CSF) when compared to the unstimulated controls. Similarly, using alveolar macrophages as representative of human tissue macrophages and a strain of HIV-1 that replicates well in these cells, a significant increase in HIV-1 replication in the presence of GM-CSF has been reported (Denis and Ghadirian, 1994). These increase effect could be abrogated by other cytokines including IL-4 and IL-13.

4.2. Suppression of HIV-1 replication in primary monocyte cultures

Whilst the majority of studies provide evidence of augmentation in HIV-1 replication, there are also reports that GM-CSF may actually suppress HIV-1 replication in monocyte-derived macrophages (Matsuda et al., 1995) or that it can cause inconsistent effects on HIV-1 expression. Matsuda et al. found that replication of HIV-1_{PAR} (a macrophage-tropic strain) in primary cultured macrophages exposed to GM-CSF was suppressed by 100-fold in contrast to HIV-1 production in macrophages incubated in the presence of M-CSF. In this study GM-CSF was added to macrophages 7 days after the isolation of monocytes, and immediately following infection with HIV-1. Our studies to date also suggest that GM-CSF causes downregulation of HIV-1_{Ba-L} replication in cells of macrophage lineage (unpublished data).

4.3. No augmentation or suppression of HIV-1 replication in monocyte-derived macrophages and alveolar macrophages

Using alveolar macrophages obtained by bronchoalveolar lavage from HIV-seronegative volunteers, Hammer et al. (1990) have investigated the role of GM-CSF on HIV-1 expression in these cells. Pretreatment of macrophages with GM-CSF (30 to 300 U/ml) for 48 to 72 h prior to HIV-1_{IIIB} infection and maintenance of the cytokine in the cultures neither had an inhibitory nor stimulatory effect on HIV-1 replication. There have been two additional studies showing that GM-CSF treatment of HIV-1 infected macrophages may give inconsistent results (Kornbluth et al., 1989; Novak et al., 1990). Novak et al., when investigating the effects of GM-CSF pretreatment of monocyte-de-

Table 1
The effect of GM-CSF on HIV-1 replication in macrophages

Reference	Cells	Strain of HIV-1	Effect on HIV-1	Source of GM-CSF	Protocol	Method of detection
Increase in HIV-1						
Folks et al., 1987	U1/U937	–	100 fold increase	Immunex 10 U/5×10^5 cells	48 h	RT activity
Koyanagi et al., 1988	MDM	JR–FL	4–90 fold increase	Unknown 100 pM–1 nM	1 day post-HIV	HIV p24 ELISA
Schuitemaker et al., 1990	MDM	Ba–L	2–5 fold increase	Sandoz 100 U/ml	5 days pre-HIV and post-HIV	HIV p24 ELISA
Perno et al., 1989	MDM	Ba–L	5–10 fold increase	Sandoz, Genetics Institute; 100 U/ml	5 days pre-HIV and post-HIV	HIV p24 ELISA
Perno et al., 1992	MDM	IIIB	7–30 fold increase	Sandoz 100 U/ml	5 days pre-HIV and post-infection	HIV p24 ELISA
Decrease in HIV-1						
Matsuda et al., 1995	MDM	Primary isolate	decrease compared to M-CSF	Schering, Plough, Kirin	7 days pre-HIV, and post-HIV	RT activity
No effect on HIV-1						
Kornbluth et al., 1989	MDM	Ba–L	Variable	Genzyme, Genetics Institute; 1–10,000 U/ml	18 h pre-HIV, and post-HIV	HIV p24 ELISA
Hammer et al., 1990	AM	IIIB	No increase/ decrease	Genetics institute; 30 U/ml	2–3 days pre-HIV, and post-HIV	RT activity
Novak et al., 1990	MDM	IIB	Variable	Endogen	5 days pre-HIV	HIV p24 ELISA

MDM: monocyte-derived macrophages.
AM: alveolar macrophages.

rived macrophages on HIV-$1_{HTLV\text{-}IIIB}$ replication, obtained variable results amongst different donors. Kornbluth et al. (1989) also reported inconsistent results showing that GM-CSF at concentrations of 1–10,000 U/ml partially protected macrophages from HIV_{Ba-L} infection in two experiments, had no effect in two other experiments, and actually upregulated HIV-1 replication in one experiment. It has been suggested that factors such as the culture conditions, source and concentration of HIV-1, frequency of replenishment of GM-CSF into the culture, HIV-1 strain as well as the stage of monocyte/macrophage maturation at the time of HIV-1 infection or cytokine exposure may contribute to such variations (Table 1).

5. Discussion

The published data suggest that GM-CSF upregulates HIV-1 expression in both primary cultured monocyte/macrophages and in promonocytic cell. However, the effects of GM-CSF on HIV-1 replication in these cells remains controversial and the mechanism by which GM-CSF might cause this effect has not been elucidated.

There are a few possible mechanisms by which GM-CSF, as a hemopoietic growth factor stimulating the differentiation and proliferation of cells of macrophage lineage might increase HIV-1 replication in these cells. Several groups have reported that both GM-CSF and M-CSF induce proliferation of a small fraction (3 to 5%) of monocyte/macrophages in vitro as determined by experiments measuring tritiated thymidine incorporation (Elliott et al., 1989; Schuitemaker et al., 1990). Walsh et al. (1991) using a macaque model, observed that GM-CSF enhanced the amount of simian immunodeficiency virus (SIV) gag protein in the culture supernatant (three- to six-fold increase), but at the same time this cytokine also significantly increased the number of simian alveolar macrophages. However, after adjusting for differences in total cell number, GM-CSF did not increase SIV replication on a per-cell basis at any of the concentrations tested, emphasising the importance of considering the total number of cells within a culture when assessing virus expression. They demonstrated that the quantification of virus in culture supernatants may not actually reflect changes in the virus replication on a per cell basis.

By stimulating the differentiation of monocytes/macrophages, GM-CSF may actually increase susceptibility of these cells to HIV-1 infection. It has been shown by several investigators that freshly isolated peripheral blood monocytes are relatively resistant to HIV-1 infection, whereas cultured monocyte-derived macrophages and tissue macrophages (such as bronchoalveolar macrophages) are fully permissive to HIV-1 infection (Rich et al., 1992; Sonza et al., 1996). Thus the maturation from monocytes to macrophages appears to correlate with increased susceptibility to HIV-1 infection. Therefore, differential regulation of HIV-1 replication by GM-CSF at various stages of monocyte maturation may explain observed increases in HIV-1 expression in cells of macrophage lineage.

In addition, GM-CSF may affect HIV-1 replication in monocyte-derived macrophages through stimulation of the binding of host-cell transcriptional factors to the HIV-1 LTR resulting in its transactivation and thus increasing HIV-1 transcription (Nabel and

Baltimore, 1987). Although this mechanism is not entirely clear, it is known that the enhanced expression of HIV-1 following pretreatment of macrophages with GM-CSF does not act through the NF-κB binding site of HIV-1 LTR. The region responsible for GM-CSF activation in cells of macrophage lineage was shown to be located slightly upstream and partially overlapping the NF-κB binding site (Zack et al., 1990).

6. GM-CSF and HIV-1 pathogenesis: summary and conclusions

Although the effects of GM-CSF on HIV-1 replication in cells of macrophage lineage in vitro are still controversial, most of the published studies have demonstrated that GM-CSF upregulates HIV-1 expression in both primary cultured macrophages and monocytic cell lines. The possible mechanism of augmentation of HIV-1 replication in these cells may involve GM-CSF-enhanced differentiation or proliferation of macrophages as well as increased production of host-cell transcriptional factors. In vivo, GM-CSF provides clinical benefit only in the presence of antiretroviral therapy, as administration of the cytokine alone to HIV-positive individuals increases HIV-1 activity in monocytes/macrophages similarly to that observed in vitro. The effects of GM-CSF on HIV-infected macrophage function remains enigmatic.

References

Antman, K.S., Griffin, J.D., Elias, A., 1988. Effect of recombinant human granulocyte–macrophage colony-stimulating factor on chemotherapy-induced myelosuppression. New Engl. J. Med. 319, 593–598.

Barre-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W., Montagnier, L., 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 22, 868–871.

Bermudez, L.E., 1994. Potential role of cytokines in disseminated mycobacterial infections. Eur. J. Clin. Microbiol. Infect. Dis. Suppl. 2, S29–S33.

Brandt, S.J., Peters, W.P., Buckner, C.D., 1988. Effect of recombinant human granulocyte–macrophage colony-stimulating factor on hematopoietic reconstitution after high dose chemotherapy and autologous bone marrow transplantation. New Engl. J. Med. 318, 869–876.

Bratton, D.L., Hamid, Q., Boguniewich, M., Doherty, D.E., Kailey, J.M., Leung, D.Y., 1995. Granulocyte–macrophage colony-stimulating factor contributes to enhanced monocyte survival in chronic atopic dermatitis. J. Clin. Invest. 95, 211–218.

Brown, C.B., Hart, C.E., Curtis, D.M., Bailey, M.C., Kaushansky, K., 1990. The neutralizing monoclonal antibodies against human granulocyte–macrophage colony-stimulating factor recognize binding domain of the molecule. J. Immunol. 144, 2184–2189.

Clark-Lewis, I., Lopez, A.F., To, L.B., Vadas, M.A., Schrader, J.W., Hood, L.E., Kent, S.B.H., 1988. Structure–function studies of human granulocyte–macrophage colony-stimulating factor. Identification of residues required for activity. J. Immunol. 141, 881–889.

Crowe, S.M., Mills, J., McGrath, M.S., 1987. Quantitative immunocytofluorographic analysis of CD4 surface antigen expression and HIV infection of human peripheral blood monocytes/macrophages. AIDS Res. Hum. Retroviruses 3, 135–145.

Denis, M., Ghadirian, E., 1994. Interleukin 13 and interleukin 4 protect bronchoalveolar macrophages from productive infection with human immunodeficiency virus type 1. AIDS Res. Hum. Retroviruses 10, 795–802.

Diederichs, K., Boone, T., Karplus, P.A., 1991. Novel fold and putative receptor binding site of granulocyte–macrophage colony-stimulating factor. Science 254, 1779–1782.

Eischen, A., Vincent, F., Bergerat, J.P., Louis, B., Faradji, A., Bohbot, A., Oberling, F., 1991. Long term cultures of human monocytes in vitro, impact of GM-CSF on survival and differentiation. J. Immunol. Meth. 143, 209–221.

Elliott, M.J., Vadas, M.A., Eglinton, J.M., Park, L.S., To, L.B., Cleland, L.G., Clark, S.C., Lopez, A.F., 1989. Recombinant human interleukin-3 (IL-3) and granulocyte–macrophage colony-stimulating factor (GM-CSF) show common biological effects and binding char-acteristics on human monocytes. Blood 74, 2349–2359.

Folks, T.M., Justement, J., Kinter, A., Dinarello, C.A., Fauci, A.S., 1987. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. Science 238, 800–802.

Gallo, R.C., Salahuddin, S.Z., Popovic, M., Shearer, G.M., Kaplan, M., Haynes, B.F., Palker, T.J., Redfield, R., Oleske, J., Safai, B., White, F., Foster, R., Markham, P.D., 1984. Frequent detection and isolation of cytopathic retroviruses detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Scienc ' 224, 500–503.

Gartner, S., Markovits, P., Markovitz, D.M., Kaplan, M.H., Gallo, R.C., Popovic, P., 1986. The role of mononuclear phagocytes in HTLV-III/LAV infection. Science 223, 215–219.

Gearing, D.P., King, J.A., Gough, N.M., Nicola, N.A., 1989. Expression cloning of a receptor for human granulocyte–macrophage colony stimulating factor. EMBO J. 8, 3667–3676.

Groopman, J.E., Mitsuyasu, R.T., DeLeo, M.J., Oette, D.H., Golde, D.W., 1987. Effect of recombinant granulocyte–macrophage colony-stimulating factor on myelopoiesis in the Acquired Immunodeficiency Syndrome. New Engl. J. Med. 317, 593–598.

Hammer, S.M., Gillis, J.M., Pinkston, P., Rose, R.M., 1990. Effect of zidovudine and granulocyte–macrophage colony-stimulating factor on human immunodeficiency virus replication in alveolar macrophages. Blood 75, 1215–1219.

Hayashida, K., Kitamura, T., Gorman, D.M., Arai, K., Yokota, T., Miyajima, A., 1990. Molecular cloning of a second subunit of the receptor for human granulocyte–macrophage colony stimulating factor (GM-CSF): reconstitution of a high affinity GM-CSF receptor. Proc. Natl. Acad. Sci. USA. 87, 9655–9659.

Hercus, T.R., Cambareri, B., Dottore, M., Woodcock, J., Bagley, C.J., Vadas, M.A., Shannon, M.F., Lopez, A.F., 1994a. Identification of residues in the first and fourth helices of human granulocyte–macrophage colony-stimulating factor involved in biologic activity and in binding to the alpha- and beta-chains of its receptor. Blood 83, 3500–3508.

Hercus, T.R., Bagley, C.J., Cambareri, B., Dottore, M., Woodcock, J.M., Vadas, M.A., Shannon, F., Lopez, A.F., 1994. Specific human granulocyte–macrophage colony-stimulating factor antagonists. Proc. Natl. Acad. Sci. U.S.A., Vol. 91, 5828–5842.

Iversen, P.O., Lo, L.B., Lopez, A.F., 1996. Apoptosis of hemopoietic cells by the human granulocyte macrophage colony-stimulating factor mutant E21R. Proc. Natl. Acad. Sci. USA. 93, 2785–2789.

Kaplan, L.D., Kahn, J.O., Crowe, S., Northfelt, D., Neville, P., Grossberg, H., Abrams, D.I., Tracey, J., Mills, J., Volberding, P.A., 1991. Clinical and virological effects of recombinant human granulocyte–macrophage colony-stimulating factor in patients receiving chemotherapy for human immunodeficiency virus-associated non-Hodgkin's lymphoma: results of randomized trial. J. Clin. Oncol. 9, 929–940.

Kornbluth, R.S., Oh, P.S., Munis, J.R., Cleveland, P.H., Richman, D.D., 1989. Interferons and bacterial lipopolysaccharide protect macrophages from productive infection. J. Exp. Med. 169, 1137–1151.

Koyanagi, Y., O'Brien, W.A., Zjao, J.Q., Golde, D.W., Gasson, J.C., Chen, I.S.Y., 1988. Cytokines alter production of HIV-1 from primary mononuclear phagocytes. Science 241, 1673–1675.

Krown, S.E., Pardes, J., Bundow, D., Polsky, B., Gold, J.W.M., Homenberg, N., 1992. Interferon-alpha, zidovudine and granulocyte macrophage colony stimulating factor: a phase I AIDS clinical trials group study in patients with Kaposi's Sarcoma associated with AIDS. J. Clin. Oncol. 10, 1344–1351.

Lafeuillade, A., Poggi, C., Tamalet, C., 1996. GM-CSF increases HIV-1 load. Lancet 347, 1123–1124.

Laursen, A.L., Moller, B., Rungby, J., Petersen, C.M., Andersen, P.L., 1994. *Pneumocystis carinii*-induced activation of the respiratory burst in human monocytes and macrophages. Clin. Exp. Immunol. 98, 196–198.

Lopez, A.F., Shannon, M.F., Hercus, T., Nicola, N.A., Cambareri, B., Dottore, M., Layton, M.J., Ellinton, L., Vadas, M.A., 1992. Residue 21 of human granulocyte–macrophage colony-stimulating factor is critical for biological activity and for high but not low affinity binding. EMBO J. 11, 909–916.

Matsuda, S., Akagawa, K., Honda, M., Yokota, Y., Takebe, Y., Takemori, T., 1995. Suppression of HIV replication in human monocyte-derived macrophages induced by granulocyte/macrophage colony-stimulating factor. AIDS Res. Hum. Retroviruses 11, 1031–1038.

McUrath, M.J., Pruett, J.E., Cohn, Z.A., 1989. Mononuclear phagocytes of blood and bone marrow: comparative roles as viral reservoirs in human immunodeficiency virus type 1 infections. Proc. Natl. Acad. Sci. USA. 86, 675–679.

Metcalf, D., 1985. The granulocyte–macrophage colony-stimulating factors. Science 229, 16–21.

Nabel, G., Baltimore, D., 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature 326, 711–713.

Nemunaitis, J., Singer, J.W., Buckner, C.D., 1988. Use of recombinant human granulocyte macrophage colony-stimulating factor in autologous marrow transplantation for lymphoid malignancies. Blood 72, 834–836.

Novak, R.M., Holzer, T.J., Kennedy, M.M., Heynen, C.A., Dawson, G., 1990. The effect of interleukin 4 (BSF-1) on infection of peripheral blood monocyte-derived macrophages with HIV-1. AIDS Res. Hum. Retroviruses 8, 973–976.

Parhar, R.S., Ernst, P., Sheth, K.V., al-Sedairy, S.T., 1992. Anti-tumor cytotoxic potential and effect on human bone marrow GM-CFU of human LAK cells generated in response to various cytokines. Eur. Cytokine Netw. 3, 299–306.

Perno, C.F., Yarchoan, R., Cooney, D.A., Hartman, N.R., Webb, D.S., Hao, Z., Mitsuya, H., Dohns, D.G., Broder, S., 1989. Replication of human immunodeficiency virus in monocytes. Granulocyte/macrophage colony-stimulating factor (GM-CSF) potentiates viral production yet enhances the antiviral effect mediated by 3'-azido-2'3'-dideoxythymidine. J. Exp. Med. 169, 933–951.

Perno, C.F., Cooney, D.A., Gao, W.Y., Hao, Z., Johns, D.G., Foli, A., Hartman, N.R., Calio, R., Broder, S., Yarchoan, R., 1992. Effects of bone marrow stimulatory cytokines on human immunodeficiency virus replication and the antiviral activity of dideoxynucleosides in cultures of monocyte/macrophages. Blood 80, 995–1003.

Petit, A.J., Terpstra, F.G., Meidema, J., 1987. Human immunodeficiency virus infection down-regulates HLA class II expression and induces differentiation in promonocytic U937 cells. J. Clin. Invest. 79, 1883.

Poli, G., Fauci, A.S., 1992. The effect of cytokines and pharmacological agents on chronic HIV infection. AIDS Res. Hum. Retroviruses 9, 191–197.

Pomerantz, R.J., Feinberg, M.B., Trono, D., Baltimore, D., 1990. Lipopolysaccharide is a potent monocyte/macrophage-specific stimulator of human immunodeficiency virus type 1 expression. J. Exp. Med. 172, 253–261.

Rich, E.A., Chen, I.S.Y., Zack, J.A., Leonard, M.L., O'Brien, W.A., 1992. Increased susceptibility of differentiated mononuclear phagocytes to productive infection with human immunodeficiency virus-1 (HIV-1). J. Clin. Invest. 89, 176–183.

Roitt, I.M., Brostoff, J., Male, D.K., 1993. Immunology. 3rd edn. Mosby, UK.

Salahuddin, S.Z., Rose, R.M., Groopman, J.E., Markham, P.D., Gallo, R.C., 1986. Human T lymphotropic virus type III infection of human alveolar macrophages. Blood 68, 281–284.

Schuitemaker, H., Kootstra, N.A., van Oers, M.H.J., van Lambalgen, R., Tersmette, M., Miedema, F., 1990. Induction of monocyte proliferation and HIV expression by IL-3 does not interfere with anti-viral activity of zidovudine. Blood 76, 1490–1493.

Smith, P.D., Lamerson, C.L., Banks, S.M., Saini, S.S., Wahl, L.M., Calderone, R.A., Wahl, S.M., 1990. Granulocyte–macrophage colony stimulating factor augments human monocyte fungicidal activity for *Candida albicans*. J. Infect. Dis. 161, 999–1005.

Sonza, S., Maerz, A., Uren, S., Violo, A., Hunter, S., Boyle, W., Crowe, S., 1995. Susceptibility of human monocytes to HIV type-1 infection in vitro is not dependent on their level of CD4 expression. AIDS Res. Hum. Retroviruses 11, 769–776.

Sonza, S., Maerz, A., Deacon, N., Meanger, J., Mills, J., Crowe, S., 1996. Human immunodeficiency virus type 1 replication is blocked prior to reverse transcription and integration in freshly isolated peripheral blood monocytes. J. Virol. 70, 3863–3869.

Vadas, M.A., Nicola, N.A., Metcalf, D., 1983. Activation of antibody-dependent cell-mediated cytotoxicity of human neutrophils and eosinophils by separate colony-stimulating factors. J. Immunol. 130, 795–799.

Walsh, D.G., Horvath, C.J., Hansen-Moosa, A., MacKey, J.J., Sehgal, P.K., Daniel, M.D., Desrosiers, R.C.,

Ringler, D.J., 1991. Cytokine influence on simian immunodeficiency virus replication within primary macrophages. Am. J. Pathol. 139, 877–887.

Yarchoan, R., Pluda, J.M., Perno, C.F., Mitsuya, H., Thomas, R.V., Wyvill, K.M., Broder, S., 1990. Initial clinical experience with dideoxynucleosides as single agents and in combination therapy. Ann. New York Acad. Sci. 616, 328–343.

Zack, J.A., Arrigo, S.J., Chen, I.S.Y., 1990. Control of expression and cell tropism of human immunodeficiency virus type 1. Adv. Virus Res. 38, 125–146.

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Research Article

Quantifying complement-mediated phagocytosis by human monocyte-derived macrophages

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Summary The present study demonstrates that SRBC can be opsonized with untreated human serum such that lysis by active complement components is minimal but sufficient opsonization occurs to permit high rates of complement-mediated phagocytosis. Phagocytosis of SRBC opsonized with 2% whole human serum by human monocyte-derived macrophages was quantified in a colourimetric assay. Ingestion of SRBC was shown to occur solely via complement receptors because no phagocytosis was observed when SRBC were coated with heat-inactivated human serum, phagocytosis was augmented by the phorbol ester, PMA, and phagocytosis was inhibited by a protein kinase C (PKC)-specific inhibitor RO 31-8220. This method was used to demonstrate directly that HIV-1 infection of human monocyte-derived macrophages inhibits complement-mediated phagocytosis and will provide a useful tool for pharmacological investigations on complement-mediated phagocytosis by adherent macrophages.

Key words: complement-mediated phagocytosis, HIV-1, human macrophages.

Introduction

The complement (C′) system, a phylogenically ancient component of the immune system, is very important in defence against bacteria.[1,2] Genetic defects resulting in deficiencies in specific C′ proteins have been linked to increased susceptibility to infections caused by bacteria such as *Neisseria meningitidis*.[3–5] Derivatives of C′ proteins, C3 and C4, are important opsonins for phagocytosis by macrophages,[6] and macrophage surface receptors that take up particles coated with these cleavage products include CR1, CR3 and CR4.[7–10] CR1 is a single transmembrane receptor, whereas CR3 and CR4 are integrin heterodimers with a common β2 chain.[7,9,10] These receptors have different affinities for different C′ products and they are thought to collaborate in mediating the uptake of C′-opsonized particles.[11,12]

C′-mediated phagocytosis has assumed increasing importance in the era of HIV/AIDS, as the AIDS-related opportunistic pathogens *Mycobacterium tuberculosis* and *Mycobacterium avium* complex are phagocytosed by macrophages predominantly via the C′ pathway.[13,14] Our observations that phagocytosis of *M. avium* complex is impaired by macrophages infected with HIV-1 *in vitro* and *in vivo*[15] suggest a defect in C′-mediated phagocytosis in macrophages as a result of HIV infection. C′ can also facilitate phagocytosis of other mycobacteria, *Legionella pneumophilia, Salmonella typhi* and *Staphylo-coccus aureus*.[16–19]

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Studies into C′-mediated phagocytosis by macrophages have been hampered by a lack of easy procedures for opsonization of target particles and convenient methods to quantify phagocytosis that can distinguish attached and phagocytosed particles. Common assays for investigation of phagocytosis by macrophages via C′ receptors usually involve tedious microscopic evaluation of internalized C′-coated targets.[20–22] Furthermore, the conventional coating protocols and targets used cannot simultaneously enable easy opsonization and convenient discrimination between attached and internalized particles.

In the present study, we describe a simple procedure using 2% human serum for C′-opsonizing SRBC as targets for phagocytosis, which can then be quantified by a modified colourimetric assay that is specific for ingestion via C′ receptors. We further show, by use of this assay, that HIV-infected monocyte-derived macrophages are defective in their ability to phagocytose complement-opsonized SRBC.

Materials and Methods

Isolation and culture of human monocytes

Human peripheral blood monocytes were isolated from buffy packs of HIV-1, hepatitis B virus (HBV), hepatitis C virus (HCV), human T-cell leukaemia virus (HTLV) and syphilis seronegative blood donors (purchased from the Red Cross Blood Bank, Melbourne, Vic., Australia) using Ficoll-Paque density gradient centrifugation followed by plastic adherence as described elsewhere.[23] Immediately after isolation, cell viability was greater than 95% as assessed by Trypan blue exclusion. The purity of monocytes was greater than 85% as determined by immunofluorescent staining with anti-CD14 mAb conjugated directly to phycoerytherin (CD14-PE; Becton

Dickinson, San Jose, CA, USA) and assessed by flow cytometry using a FACS Star Plus flow cytometer (Becton Dickinson). Monocytes were resuspended and cultured in Iscove's medium (GibcoBRL, Life Technologies, NY, USA) supplemented with 10% heat-inactivated human AB-positive serum, 2 mmol/L L-glutamine (GibcoBRL) and 24 μg/mL gentamicin (Delta West, Bentley, WA, Australia) at a concentration of 0.5–1 × 10^6 cells/mL in polytetrafluorethylene (Teflon) jars (Savillex, Minnetonka, MO, USA).

Preparation of C'-opsonized sheep red blood cells

Sheep red blood cells (2 × 10^9) (ICN-Cappel, Aurora, OH, USA) were washed three times in cold PBS (1500 *g*, 7 min), centrifuged and opsonized for 30 min at room temperature immediately prior to the phagocytosis assay using prewarmed (37°C) 2% AB-negative untreated human whole serum (NHS) from HIV-negative donors (a source of human C' components). To terminate opsonization, SRBC were pelleted by centrifugation at 660 *g* for 10 min at 4°C. Supernatant was collected and the concentration of released haemoglobin (Hb) was measured at 540 nm to determine the lysis of the SRBC resulting from destruction by C' components. Sheep red blood cells were then washed three times in cold PBS and resuspended at a concentration of 1 × 10^8/mL. Sheep red blood cells used as controls were prepared using the same procedures but using heat-inactivated serum (iNHS); iNHS was derived from serum prepared from the same donor, heat-inactivated at 56°C for 45 min (iNHS) and clarified by centrifugation (20 000 *g*, 10 min). Freshly prepared NHS and iNHS were aliquoted into microcentrifuge tubes, typically in a volume of 50–100 μL, snap-frozen in liquid nitrogen and stored at −80°C. Serum stored in this manner could be used for up to 12 months after preparation.

For inhibition studies of Fc_γR-mediated phagocytosis, SRBC were opsonized with IgG by incubation with rabbit antiserum to SRBC (ICN Cappel) at a dilution of 1 : 300 for 30 min at room temperature.

Gel electrophoresis and western blotting

For analysis of C3 derivatives (major C' opsonins) on opsonized SRBC preparations, SRBC samples were lysed in cold 0.2% NaCl (Merck, Kilsyth, Vic., Australia) solution, and SRBC membranes pelleted by centrifuging at 20 800 *g* for 5 min at 4°C. The SRBC membrane fragments were washed once with cold 0.2% NaCl solution and the pellet was boiled in SDS sample buffer (10 mmol/L Tris pH 8.0, 2 mmol/L EDTA, 1% SDS, 5% β-mercaptoethanol, 5% glycerol) at 95°C. Samples were analysed by SDS-PAGE using the discontinuous buffer system at 100 V. Proteins were transferred to nitrocellulose (Amersham International, Buckinghamshire, UK) at 70 V for 2 h. The nitrocellulose filters were blocked in 5% skim milk in Tris-buffered saline containing 0.3% Tween-20 (Astral, Gymea, NSW, Australia) for 2 h at 37°C and then probed with goat polyvalent antiserum against human C3 (ICN-Cappel) at 4°C for 16 h. Following five washes in 1 × TBST, blots were probed with a rabbit-antigoat antibody conjugated with horseradish peroxidase (Dako, Carpinteria, CA, USA), followed by three washes in 1 × TBST, and developed for enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham International).

C'-mediated phagocytosis assay

On days 5–14 of postisolation, monocyte-derived macrophages (MDM) were adhered in quadruplicate wells onto flat-bottomed 96-well plates at 5 × 10^4 cells/well in 200 μL of supplemented Iscove's medium for 24 h at 37°C in a 5% CO_2 humidified incubator. The volume of the medium was adjusted to 85 μL before the addition of 5 μL of phorbol-12-myristate-13-acetate (PMA; Sigma) in 10% dimethylsulfoxide (Merck) (final concentration of PMA of 200 nmol/L per well) for 10 min prior to the assay. Phagocytic targets (SRBC coated with NHS or iNHS) were added to MDM at the ratio of 20 SRBC to 1 MDM and centrifuged at 57 *g* for 5 min at 4°C onto the adherent MDM. The plate was placed at 37°C in 5% CO_2 for phagocytosis to proceed. Phagocytosis was terminated after 60 min by placing the plate on ice and washing non-phagocytosed SRBC away with cold (4°C) PBS. The amount of internalized SRBC was measured using a colourimetric assay.[24] Briefly, unbound SRBC were washed off the wells with cold PBS, whereas adhered but non-phagocytosed SRBC were lysed with 0.2% NaCl solution for 3 min and removed by washing three times with prewarmed (37°C) Iscove's medium. Monocyte-derived macrophages were lysed with 6 mol/L urea solution containing 0.2 mol/L Tris-HCl buffer (pH 7.4). The number of phagocytosed SRBC was determined by measuring the amount of fluorene blue that had been converted from 2,7-diaminofluorene (Sigma) by the pseudoperoxidase activity of Hb.

Absorbance was determined at 620 nm in an ELISA plate reader (Labsystems, Helsinki, Finland) and corrected against a blank, which contained only 2,7-diaminofluorene solution and urea–Tris buffer. The adjusted absorbance was compared to a standard curve, which was generated for each assay plate using known numbers of SRBC (twofold dilutions in triplicate ranging from 8 × 10^5 to 1 × 10^6 per well) and fitted by four-parameter analysis using a microplate analysis program (Deltasoft 3, BioMetallics Inc., Princeton, NJ, USA). The number of SRBC determined from this standard curve was divided by the number of MDM in the well (5 × 10^4) and multiplied by 100 to obtain the phagocytic index (the number of SRBC phagocytosed per 100 MDM). The data from quadruplicate wells were averaged and expressed as mean ± SD.

Measurement of C'-mediated phagocytosis in HIV-infected monocyte-derived macrophages

Monocyte-derived macrophages prepared from individual donors' monocytes were divided into two and either infected with $HIV\text{-}1_{Ba\text{-}L}$ or mock-infected, as described elsewhere[15] on day 5 of postisolation. Infected cells were cultured for a further 6 days and then plated for 24 h onto 96-well plates for the measurement of C'- and F_c-mediated phagocytosis. Infection was confirmed by the measurement of reverse transcriptase activity in culture supernatants on day 7 of postinfection. Experiments were carried out under strict PC3 containment until the virus was inactivated by the urea lysis step, after which the plates were transferred to the PC2 laboratory for colourimetric determination of released Hb.

Statistical analysis

The significance of the data from various phagocytosis experiments was assessed using a paired Student's *t*-test; significance depended upon a *P*-value of ≤ 0.05, two-tailed.

Results

Sensitivity of the colourimetric assay for sheep red blood cells

In order to quantify the number of SRBC phagocytosed by macrophages, Hb released following lysis of cells was determined by reaction with 2,7-diaminofluorene and the fluorene blue product was measured spectrophotometrically.[24] To determine the relationship between the fluorene blue generated

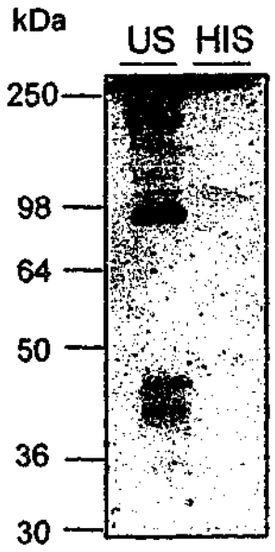

Figure 1 Deposition of C′ opsonins on SRBC targets. Sheep red blood cell ghosts were obtained by lysing SRBC with 0.2% NaCl solution. C3 opsonins were detected by immunoblotting with a polyclonal goat-antiserum against human C3 following enhanced chemiluminescence detection, and were present in SRBC opsonized with untreated serum (US) but not in SRBC samples treated with heat-inactivated serum (HIS).

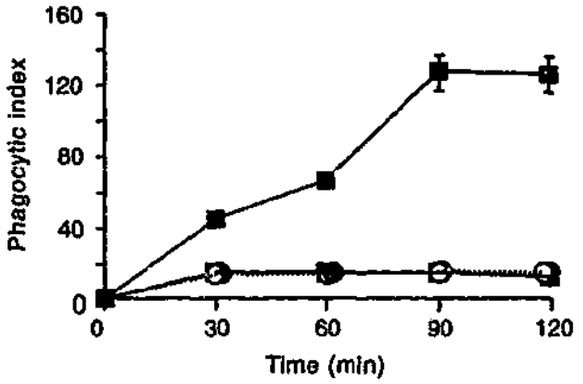

Figure 2 Kinetics of C′-mediated phagocytosis by monocyte-derived macrophages (MDM) measured using the colourimetric assay. Monocyte-derived macrophages were prestimulated with PMA (■,□) or DMSO control (●,○) for 10 min at 37°C. Sheep red blood cells (opsonized with untreated serum (NHS) (■,●), or heat-inactivated serum (iNHS) (□,○)) were added at a MDM to target ratio of 1 : 20, and phagocytosis was allowed to proceed for the times indicated. Phagocytosis was then quantified as described in Materials and Methods. The figure is a representative of three independent experiments. Each data point was performed in quadruplicate and error bars indicate standard deviation. Phagocytic indices from 30 min onwards that were below the lowest point on the standard curve are arbitrarily defined as 15 (the phagocytic index equivalent to the lowest limit of the standard curve).

and the number of SRBC, a standard curve was constructed with SRBC ranging from 8×10^3 to 1×10^6 per well. Under the conditions used, colour development was not saturated up to 4.5×10^5 SRBC per well (data not shown). The range of values that could be read from the standard curve (from the first data point significantly above the background to the point of saturation) equated to a phagocytic index of between 15 and 900 in experiments using 5×10^4 MDM plated per well.

Effect of variables on phagocytosis of sheep red blood cell targets

Conditions for opsonization of targets The $Fc_\gamma R$-mediated phagocytosis assay described by Gebran and coworkers[24] was modified to measure C′-mediated phagocytosis without the requirement of target opsonization by purified C′ components. Untreated whole serum (NHS) was evaluated as a source of C′ components. Various serum concentrations were tested to achieve adequate opsonization with insignificant lysis of the SRBC targets. When MDM were incubated with SRBC for 60 min, a similar extent of phagocytosis of target particles was achieved using targets opsonized at room temperature at final serum concentrations of 2% (phagocytic index ± SD = 389 ± 33.0) and 5% (phagocytic index ± SD = 433 ± 53.1); however, the latter concentration resulted in significantly more lysis of the target SRBC (62% *vs* 14%, respectively). Incubation periods of 30 min, 1 h and 2 h were evaluated with incubation at 4°C and/or room temperature (20–25°C); the optimal incubation conditions were found to be 30 min at room temperature (data not shown). On the basis of these results, the target opsonization parameters were set at 2% serum at room temperature for 30 min. This concentration was subsequently used successfully with AB serum prepared from several individuals, but should be optimized for each serum batch.

Deposition of C′ on SRBC opsonized under the optimal conditions was subsequently demonstrated by the detection of C3 products on SRBC ghosts using SDS-PAGE followed by immunoblotting with polyclonal goat-antiserum against human C3 components. Multiple bands between 40 kDa and 200 kDa (C3 products) were detected on SRBC opsonized with NHS but not iNHS (Fig. 1).

Conditions for phagocytosis assay incubation time for phagocytosis To determine the optimal incubation time for phagocytosis, the kinetics of C′-mediated phagocytosis (0–120 min) by MDM were assessed using a target to MDM ratio of 20 : 1. The number of phagocytosed SRBC, as assessed by the content of internalized Hb, increased as a function of time, reached a maximum by 90 min and plateaued thereafter (Fig. 2). *Adherence time of MDM prior to phagocytosis assay* As macrophage function, including phagocytosis, might be augmented as a result of activation induced by adherence,[25] the effect of altering the adherence period prior to conducting a phagocytic assay was examined. Monocyte-derived macrophages from three donors were adhered to plastic 96-well plates for 24 h prior to phagocytosis assay and compared to control MDM from the same donors adhered for only 2 h (as described for $Fc_\gamma R$-mediated phagocytosis[24]). Adherence for 24 h resulted in higher levels of phagocytosis than 2 h of adherence (86% increase; Fig. 3).

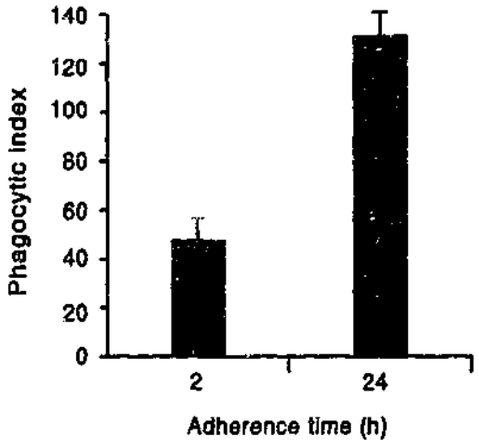

Figure 3 Phagocytic index is increased in monocyte-derived macrophages (MDM) that have adhered for 24 h in comparison to those that have adhered for only 2 h prior to phagocytosis assay. Phagocytosis assays were performed on MDM cultured for 12 days in Teflon pots after their initial isolation and then adhered to plastic for 2 h or 24 h prior to assay. The figure is representative of three independent experiments using cells from three different donors (mean ± SD).

Increasing the adherence time to 48 h and 72 h did not result in higher levels of phagocytosis when compared to 24 h (D Doischer, pers. comm., 2000).

Specificity of C'-mediated phagocytosis assay

Sheep red blood cells treated with NHS as described earlier were verified to be phagocytosed via a C'-mediated pathway. First, phagocytosis was enhanced by PMA, a known activator of C' receptors (Fig. 4a, see also 4b,c). There was no phagocytosis of SRBC targets when the SRBC were treated with iNHS (Fig. 4a–c). In addition, phagocytosis measured by this assay was inhibited by a specific PKC inhibitor, RO 31-8220 (Fig. 4b), but not the tyrosine kinase (PTK)-specific inhibitor, genistein (Fig. 4c). The PKC inhibitor did not inhibit the $Fc_{\gamma}R$-mediated phagocytosis of Ig-opsonized SRBC (Fig. 4d).

Quantification of phagocytosis of C'-opsonized sheep red blood cell targets by monocyte-derived macrophages

C'-mediated phagocytosis by MDM was assessed under the conditions established using cells from eight different donors cultured for a mean of 12 days (range 6–15 days) after isolation. As shown in Table 1, the mean phagocytic index of

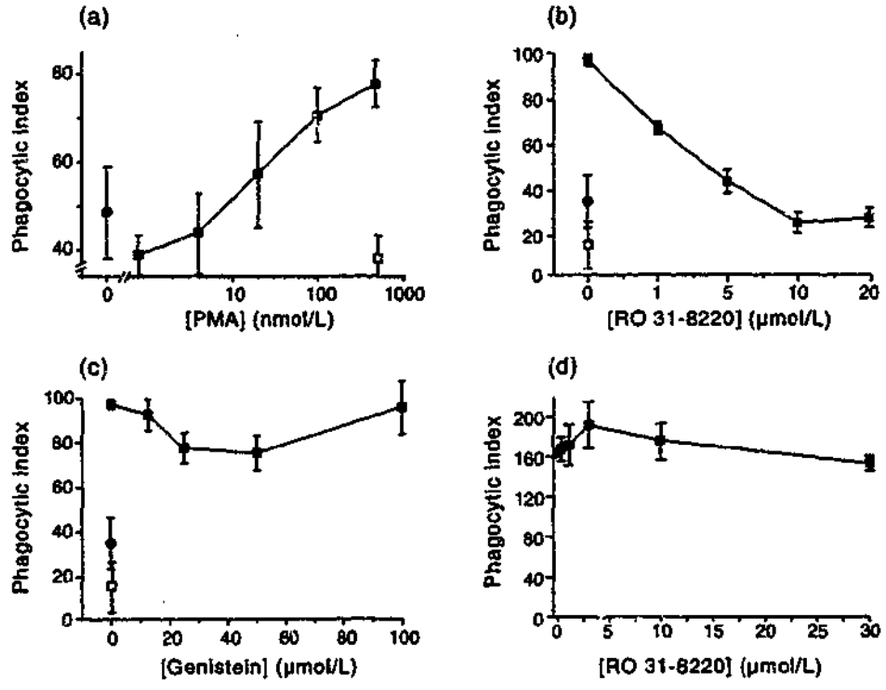

Figure 4 Specificity of C'-mediated phagocytosis assay. (a) Monocyte-derived macrophages (MDM) were pre-incubated with the indicated concentrations of PMA in 0.5% DMSO (■,□ and ●) 10 min prior to the addition of SRBC coated with untreated serum (NHS; ■) or heat-inactivated serum (iNHS; □). Data represent mean ± SD from a single experiment performed in triplicate. For all subsequent experiments, a PMA concentration of 200 nmol/L was used. (b,c) Adhered MDM were treated with varying concentrations of the indicated kinase inhibitors for 10 min. After a further 10 min of incubation with PMA (■,□) or the DMSO vehicle control (●), opsonized SRBC (■,●) or iNHS-treated SRBC (□) were added to the MDM for phagocytosis at 37°C for 1 h. Quantification of ingested SRBC was performed as described in Materials and Methods. Shown here are results (mean ± SD) from one experiment. Each data point was performed in triplicate and error bars indicate standard deviations. (d) Monocyte-derived macrophages were plated for 2 h prior to assay. Immunoglobulin-opsonized SRBC (■) were then added to MDM for phagocytosis at 37°C for 30 min. Quantification of ingested SRBC was performed as described in Materials and Methods. Shown here are representative results (mean ± SD) from three independent experiments.

MDM was 199 ± 59 (mean ± SEM; range, 47–490). For all experiments, iNHS-coated SRBC controls were below or very close to the lower limit of detection of the assay (data not shown).

Inhibition of C′- and Fc-mediated phagocytosis by HIV-1 infection of monocyte-derived macrophages

Monocyte-derived macrophages from three separate donors were infected with HIV-$1_{Ba\text{-}L}$, which is an M-tropic laboratory-adapted strain of HIV-1. Infected and mock-infected cells were then assayed at the same time for their ability to phagocytose either C′-opsonized or IgG-opsonized SRBC. The data show that C′-mediated phagocytosis was strongly inhibited by HIV-1 infection, as was Fc-mediated phagocytosis (Table 2).

Discussion

Phagocytosis assays for monocytes often use non-adherent cells cultured in suspension and flow cytometric assessment of internalization of fluorescently labelled targets.[26,27] Adherent cells may better reflect the *in vivo* physiological environment of macrophages as effector cells. Conventional assays using adherent cells usually involve microscopic analysis of internalized particles,[20–22,28] and investigator bias can diminish their usefulness. The C′-mediated phagocytosis assay reported in the present study employs an adaptation of a colourimetric method described previously by Gebran and coworkers[24] for $Fc_\gamma R$-mediated phagocytosis, eliminating the need for manual microscopic counting, and is highly reproducible and independent of investigator bias. The protocol also enables the measurement of only ingested targets and not attached particles. In addition, a simple and inexpensive method to produce C′-opsonized SRBC as phagocytic targets is provided.

Commonly used targets for measuring C′-mediated phagocytosis are C′-coated zymosan, latex beads and erythrocytes.[21,22,26] Zymosan is resistant to lysis by C′ components and can be opsonized adequately using NHS.[29,30] Latex beads are also resistant to lysis but cannot be fixed directly with C′ and they must be coated by BSA and anti-BSA Ig prior to incubation with serum.[26] Despite the relative ease of opsonization of these lysis-resistant particles, their use in assays is not ideal because attached and engulfed particles cannot be differentiated easily. The extent of attachment must be additionally measured (e.g. by using cytochalasin D to inhibit microtubules required for phagocytosis but not the attachment of particles[26]). Another commonly used procedure is to use fluorescently labelled targets followed by quenching of the attached fluorescent targets using a non-cell permeable quenching agent.[29] However, the reliability of quenching is debatable and, in our experience, these methods may be associated with a high background.[27]

In contrast, attached erythrocytes can be hypotonically lysed under conditions that do not lyse macrophages, thus allowing precise quantification of internalized particles.[20,21,31] As SRBC are susceptible to C′ lysis, the published protocols for opsonizing SRBC use purified C′ components (e.g. C3, factor I, factor H), or sera deficient in one of the later C′ components that constitute the membrane attack complex that is responsible for lysis[20,21] instead of whole serum. The present study reports for the first time conditions that allow opsonization of SRBC by untreated whole serum without generating substantial lysis, a rapid and inexpensive protocol for the production of C′-opsonized targets.

Because C′ is heat labile,[5] iNHS was used to coat SRBC as a negative control. The negligible phagocytosis of iNHS-treated SRBC, as well as the PKC dependency of NHS-opsonized SRBC phagocytosis observed in the present study, confirms the specificity of the assay and demonstrates that opsonization of SRBC by xenoantibodies present in human serum[32] does not result in significant phagocytosis via Fc-mediated pathways under the conditions used here for opsonization and phagocytosis. The dependence of phagocytosis on PMA stimulation (and thus PKC activity) and inhibition with the PKC inhibitor RO 31-8220, but not the tyrosine kinase inhibitor genistein, are in agreement with earlier studies by other investigators showing the dependence

Table 1 C′-mediated phagocytosis by monocyte-derived macrophages

Macrophage donor*	Phagocytic index ± SD
1	490 ± 33
2	131 ± 10
3	47 ± 4
4	61 ± 16
5	113 ± 4
6	286 ± 31
7	386 ± 24
8	81 ± 12
Mean (n = 8) ± SEM	199 ± 59

*Experiments were performed in triplicates using monocyte-derived macrophages from eight different donors.

Table 2 Inhibition of C′- and Fc-mediated phagocytosis by HIV-1 infection of monocyte-derived macrophages

Donor	C′-mediated Mock-infected	C′-mediated HIV-infected	C′-mediated % Inhibition	Fc-mediated Mock-infected	Fc-mediated HIV-infected	Fc-mediated % Inhibition
A	113.4	4.2	96.3	161.3	17.2	89.3
B	285.8	31.4	89.0	268.3	48.1	82.1
C	386.1	23.8	93.8	204.6	7.8	96.2
Mean	261.8	19.8	93.0*	211.4	24.4	89.2**
SEM	79.6	8.1	2.1	31.1	12.2	4.1

*P = 0.00053; **P = 0.0021.

of C'-mediated phagocytosis on PKC activation.[22,33,34] This assay is particularly useful for such pharmacological investigations because the protocol employs 96-well plates, allowing a comparison of C'-mediated phagocytosis of MDM from the same donor with numerous different treatments.

C'-mediated phagocytosis requires C' receptors to be activated. Despite pre-incubation of MDM with PMA to activate C' receptors, the phagocytic index remained variable and low. Because it has been reported that lengthy adherence of MDM to either plastic or glass can activate C' receptors for phagocytosis,[21] the protocol has been modified to adhere cells for 24 h instead of 2 h prior to assay.

We have shown previously that HIV-infection of human MDM impairs their ability to phagocytose *M. avium* complex,[15] implying that complement-mediated phagocytosis is impaired in MDM because *M. avium* complex is principally ingested via a complement-dependent path-way.[13,14] In the present study, we show directly, using specifically opsonized target particles, that complement-mediated phagocytosis is impaired when MDM are infected with HIV-$1_{Ba\text{-}L}$ and that the degree of inhibition is at least as strong as that of Fc-mediated phagocytosis. We are currently using this assay in the laboratory to investigate the signalling pathways involved in C'-mediated phagocytosis by macrophages and the effect of HIV-1 infection on this function. In addition, this assay provides a useful tool for the examination of the effects of therapeutic agents on C'-mediated phagocytosis and, thus, may assist in assessing new adjunctive therapies for restoration of activity against pathogens entering cells via this pathway (e.g. *M. avium* complex) in immunodeficient patients.

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References

1 Ross SC, Densen P. Complement deficiency states and infection: Epidemiology, pathogenesis and consequences of Neisserial and other infections in an immune deficiency. *Medicine* 1984; **63**: 243–73.

2 Figueroa JE, Densen P. Infectious diseases associated with complement deficiencies. *Clin. Microbiol. Rev.* 1991; **4**: 359–95.

3 Ellison III RT, Kohler PF, Curd JG, Judson FN, Reller LB. Prevalence of congenital or acquired complement deficiency in patients with sporadic meningococcal disease. *N. Engl. J. Med.* 1983; **308**: 913–16.

4 Merino J, Rodriguez-Valverde V, Lamelas JA, Riestra JL, Casanueva B. Prevalence of deficits of complement components in patients with recurrent meningococcal infections. *J. Infect. Dis.* 1983; **148**: 331.

5 Nielsen HE, Koch C, Magnussen P, Lind I. Complement deficiencies in selected groups of patients with meningococcal disease. *Scand. J. Infect. Dis.* 1989; **21**: 389–96.

6 Prodinger WM, Würzner R, Erdei A, Dierich MP. Complement. In: Paul WE (ed.). *Fundamental Immunology*. Philadelphia: Lippincott-Raven Publishers, 1999; 967–96.

7 Fearon DT. Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte, and monocyte. *J. Exp. Med.* 1980; **152**: 20–30.

8 Schneider RJ, Kulczycki AJ, Law SK, Atkinson JP. Isolation of a biologically active macrophage receptor for the third component of complement. *Nature* 1981; **290**: 789–92.

9 Wright SD, Rao PE, Van Voorhis WC *et al.* Identification of the C3bi receptor of human monocytes and macrophages by using monoclonal antibodies. *Proc. Natl Acad. Sci. USA* 1983; **80**: 5699–703.

10 Myones BL, Dalzell JG, Hogg N, Ross GD. Neutrophil and monocyte cell surface p150,95 has iC3b-receptor (CR4) activity resembling CR3. *J. Clin. Invest.* 1988; **82**: 640–51.

11 Fallman M, Andersson R, Andersson T. Signaling properties of CR3 (CD11b/CD18) and CR1 (CD35) in relation to phagocytosis of complement-opsonized particles. *J. Immunol.* 1993; **151**: 330–8.

12 Aderem A, Underhill D. Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.* 1999; **17**: 593–623.

13 Schlesinger LS, Bellinger-Kawahara CG, Payne NR, Horwitz MA. Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. *J. Immunol.* 1990; **144**: 2771–80.

14 Bermudez L, Young L, Enkel H. Interaction of *Mycobacterium avium* complex with human macrophages; roles of membrane receptors and serum proteins. *Infect. Immunol.* 1991; **59**: 1679–702.

15 Kedzierska K, Mak J, Mijch A *et al.* Granulocyte-macrophage colony-stimulating factor augments phagocytosis of *Mycobacterium avium* compex by human immunodeficiency virus type 1-infected monocytes/macrophages *in vitro* and *in vivo*. *J. Infect. Dis.* 2000; **181**: 390–4.

16 Payne NR, Horwitz MA. Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. *J. Exp. Med.* 1987; **166**: 1377–89.

17 Schlesinger LS, Horwitz MA. Phagocytosis of *Mycobacterium leprae* by human monocyte-derived macrophages is mediated by complement receptors CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) and IFN-gamma activation inhibits complement receptor function and phagocytosis of this bacterium. *J. Immunol.* 1991; **147**: 1983–94.

18 Ishibashi Y, Arai T. Roles of the complement receptor type 1 (CR1) and type 3 (CR3) on phagocytosis and subsequent phagosome–lysosome fusion in Salmonella-infected murine macrophages. *FEMS Microbiol. Immunol.* 1990; **2**: 89–96.

19 Verbrugh HA, Peterson PK, Nguyen BY, Sisson SP, Kim Y. Opsonization of encapsulated *Staphylococcus aureus*: The role of specific antibody and complement. *J. Immunol.* 1982; **129**: 1681–7.

20 Wright SD, Tobias PS, Ulevitch RJ, Ramos RA. Lipopolysaccharide (LPS) binding protein opsonizes LPC-bearing particles for recognition by a novel receptor on macrophages. *J. Exp. Med.* 1989; **170**: 1231–41.

21 Newman SL, Mikus LK, Tucci MA. Differential requirements for cellular cytoskeleton in human macrophage complement receptor- and Fc receptor-mediated phagocytosis. *J. Immunol.* 1991; **146**: 967–74.

22 Allen LA, Aderem A. Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. *J. Exp. Med.* 1996; **184**: 627–37.

23 Kedzierska K, Crowe SM. Culture of HIV in monocytes and macrophages. In: Coico R (ed.). *Current Protocols in Immunology*. New York: Greene Publishing Associates, Inc. & John Wiley & Sons, Inc., 2001; 12.4.1–12.4.11.

24 Gebran SJ, Romano EL, Pons HA, Cariani L, Soyano AN. A modified colorimetric method for the measurement of phagocytosis and antibody-dependent cell cytotoxicity using 2,7-diaminofluorene. *J. Immunol. Methods* 1992; **151**: 255–60.

25 Newman SL, Devery-Pocius JE, Ross GD, Henson PM. Phagocytosis by human monocyte-derived macrophages: Independent function of receptors for C3b (CR1) and iC3b (CR3). *Complement* 1984; **1**: 213–27.

26 Andoh A, Fujiyama Y, Kitoh K, Hodohara K, Bamba T, Hosoda S. Flow cytometric assay for phagocytosis of human monocytes mediated via Fc gamma-receptors and complement receptor CR1 (CD35). *Cytometry* 1991; **12**: 677–86.

27 Hewish MJ, Meikle AM, Hunter SD, Crowe SM. Quantifying phagocytosis of *Mycobacterium avium* complex by human monocytes in whole blood. *Immunol. Cell Biol.* 1996; **74**: 306–12.

28 Mills J. Effects of sendai virus infection on function of cultured mouse alveolar macrophages. *Am. Rev. Res. Dis.* 1979; **120**: 1239–44.

29 Hed J, Stendahl O. Differences in the ingestion mechanisms of IgG and C3b particles in phagocytosis by neutrophils. *Immunology* 1982; **45**: 727–36.

30 Newman SL, Mikus LK. Deposition of C3b and iC3b onto particulate activators of the human complement system: Quantitation with monoclonal antibodies to human C3. *J. Exp. Med.* 1985; **161**: 1414–31.

31 Pommier C, Inada S, Fries LF, Takahashi T, Frank MM, Brown EJ. Plasma fibronectin enhances phagocytosis of opsonized particles by human peripheral blood monocytes. *J. Exp. Med.* 1983; **157**: 1844–54.

32 Strokan V, Rydberg L, Hallberg EC, Molne J, Breimer ME. Characterisation of human natural anti-sheep xenoantibodies. *Xenotransplantation* 1998; **5**: 111–21.

33 Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 1982; **257**: 7847–51.

34 Wright SD, Silverstein SC. Tumor-promoting phorbol esters stimulate C3b and C3b' receptor-mediated phagocytosis in cultured human monocytes. *J. Exp. Med.* 1982; **156**: 1149–64.

BIBLIOGRAPHY

Achim, C.L., Heyes, M.P. and Wiley, C.A. (1993) Quantitation of human immunodeficiency virus, immune activation factors, and quinolinic acid in AIDS brains. *J Clin Invest*, **91:**2769-2775.

Adams, D.O. and Hamilton, T.A. (1984) The cell biology of macrophage activation. *Annu Rev Immunol*, **2:**283-318.

Adams, D.O. and Hamilton, T.A. (1987) Molecular transductional mechanisms by which IFN gamma and other signals regulate macrophage development. *Immunol Rev*, **97:**5-27.

Aderem, A. (1995) The MARCKS family of protein kinase-C substrates. *Biochem Soc Trans*, **23:**587-591.

Aderem, A. and Underhill, D. (1999) Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol*, **17:**593-623.

Aiken, C. and Trono, D. (1995) Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. *J Virol*, **69:**5048-5056.

Akiyama, Y., Griffith, R., Miller, P., Stevenson, G.W., Lund, S., Kanapa, D.J. and Stevenson, H.C. (1988) Effects of adherence, activation and distinct serum proteins on the *in vitro* human monocyte maturation process. *J Leukoc Biol*, **43:**224-231.

Alkhatib, G., Combadiere, C., Broder, C.C., Feng, Y., Kennedy, P.E., Murphy, P.M. and Berger, E.A. (1996) CC CKR5; a RANTES, MIP-1 alpha, MIP-1 beta receptor as a fusion cofactor for macrophage tropic HIV-1. *Science*, **272:**1955-1958.

Akridge, R.E. and Reed, S.G. (1996) Interleukin-12 decreases human immunodeficiency virus type-1 replication in human macrophage cultures reconstituted with autologous peripheral blood mononuclear cells. *J Infect Dis*, **173:**559-564.

Allen, L.A. and Aderem, A. (1996) Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. *J Exp Med*, **184:**627-637.

Ambrus, J.L., Poiesz, B.J., Lillie, M.A., Stadler, I., Di Berardino, L.A. and Chadha, K.C. (1989) Interferon and interferon inhibitor levels in patients infected with varicella-zoster virus, acquired immunodeficiency syndrome, acquired immunodeficiency syndrome-related complex, or Kaposi's sarcoma, and in normal individuals. *Am J Med*, **87**:405-407.

Amiel, C., Darcissac, E., Truong, M.J., Dewulf, J., Loyens, M., Mouton, Y., Capron, A. and Bahr, G.M. (1999) Interleukin-16 (IL-16) inhibits human immunodeficiency virus replication in cells from infected subjects, and serum IL-16 levels are with disease progression. *J Infect Dis*, **179**:83-91.

Anderson, S.J., Lenburg, M., Landau, N.R. and Garcia, J.V. (1994) The cytoplasmic domain of CD4 is sufficient for its down-regulation from the cell surface by human immunodeficiency virus type 1 Nef. *J Virol*, **68**:3092-3101.

Andreesen, R., Mackensen, A., Osterholz, J., Brugger, W. and Lohr, G.W. (1988) Microculture assay for human macrophage maturation *in vitro*. Cell ELISA analysis of differentiation antigen expression. *Int Arch Allergy Appl Immunol*, **86**:281-287.

Angel, J.B., High, K., Rhame, F., Brand, D., Whitmore, J.B., Agosti, J.M., Gilbert, M.J. and Deresinski, S.C. (2000) Phase III study of granulocyte-macrophage colony-stimulating factor in advanced HIV disease: effect on infections, CD4 cell counts and HIV suppression. *AIDS*, **14**:387-395

Anstey, N.M., Weinberg, J.B., Hassanali, M.Y., Mwaikambo, E.D., Manyenga, D., Misukonis, M., Arnelle, D.R., Hollis, L., McDonald, M.I. and Granger, D.L. (1996) Nitric oxide in Tanzanian children with malaria: inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. *J Exp Med*, **184**:557-567.

Antman, K.S., Griffin, J.D. and Elias, A. (1988). Effect of recombinant human granulocyte-macrophage colony-stimulating factor on chemotherapy-induced myelosuppression. *N Engl J Med*, **319**:593-598.

Araki, N., Johnson, M.T. and Swanson, J.A. (1996) A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J Cell Biol*, **135**:1249-1260.

Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O. and Caroni, P. (1998) Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature*, **393:**805-809.

Armitage, J. (1998) Emerging applications of recombinant human granulocyte-macrophage colony-stimulating factor. *Blood*, **92:**4491-4508.

Arthos, J., Deen, K.C., Chaikin, M.A., Fornwald, J.A., Sathe, G., Sattentau, Q.J., Clapham, P.R., Weiss, R.A., McDougal, J.S., Pietropaolo, C., Axel, R., Truneh, A., Maddon, P. and Sweet, R. (1989) Identification of the residues in human CD4 critical for the binding of HIV. *Cell*, **57:**469-481.

Astier, A., Avraham, H., Manie, S.N., Groopman, J., Canty, T., Avraham, S. and Freedman, A.S. (1997) The related adhesion focal tyrosine kinase is tyrosine-phosphorylated after beta1-integrin stimulation in B cells and binds to p130cas. *J Biol Chem*, **272:**228-232.

Auger, M. and Ross, J. (1992) The biology of the macrophage. The Natural Immune System. In Lewis, C.E. and McGee, J.D. (eds.), *The Macrophage*. RL Press, Oxford University Press, Oxford., pp. 3-74.

Avraham, S., London, R., Fu, Y., Ota, S., Hiregowdara, D., Li, J., Jiang, S., Pasztor, L.M., A., W.R., Groopman, J.E. and Avraham, H. (1995) Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. *J Biol Chem*, **270:**27742-27751.

Bailer, R.T., Holloway, A., Sun, J., Margolick, J.B., Martin, M., Kostman, J. and Montaner, L.J. (1999) IL-13 and IFN-gamma secretion by activated T cells in HIV-1 infection associated with viral suppression and a lack of disease progression. *J Immunol*, **162:**7534-7542.

Baldwin, G.C., Fleischmann, J., Chung, Y., Koyanagi, Y., Chen, I.S.Y. and Golde, D.W. (1990) Human immunodeficiency virus causes mononuclear phagocyte dysfunction. *Proc Natl Acad Sci USA*, **87:**3933-3937.

Baltimore, D. (1970) Viral RNA-dependent DNA polymerase. *Nature*, **392:**245-252.

Baqui, A., Jabra-Rizk, M., Kelley, J., Zhang, M., Falkler, W.J. and Meiller, T. (2000) Enhanced interleukin-1beta, interleukin-6 and tumor necrosis factor-alpha

production by LPS stimulated human monocytes isolated from HIV+ patients. *Immunopharmacol Immunotoxicol 2000*, **22:**401-421.

Baqui, A.A., Meiller, T.F., Zhang, M. and Falkler, W.A.J. (1999) The effects of HIV viral load on the phagocytic activity of monocytes activated with lipopolysaccharide from oral microorganisms. *Immunopharmacol Immunotoxicol*, **21:**421-438.

Barcellini, W., Rizzardi, G., Borghi, M., Fain, C., Lazzarin, A. and Meroni, P. (1994) TH1 and TH2 cytokine production by peripheral blood mononuclear cells from HIV-infected patients. *AIDS*, **8:**757-762.

Barre-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W. and Montagnier, L. (1983) Isolation of a T-lymphocytotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science*, **220:**868-871.

Barre-Sinoussi, F. (1996) HIV as the cause of AIDS. *Lancet*, **348:**31-35.

Basham, T. and Mengan, T. (1983) Recombinant interferon-gamma increases HLA-DR synthesis and expression. *J Immunol*, **130:**1492-1494.

Belosevic, M., Davis, C.E., Meltzer, M.S. and Nacy, C.A. (1988) Regulation of activated macrophage antimicrobial activities. Identification of lymphokines that cooperate with IFN-gamma for induction of resistance to infection. *J Immunol*, **141:**890-896.

Bender, B., Davidson, B., Kline, R., Brown, C. and Quinn, T. (1988) Role of the mononuclear phagocyte system in the immunopathogenesis of human immunodeficiency virus infection and the acquired immunodeficiency syndrome. *Rev Infect Dis*, **10:**1142-1154.

Bermudez, L. and Young, L. (1990) Recombinant granulocyte macrophage colony stimulating factor activates human macrophages to inhibit grown or kill *Mycobacterium avium* complex. *J Leukoc Biol*, **48:**67-73.

Bermudez, L., Martinelli, J., Petrofsky, M., Kolonoski, P. and Young, L. (1994) Recombinant granulocyte macrophage colony stimulating factor enhances the effects of antibiotics against *Mycobacterium avium* complex infection in the beige mouse model. *J Infect Dis*, **169:**575-580.

Bernstein, M.S., Tong-Starksen, S.E. and Locksley, R.M. (1991) Activation of human monocyte derived macrophages with lipopolysaccharide decreases human immunodeficiency virus replication *in vitro* at the level of gene expression. *J Clin Invest*, **88:**540-545.

Biggs, B.A., Hewish, M., Kent, S., Hayes, K. and Crowe, S.M. (1995) HIV-1 infection of human macrophages impairs phagocytosis and killing of *Toxoplasma gondii*. *J Immunol*, **154:**6132-6139.

Birx, D., Redfield, R., Tencer, K., Fowler, A., Burke, D. and Tosato, G. (1990) Induction of interleukin-6 during human immunodeficiency virus infection. *Blood*, **76:**2303-2310.

Biswas, P., Poli, G., Kinter, A.L., Justement, J.S., Stanley, S.K., Maury, W.J., Bressler, P., Orenstein, J.M. and Fauci, A.S. (1992) Interferon gamma induces the expression of human immunodeficiency virus in persistently infected promonocytic cells (U1) and redirects the production of virions to intracytoplasmic vacuoles in phorbol myristate acetate-differentiated U1 cells. *J Exp Med*, **176:**739-750.

Bober, L., Grace, M., Pugliese-Sivo, C., Rojas-Triana, A., Sullivan, L. and Narula, S. (1995) The effects of colony stimulating factors on human monocyte cell function. *Int J Immunopharmacol*, **17:**385-392.

Bofill, M., Gombert, W., Borthwick, N.J., Akbar, A.N., McLaughlin, J.E., Lee, C.A., Johnson, M.A., Pinching, A.J. and Janossy, G. (1995) Presence of CD3+CD8+Bcl-2(low) lymphocytes undergoing apoptosis and activated macrophages in lymph nodes of HIV-1+ patients. *Am J Pathol*, **146:**1542-1555.

Bonnerot, C., Briken, V., Brachet, V., Lankar, D., Cassard, S., Jabri, B. and Amigorena, S. (1998) Syk protein tyrosine kinase regulates Fc receptor gamma-chain-mediated transport to lysosomes. *EMBO J 1998*, **17:**4606-4616.

Borghi, P., Fantuzzi, L., Varano, B., Gessani, S., Puddu, P., Conti, L., Capobianchi, M.R., Ameglio, F. and Belardelli, F. (1995) Induction of interleukin 10 by human immunodeficiency virus type 1 and its gp120 protein in human monocytes macrophages. *J Virol*, **69:**1284-1287.

Brandt, S.J., Peters, W.P. and Buckner, C.D. (1988) Effect of recombinant human granulocyte-macrophage colony-stimulating factor on hematopoietic

reconstitution after high dose chemotherapy and autologous bone marrow transplantation. *N Engl J Med*, **318**:869-876.

Bravo-Cuellar, A., Nowacki, W., Vuillier, F., de Saint-Martin, J. and Orbach-Arbouys, S. (1992) The bactericidal capacity of peripheral blood monocytes from HIV positive patients may collapse very soon after the infection. *Immunol Lett*, **31**:297-299.

Brigino, E., Haraguchi, S., Koutsonikolis, A., Cianciolo, G.J., Owens, U., Good, R.A. and Day, N.K. (1997) Interleukin 10 is induced by recombinant HIV-1 Nef protein involving the calcium/calmodulin-dependent phosphodiesterase signal transduction pathway. *Proc Natl Acad Sci USA*, **94**:3178-3182.

Brites, C., Gilbert, M.J., Pedral-Sampaio, D., Bahia, F., Pedroso, C., Alcantara, A.P., Sasaki, M.D., Matos, J., Renjifo, B., Essex, M., Whitmore, J.B., Agosti, J.M. and Badaro, R. (2000) A randomized, placebo-controlled trial of granulocyte-macrophage colony-stimulating factor and nucleoside analogue therapy in AIDS. *J Infect Dis*, **182**:1531-1535.

Brown, C.B., Hart, C.E., Curtis, D.M., Bailey, M.C. and Kaushansky, K. (1990) The neutralizing monoclonal antibodies against human granulocyte-macrophage colony-stimulating factor recognize binding domain of the molecule. *J Immunol*, **144**:2184-2189.

Bryant, M. and Ratner, L. (1990) Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc Natl Acad Sci USA*, **87**:523-527.

Bubeck Wardenburg, J., Pappu, R., By, J.Y., Mayer, B., Chernoff, J., Straus, D. and Chan, A.C. (1998) Regulation of PAK activation and the T cell cytoskeleton by the linker protein SLP-76. *Immunity*, **9**:607-616.

Buchbinder, S.P., Katz, M.H., Hessol, N.A., O'Malley, P.M. and Holmberg, S.D. (1994) Long-term HIV-1 infection without immunologic progression. *AIDS 1994*, **8**:1123-1128.

Burridge, K. and Fath, K. (1989) Focal contacts: transmembrane links between the extracellular matrix and the cytoskeleton. *Bioessays*, **10**:104-108.

Burridge, K., Nuckolls, G., Otey, C., Pavalko, F., Simon, K. and Turner, C. (1990) Actin-membrane interaction in focal adhesions. *Cell Differ Dev*, **32**:337-342.

Bustelo, X.R. (2000) Regulatory and signalling properties of the Vav family. *Mol Cell Biol*, **20**:1461-1477.

Bustelo, X.R., Suen, K.L., Leftheris, K., Meyers, C.A. and Barbacid, M. (1994) Vav cooperates with Ras to transform rodent fibroblasts but is not a Ras GDP/GTP exchange factor. *Oncogene*, **9**:2405-2413.

Cameron, P.U., Freudenthal, P.S., Barker, J.M., Gezelter, S., Inaba, K. and Steinman, R.M. (1992) Dendritic cells exposed to human immunodeficiency virus type-1 transmit a vigorous cytopathic infection to CD4+ T cells. *Science*, **257**:383-387.

Cameron, P.U., Granger, D.L., Matthews, T.J. and Weinberg, J.B. (1993) Human immunodeficiency virus (HIV)-infected human blood monocytes and peritoneal macrophages have reduced anticryptococcal activity whereas HIV-infected alveolar macrophages retain normal activity. *J Infect Dis*, **170**:60-67.

Canque, B., Bakri, Y., Camus, S., Yagello, M., Benjouad, A. and Gluckman, J.C. (1999) The susceptibility to X4 and R5 human immunodeficiency virus-1 strains of dendritic cells derived in vitro from CD34(+) hematopoietic progenitor cells is primarily determined by their maturation stage. *Blood*, **93**:3866-3875.

Cao, Y., Qin, L., Zhang, L., Safrit, J. and Ho, D.D. (1995) Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N Engl J Med*, **332**:201-208.

Capobianchi, M. (1996) Induction of lymphomonocyte activation by HIV-1 glycoprotein gp120. Possible role in AIDS pathogenesis. *J Biol Regul Homeost Agents*, **10**:83-91.

Capobianchi, M.R., Abbate, I., Antonelli, G., Turriziani, O., Dolei, A. and Dianzani, F. (1998) Inhibition of HIV type-1 BaL replication by MIP-1alpha, MIP-1beta, and RANTES in macrophages. *AIDS Res Hum Retroviruses*, **14**:233-240.

Capsoni, F., Minonzio, F., Ongari, A.M., P, R.G., Lazzarin, A. and Zanussi, C. (1992) Monocyte-derived macrophage function in HIV-infected subjects: *in vitro* modulation by rIFN-gamma and rGM-CSF. *Clin Immunol Immunopathol*, **62**:176-182.

Capsoni, F., Minonzio, F., Ongari, A.M., Bonara, P., Pinto, G., Carbonelli, V., Lazzarin, A. and Zanussi, C. (1994) Fc receptors expression and function in

mononuclear phagocytes from AIDS patients; modulation by IFN-gamma. *Scand J Immunol*, **39:**45-50.

Caron, E. and Hall, A. (1998) Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science*, **282:**1717-1721.

Carpenter, C.C., Cooper, D.A., Fischl, M.A., Gatell, J.M., Gazzard, B.G., Hammer, S.M., Hirsch, M.S., Jacobsen, D.M., Katzenstein, D.A., Montaner, J.S., Richman, D.D., Saag, M.S., Schechter, M., Schooley, R.T., Thompson, M.A., Vella, S., Yeni, P.G. and Volberding, P.A. (2000) Antiretroviral therapy in adults: updated recommendations of the International AIDS Society-USA Panel. *JAMA*, **283:**381-390.

Cavert, W., Notermans, D.W., Staskus, K., Wietgrefe, S.W., Zupancic, M., Gebhard, K., Henry, K., Zhang, Z.Q., Mills, R., McDade, H., Schuwirth, C.M., Goudsmit, J., Danner, S.A. and Haase, A.T. (1997) Kinetics of response in lymphoid tissues to antiretroviral therapy of HIV-1 infection. *Science*, **276:**960-964.

Chan, D.C., Fass, D., Berger, J.M. and Kim, P.S. (1997) Core structure of gp41 from the HIV envelope glycoprotein. *Cell*, **89:**263-273.

Chan, H.T., Kedzierska, K., O'Mulliane, J., Crowe, S.M. and Jaworowski, A. (2001) Quantifying complement-mediated phagocytosis by human monocyte-derived macrophages. *Immunol Cell Biol*, **79:**429-435.

Chatila, T., Geha, R. and Arnaout, M. (1989) Constitutive and stimulus-induced phosphorylation of CD11b/CD18 leukocyte adhesion molecules. *J Cell Biol*, **109:**3435-3444.

Chaturvedi, S., Frame, P. and Newman, S.L. (1995) Macrophages from human immunodeficiency virus positive persons are defective in host defence against *Histoplasma capsulatum*. *J Infect Dis*, **171:**320-327.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P.D., Wu, L., Mackay, C.R., LaRosa, G., Newman, W., Gerard, N., Gerard, C. and Sodroski, J. (1996) The beta chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell*, **85:**1135-1148.

Chowers, M.Y., Pandori, M.W., Spina, C.A., Richman, D.D. and Guatelli, J.C. (1995) The growth advantage conferred by HIV-1 nef is determined at the level

of viral DNA formation and is independent of CD4 downregulation. *Virology*, **212:**451-457.

Chun, T.W., Stuyver, L., Mizell, S.B., Ehler, L.A., Mican, J.A., Baseler, M., Lloyd, A.L., Nowak, M.A. and Fauci, A.S. (1997) Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc Natl Acad Sci USA*, **94:**13193-13197.

Chun, T.W., Engel, D., Berrey, M., Shea, T., Corey, L. and Fauci, A.S. (1998) Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. *Proc Natl Acad Sci USA*, **95:**8869-8873.

Clapham, P.R., Weiss, R.A., Dalgleish, A.G., Exley, M., Whitby, D. and Hogg, N. (1987) Human immunodeficiency virus infection of monocytic and T lymphocytic cells; receptor modulation and differentiation induced by phorbol ester. *Virology*, **158:**44-51.

Clark, S., Saag, M.S., Decker, D., Campbell-Hill, S., Roberson, J.L., Veldkamp, P.J., Kappes, J.C., Hahn, B.H. and Shaw, G.M. (1991) High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. *N Engl J Med*, **324**.

Clarke, J.R., Krishnan, V., Bennett, J., Mitchell, D. and Jeffries, D.J. (1990) Detection of HIV-1 in human lung macrophages using the polymerase chain reaction. *AIDS*, **4:**1133-1136.

Clark-Lewis, I., Lopez, A.F., To, L.B., Vadas, M.A., Schrader, J.W., Hood, L.E. and Kent, S.B. (1988) Structure-function studies of human granulocyte-macrophage colony-stimulating factor. Identification of residues required for activity. *J Immunol*, **141:**881-889.

Clavel, F., Guyader, M., Guetard, D., Salle, M., Montagnier, L. and Alizon, M. (1986) Molecular cloning and polymorphism of the human immune deficiency virus type 2. *Nature*, **324:**691-695.

Clerici, M. and Shearer, G. (1993) A TH1-->TH2 switch is a critical step in the etiology of HIV infection. *Immunol Today*, **14:**107-111.

Cocchi, F., DeVico, A., Garzino-Demo, A., Arya, S., Gallo, R. and Lusso, P. (1995) Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science*, **270:**1811-1815.

Cocchi, F., DeVico, A.L., Garzino-Demo, A., Cara, A., Gallo, R.C. and Lusso, P. (1996) The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection. *Nat Med*, **2:**1244-1247.

Coffin, J. (1992) Structure and classification of retroviruses. *In: Levy JA Ed. The Retroviridae, New York, Plenum Press*19-50.

Coleman, D.L., Chodakewitz, J.A., Bartiss, A.H. and Mellors, J.W. (1988) Granulocyte-macrophage colony-stimulating factor enhances selective effector functions of tissue-derived macrophages. *Blood*, **72:**573-578.

Collette, Y., Chang, H.L., Cerdan, C., Chambost, H., Algarte, M., Mawas, C., Imbert, J., Burny, A. and Olive, D. (1996a) Specific Th1 cytokine down-regulation associated with primary clinically derived human immunodeficiency virus type 1 Nef gene-induced expression. *J Immunol*, **156:**360-370.

Collette, Y., Dutartre, H., Benziane, A., Ramos-Morales, Benarous, R., Harris, M. and Olive, D. (1996b) Physical and functional interaction of Nef with Lck. HIV-1 Nef-induced T-cell signalling defects. *J Biol Chem*, **271:**6333-6341.

Collins, H.L. and Bancroft, G.J. (1992) Cytokine enhancement of complement-dependent phagocytosis by macrophages: synergy of tumor necrosis factor-alpha and granulocyte-macrophage colony-stimulating factor for phagocytosis of *Cryptococcus neoformans*. *Eur J Immunol*, **22:**1447-1454.

Collman, R., Hassan, N.F., Walker, R., Godfrey, B., Cutilli, J., Hastings, J.C., Friedman, H., Douglas, S.D. and Nathanson, N. (1989) Infection of monocyte-derived macrophages with human immunodeficiency virus type 1 (HIV-1). Monocyte tropic and lymphocyte tropic strains of HIV-1 show distinctive patterns of replication in a panel of cell types. *J Exp Med*, **170:**1149-1163.

Collman, R., Balliet, J.W., Gregory, S.A., Friedman, H., Kolson, D.L., Nathanson, N. and Srinivasan, A. (1992) An infectious molecular clone of an unusual macrophage tropic and highly cytopathic strain of human immunodeficiency virus type 1. *J Virol*, **66:**7517-7521.

Connor, R., Mohri, H., Cao, Y. and Ho, D. (1993) Increased viral burden and cytopathicity correlate temporally with CD4+ T lymphocyte decline and clinical progression in human immunodeficiency virus type-1 infected individuals. *J Virol*, **67:**1772-1777.

Cooper, D.A., Gold, J., Maclean, P., Donovan, B., Finlayson, R., Barnes, T.G., Michelmore, H.M., Brooke, P. and Penny, R. (1985) Acute AIDS retrovirus infection; definition of a clinical illness associated with seroconversion. *Lancet*, **1**:537-540.

Corey, S.J. and Anderson, S.M. (1999) Src-related protein tyrosine kinases in hematopoiesis. *Blood*, **93**:1-14.

Cox, D., Chang, P., Kurosaki, T. and Greenberg, S. (1996) Syk tyrosine kinase is required for immunoreceptor tyrosine activation motif-dependent actin assembly. *J Biol Chem*, **271**:16597-16602.

Cox, D., Chang, P., Zhang, Q., Reddy, P.G., Bokoch, G.M. and Greenberg, S. (1997) Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. *J Exp Med*, **186**:1487-1494.

Cox, D., Tseng, C.C., Bjekic, G. and Greenberg, S. (1999) A requirement for phosphatidylinositol 3-kinase in pseudopod extension. *J Biol Chem*, **274**:1240-1247.

Crespo, P., Schuebel, K.E., Ostrom, A.A., Gutkind, J.S. and Bustelo, X.R. (1997) Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. *Nature*, **385**:169-172.

Crowe, S.M., Mills, J. and McGrath, M.S. (1987) Quantitative immunocytofluorographic analysis of CD4 surface antigen expression and HIV infection of human peripheral blood monocyte/macrophages. *AIDS Res Hum Retroviruses*, **3**:135-145.

Crowe, S.M., Vardaxis, N.J., Kent, S.J., Maerz, A.L., Hewish, M.J., McGrath, M.S. and Mills, J. (1994) HIV infection of monocyte-derived macrophages *in vitro* reduces phagocytosis of *Candida albicans*. *J Leukoc Biol*, **56**:318-327.

Crowe, S.M. (1995) Role of macrophages in the pathogenesis of human immunodeficiency virus (HIV) infection. *Aust NZ J Med*, **25**:777-783.

Crowe, S.M. and Lopez, A. (1997) GM-CSF and its effects on replication of HIV-1 in cells of macrophage lineage. *J Leukoc Biol*, **62**:41-48.

Crowe, S.M. and Sonza, S. (2000) HIV-1 can be recovered from a variety of cells including peripheral blood monocytes of patients receiving highly active

antiretroviral therapy: a further obstacle to eradication. *J Leukoc Biol*, **68:**345-350.

Crowle, A., Ross, E.R., Cohn, D.L., Gilden, J. and May, M.H. (1992) Comparison of the abilities of *Mycobacterium avium* and *Mycobacterium intracellulare* to infect and multiply in cultured human macrophages from normal and human immunodeficiency virus infected subjects. *Infect Immun*, **60:**3697-3703.

Crowley, M.T., Costello, P.S., Fitzer-Attas, C.J., Turner, M., Meng, F., Lowell, C., Tybulewicz, V.L. and DeFranco, A.L. (1997) A critical role for Syk in signal transduction and phagocytosis mediated by Fc gamma receptors on macrophages. *J Exp Med*, **186:**1027-1039.

Daeron, M. (1997) Fc receptor biology. *Annu Rev Immunol*, **15:**203-234.

Darby, C., Geahlen, R. and Schreiber, A. (1994) Stimulation of macrophage Fc gamma RIIIA activates the receptor-associated protein tyrosine kinase Syk and induces phosphorylation of multiple proteins including p95Vav and p62/GAP-associated protein. *J Immunol*, **152:**5429-5437.

Deacon, N.J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D.J., McPhee, D.A., Greenway, A.L., Ellett, A., Chatfield, C., Lawson, A., Crowe, S.M., Maerz, A., Sonza, S., Learmont, J., Sullivan, S., Cunningham, A., Dwyer, D., Dowton, D. and Mills, J. (1995) Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science*, **270:**988-991.

Dean, M., Carrington, M., Winkler, C., Huttley, G., Smith, M., Allikmets, R., Goedert, J., Buchbinder, S., Vittinghoff, E., Gomperts, E., Donfield, S., Vlahov, D., Kaslow, R., Saah, R., Rinaldo, C., Detels, R. and O'Brien, S. (1996) Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science*, **273:**1856-1862.

Delemarre, F.G., Stevenhagen, A., Kroon, F.P. and van Furth, R. (1998) Reduced toxoplasmastatic activity of monocytes from AIDS patients: a role for granulocyte-macrophage colony-stimulating factor. *Scand J Immunol*, **47:**163-166.

Denis, M. and Ghadirian, E. (1994) Dysregulation of interleukin-8, interleukin-10, and interleukin-12 release by alveolar macrophages from HIV type 1-infected subjects. *AIDS Res Hum Retroviruses*, **10:**1619-1627.

DeGroote, M.A. and Fang, F.C. (1995) NO inhibitions: antimicrobial properties of nitric oxide. *Clin Infect Dis*, **21:**S162-165.

Delemarre, F., Stevenhagen, A., Kroon, F., van Eer, M., Meenhorst, P. and Furth, R. (1995) Reduced toxoplasmastatic activity of monocytes and monocyte-derived macrophages from AIDS patients is mediated via prostaglandin E2. *AIDS*, **9:**441-445.

Desjardins, M., Celis, J.E., van Meer, G., Dieplinger, H., Jahraus, A., Griffiths, G. and Huber, L.A. (1994) Molecular characterisation of phagosomes. *J Biol Chem*, **269:**32194-32200.

Dharmawardhane, S., Brownson, D., Lennartz, M. and Bokoch, G.M. (1999) Localization of p21-activated kinase 1 (PAK1) to pseudopodia, membrane ruffles, and phagocytic cups in activated human neutrophils. *J Leukoc Biol*, **66:**521-527.

Di Marzio, P., Tse, J. and Landau, N. (1998) Chemokine receptor regulation and HIV type 1 tropism in monocyte-macrophages. *AIDS Res Hum Retroviruses*, **14:**129-138.

Diederichs, K., Boone, T. and Karplus, P.A. (1991) Novel fold and putative receptor binding site of granulocyte-macrophage colony-stimulating factor. *Science*, **254:**1779-1782.

Dimitrov, D.S. (1997) How do viruses enter cells? The HIV coreceptors teach us a lesson of complexity. *Cell*, **91:**721-730.

Dobmeyer, T.S., Raffel, B., Dobmeyer, J.M., Findhammer, S., Klein, S.A., Kabelitz, D., Hoelzer, D., Helm, E.B. and Rossol, R. (1995) Decreased function of monocytes and granulocytes during HIV-1 infection correlates with CD4 cell counts. *Eur J Med Res*, **1:**9-15.

Dougherty, G.J. and McBride W.H. (1989) Monocyte differentiation in vitro. In Zembala, M. and Asherson, G.L. (eds.), *Human monocytes*. Academic Press, London, UK, pp. 89-97.

Doultree, J.C., Maerz, A.L., Ryan, N.J., Baird, R.W., Wright, E., Crowe, S.M. and Marshall, J.A. (1995) In vitro growth of the microsporidian *Septata intestinalis* from an AIDS patient with disseminated illness. *J Clin Microbiol*, **33**:463-470.

Dragic, T., Litwin, V., Allaway, G.P., Martin, S.R., Huang, Y., Nagashima, K.A., Cayanan, C., Maddon, P.J., Koup, R.A., Moore, J.P. and Paxton, P.A. (1996) HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature*, **381**:667-673.

Drancourt, M., Bonnet, E., Gallais, H., Peloux, Y. and Raoult, D. (1992) *Rhodococcus equi* infection in patients with AIDS. *J Infect*, **24**:123-131.

Dunne, J., Feighery, C. and Whelan, A. (1996) Beta-2-microglobulin, neopterin and monocyte Fc gamma receptors in opportunistic infections of HIV-positive patients. *Br J Biomed Sci*, **53**:263-269.

Ducng, L.T., Lakkakorpi, P.T., Nakamura, I., Machwate, M., Nagy, R.M. and Rodan, G.A. (1998) Pyk2 in osteoclasts is an adhesion kinase, localized in the sealing zone, activated by ligation of alpha/beta integrin, and phosphorylated by Src kinase. *J Clin Invest*, **102**:881-892.

Eales, L.J., Moshtael, O. and Pinching, A.L. (1987) Microbicidal activity of monocyte derived macrophages in AIDS and related disorders. *Clin Exp Immunol*, **67**:227-235.

Edberg, J., Yee, A., Rakshit, D., Chang, D., Gokhale, J., Indik, Z., Schreiber, A. and Kimberly, R. (1999) The cytoplasmic domain of human Fc gamma RIa alters the functional properties of the Fc gamma RI gamma-chain receptor complex. *J Biol Chem*, **274**:30328-30333.

Eischen, A., Vincent, F., Bergerat, J., Louis, B., Faradji, A., Bohbot, A. and Oberline, F. (1991) Long term cultures of human monocytes in vitro. Impact of GM-CSF on survival and differentiation. *J Immunol Methods*, **143**:209-221.

Elbim, C., Pillet, S., Prevost, M.H., Preira, A., Girard, P.M., Rogine, N., Matusani, H., Hakim, J., Israel, N. and Gougerot-Pocidalo, M.A. (1999) Redox and activation status of monocytes from human immunodeficiency virus-infected patients: relationship with viral load. *J Virol 1999*, **73**:4561-4566.

Elliott, M.J., Vadas, M.A., Eglinton, J.M., Park, L.S., To, L.B., Cleland, L.G., Clark, S.C. and Lopez, A.F. (1989) Recombinant human interleukin-3 (IL-3) and

granulocyte-macrophage colony-stimulating factor (GM-CSF) show common biological effects and binding characteristics on human monocytes. *Blood*, **74**:2349-2359.

Embretson, J., Zupancic, M., Ribas, J.L., Burke, A., Racz, P., Tenner-Racz, K. and Haase, A. (1993) Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature*, **362**:359-362.

Emerman, M. and Malim, M.H. (1998) HIV-1 regulatory/accessory genes: keys to unravelling viral and host cell biology. *Science*, **280**:1880-1884.

Ennen, J., Ernst, M. and Flad, H.D. (1989) The effect of interleukin-2 on Fc gamma receptor function of human monocytes requires specific intercellular interaction. *Immunobiology*, **179**:17-32.

Esser, R., von Briesen, H., Brugger, M., Ceska, M., Glienke, W., Muller, S., Rehm, A., Rubsamen-Waigmann, H. and Andreesen, R. (1991) Secretory repertoire of HIV infected human monocytes/macrophages. *Pathobiology*, **59**:219-222.

Esser, R., Glienke, W., von Briesen, H., Rubsamen-Waigmann, H. and Andreesen, R. (1996) Differential regulation of proinflammatory and hematopoietic cytokines in human macrophages after infection with human immunodeficiency virus. *Blood*, **88**:3474-3481.

Esser, R., Glienke, W., Andreesen, R., Unger, R., Kreutz, M., Rubsamen-Waigmann, H. and von Briesen, H. (1998) Individual cell analysis of the cytokine repertoire in human immunodeficiency virus-1-infected monocytes/macrophages by a combination of immunocytochemistry and in situ hybridization. *Blood*, **91**:4752-4760.

Estevez, M.E., Ballart, I.J., Diez, R.A., Planes, D.N., Scaglione, C. and Sen, L. (1986) Early defect of phagocytic cell function in subjects at risk for acquired immunodeficiency syndrome. *Scand J Immunol*, **24**:215-221.

Evans, L.A. and Levy, J.A. (1989) Characteristics of HIV infection and pathogenesis. *Biochimica Biophysica Acta*, **989**:237-254.

Eversole, L.R., Fleischmann, J., Baldwin, G.C. and Sapp, J.P. (1994) The effects of human immunodeficiency virus infection on macrophage phagocytosis of Candida. *Oral Microbiol Immunol*, **9**:55-59.

Ezekowitz, R. and Gordon, S. (1982) Surface properties of activated macrophages; sensitized lymphocytes, specific antigen and lymphokines reduce expression of antigen F4/80 and FC and mannose/fucosyl receptors, but induce Ia. *Adv Exp Med Biol*, **155:**401-407.

Fackler, O., Kienzle, N., Kremmer, E., Boese, A., Schramm, B., Klimkait, T., Kucherer, C. and Mueller-Lantzsch, N. (1997) Association of human immunodeficiency virus Nef protein with actin is myristoylation dependent and influences its subcellular localization. *Eur J Biochem*, **247:**843-851.

Fackler, O.T., Lu, X., Frost, J.A., Geyer, M., Jiang, B., Luo, W., Abo, A., Alberts, A.S. and Peterlin, B.M. (2000) p21-activated kinase 1 plays a critical role in cellular activation by Nef. *Mol Cell Biol*, **20:**2619-2627.

Fackler, O.T., Luo, W., Geyer, M., Alberts, A.S. and Peterlin, B.M. (1999) Activation of Vav by Nef induces cytoskeletal rearrangements and downstream effector functions. *Mol Cell*, **3:**729-739.

Fan, J., Li, P., Kok, T. and Burrell, C. (1997) AZT blocks down-regulation of IL-2 and IFN-gamma gene expression in HIV acutely infected cells. *Arch Virol*, **142:**1035-1043.

Fantuzzi, L., Gessani, S., Borghi, P., Varano, B., Conti, L., Puddu, P. and Belardelli, F. (1996) Induction of interleukin-12 (IL-12) by recombinant glycoprotein gp120 of human immunodeficiency virus type 1 in human monocytes/macrophages: requirement of gamma interferon for IL-12 secretion. *J Virol*, **70:**4121-4124.

Fantuzzi, L., Conti, L., Gauzzi, M.C., Eid, P., Del Corno, M., Varano, B., Canini, I., Belardelli, F. and S, G. (2000) Regulation of chemokine/cytokine network during in vitro differentiation and HIV-1 infection of human monocytes: possible importance in the pathogenesis of AIDS. *J Leukoc Biol*, **68:**391-399.

Fantuzzi, L., Canini, I., Belardelli, F. and Gessani, S. (2001) HIV-1 gp120 stimulates the production of beta-chemokines in human peripheral blood monocytes through a CD4-independent mechanism. *J Immunol*, **166:**5381-5387.

Fear, W., Kesson, A., Naif, H., Lynch, G. and Cunningham, A. (1998) Differential tropism and chemokine receptor expression of human immunodeficiency virus type-1 in neonatal monocytes, monocyte-derived macrophages, and placental macrophages. *J Virol*, **72:**1334-1344.

Fearon, D.T. (1980) Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte, and monocyte. *J Exp Med*, **152:**20-30.

Feng, Y., Broder, C.C., Kennedy, P.E. and Berger, E.A. (1996) HIV-1 entry cofactor; functional cDNA cloning of a seven transmembrane, G protein-coupled receptor. *Science*, **272:**872-877.

Fenyo, E.M., Albert, J. and Asjo, B. (1989) Replicative capacity, cytopathic effect and cell tropism of HIV. *AIDS*, **3:**S5-12.

Finzi, D., Hermankova, M., Pierson, T., Carruth, L.M., Buck, C., Chaisson, R.E., Quinn, T.C., Chadwick, K., Margolick, J., Brookmeyer, R., Gallant, J., Markowitz, M., Ho, D.D., Richman, D.D. and Siliciano, R.F. (1997) Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science*, **278:**1295-1300.

Fitzer-Attas, C.J., Lowry, M., Crowley, M.T., Finn, A.J., Meng, F., DeFranco, A.L. and Lowell, C.A. (2000) Fc gamma receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn. *J Exp Med*, **191:**669-682.

Foli, A., Saville, M.W., Baseler, M.W. and Yarchoan, R. (1995) Effects of the Th1 and Th2 stimulatory cytokines interleukin-12 and interleukin-4 on human immunodeficiency virus replication. *Blood*, **85:**2114-2123.

Foli, A., Saville, M., May, L., Webb, D. and Yarchoan, R. (1997) Effects of human immunodeficiency virus and colony-stimulating factors on the production of interleukin-6 and tumor necrosis factor alpha by monocyte/macrophages. *AIDS Res Hum Retroviruses*, **13:**829-839.

Folks, T., Clouse, K., Justement, J., Rabson, A., Duh, E., Kehrl, J. and Fauci, A. (1989) Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proc Natl Acad Sci USA*, **86:**2365-2368.

Folks, T.M., Justement, J., Kinter, A., Dinarello, C.A. and Fauci, A.S. (1987) Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science*, **238:**800-802.

Folks, T.M., Justement, J., Kinter, A., Schnittman, S., Orenstein, J., Poli, G. and Fauci, A.S. (1988) Characterisation of a promonocyte clone chronically infected with HIV and inducible by 13-phorbol-12-myristate acetate. *J Immunol*, **140:**1117-1122.

Gabuzda, D.H., Ho, D.D., de la Monte, S.M., Hirsch, M.S., Rota, T.R. and Sobel, R.A. (1986) Immunohistochemical identification of HTLV-III antigen in brains of patients with AIDS. *Ann Neurol*, **20:**289-295.

Gallo, P., De Rossi, A., Sivieri, S., Chieco-Bianchi, L. and Tavolato, B. (1994) M-CSF production by HIV-1-infected monocytes and its intrathecal synthesis. Implications for neurological HIV-1-related disease. *J Neuroimmunol*, **51:**193-198.

Gallo, R.C., Sarin, P.S., Gelmann, E.P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V.S., Mann, D. and Sidhu, G.D. (1983) Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science*, **220:**865-867.

Garcia, F., Niebla, G., Romeu, J., Vidal, C., Plana, M., Ortega, M., Ruiz, L., Gallart, T., Clotet, B., Miro, J.M., Pumarola, T. and Gatell, J.M. (1999) Cerebrospinal fluid HIV-1 RNA levels in asymptomatic patients with early stage chronic HIV-1 infection: support for the hypothesis of local virus replication. *AIDS*, **13:**1491-1496.

Garcia, J.V., Alfano, J. and Miller, A.D. (1993) The negative effect of human immunodeficiency virus type-1 Nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4. *J Virol*, **67:**1511-1516.

Gartner, S.P., Markovits, D.M., Markovitz, M.H., Kaplan, M.H., Gallo, R.C. and Popovic, M. (1986) The role of mononuclear phagocytes in HTLV-III/LAV infection. *Science*, **233:**215-219.

Gartner, S. and Popovic, M. (1990) Macrophage tropism of HIV-1. *AIDS Res Human Retrovirus*, **6:**1017-1021.

Garzino-Demo, A., Moss, R.B., Margolick, J.B., Cleghorn, F., Sill, A., Blattner, W.A., Cocchi, F., Carlo, D.J., DeVico, A.L. and Gallo, R.C. (1999) Spontaneous and antigen-induced production of HIV-inhibitory beta-

chemokines are associated with AIDS-free status. *Proc Natl Acad Sci USA*, **96:**11986-11991.

Gearing, D., King, J., Gough, N. and Nicola, N. (1989) Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. *EMBO J*, **8:**3667-3676.

Gebran, S.J., Romano, E.L., Pons, H.A., Cariani, L. and Soyano, A.N. (1992) A modified colorimetric method for the measurement of phagocytosis and antibody-dependent cell cytotoxicity using 2,7-diaminofluorene. *J Immunol Methods*, **151:**255-260.

Geertsma, M., van Wengen-Stevenhagen, A., van Dam, E., Risberg, K., Kroon, F., Groeneveld, P. and Nibbering, P. (1999) Decreased expression of zeta molecules by T lymphocytes is correlated with disease progression in human immunodeficiency virus-infected persons. *J Infect Dis*, **180:**649-658.

Geijtenbeek, T.B., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Adema, G.J., van Kooyk, Y. and Figdor, C.G. (2000) Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell*, **100:**575-585.

Gendelman, H.E., Orenstein, J.M., Martin, M.A., Ferrua, C., Mitra, R., Phipps, T., Wahl, L.A., Lane, H.C., Fauci, A.S., Burke, D.S., Skillman, D. and Meltzer, M. (1988) Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor-1 treated monocytes. *J Exp Med*, **167:**1428-1441.

Gendelman, H.E., Baca, L.M., Turpin, J., Kalter, D.C., Hansen, B., Orenstein, J.M., Dieffenbach, C.W., Friedman, R.M. and Meltzer, M.S. (1990) Regulation of HIV replication in infected monocytes by IFN-alpha. Mechanisms for viral restriction. *J Immunol*, **145:**2669-2676.

Genis, P., Jett, M., Bernton, E.W., Boyle, T., Gelbard, H.A., Dzenko, K., Keane, R.W., Resnick, L., Mizrachi, Y. and Volsky, D.J. (1992) Cytokines and arachidonic metabolites produced during human immunodeficiency virus (HIV) infected macrophage astroglia interactions implications for the neuropathogenesis of HIV disease. *J Exp Med*, **176:**1703-1718.

Gessani, S., Puddu, P., Varano, B., Borghi, P., Conti, L., Fantuzzi, L. and Belardelli, F. (1994) Induction of beta interferon by human immunodeficiency

virus type 1 and its gp120 protein in human monocytes-macrophages: role of beta interferon in restriction of virus replication. *J Virol*, **68:**1983-1986.

Gessani, S., Borghi, P., Fantuzzi, L., Varano, B., Conti, L., Puddu, P. and Belardelli, F. (1997) Induction of cytokines by HIV-1 and its gp120 protein in human peripheral blood monocyte/macrophages and modulation of cytokine response during differentiation. *J Leukoc Biol*, **62:**49-53.

Ghassemi, M., Andersen, B.R., Reddy, V.M., Gangadharam, P.R., Spear, G.T. and Novak, R.M. (1995) Human immunodeficiency virus and *Mycobacterium avium* complex coinfection of monocytoid cells results in reciprocal enhancement of multiplication. *J Infect Dis*, **171:**68-73.

Ghazizadeh, S., Bolen, J. and Fleit, H. (1994) Physical and functional association of Src-related protein tyrosine kinases with Fc gamma RII in monocytic THP-1 cells. *J Biol Chem*, **269:**8878-8884.

Ghazizadeh, S., Bolen, J. and Fleit, H. (1995) Tyrosine phosphorylation and association of Syk with Fc gamma RII in monocytic THP-1 cells. *Biochem J*, **305:**669-674.

Ghorpade, A., Nukuna, A., Che, M., Haggerty, S., Persidsky, Y., Carter, E., Carhart, L., Shafer, L. and Gendelman, H.E. (1998) Human immunodeficiency virus neurotropism: an analysis of viral replication and cytopathicity for divergent strains in monocytes and microglia. *J Virol 1998*, **72:**3340-3350.

Glass, J.D., Wesselingh, S.L., Selnes, O.A. and McArthur, J.C. (1993) Clinical-neuropathologic correlation in HIV-associated dementia. *Neurology*, **43:**2230-2237.

Goff, S.P. (1990) Retroviral reverse transcriptase: synthesis, structure and function. *J Acquired Immune Defic Syndr*, **3:**817-831.

Gordon, S. (1978) Regulation of enzyme secretion by mononuclear phagocytes: studies with macrophage plasminogen activator and lysozyme. *Fed Proc*, **37:**2754-2758.

Gordon, S. (1986) Biology of the macrophage. *J Cell Sci*, **4:**267-286.

Gorry, P.R., Howard, J.L., Churchill, M.J., Anderson, J.L., Cunningham, A., Adrian, D., McPhee, D.A. and Purcell, D.F. (1999) Diminished production of human immunodeficiency virus type 1 in astrocytes results from inefficient

translation of gag, env, and nef mRNAs despite efficient expression of Tat and Rev. *J Virol*, **73**:352-361.

Gottlieb, M.S., Schroff, R., Schanker, H.M., Weisman, J.D., Fan, P.T., Wolf, R.A. and Saxon, A. (1981) *Pneumocystis carinii* pneumonia andmucosal condidiasis in previously healthy homosexual men; evidence of a new acquired cellular immunodeficiency. *N Engl J Med*, **305**:1425-1431.

Granowitz, E., Saget, B., Wang, M., Dinarello, C. and Skolnik, P. (1995) Interleukin 1 induces HIV-1 expression in chronically infected U1 cells: blockade by interleukin-1 receptor antagonist and tumor necrosis factor binding protein type 1. *Mol Med*, **1**:667-677.

Grassi, G., Pozzato, G., Moretti, M. and Giacca, M. (1995) Quantitative analysis of hepatitis C virus RNA in liver biopsies by competitive reverse transcription and polymerase chain reaction. *J Hepatol*, **23**:403-411.

Greco, G., Fujimura, S.H., Mourich, D.V. and Levy, J.A. (1999) Differential effects of human immunodeficiency virus isolates on beta-chemokine and gamma interferon production and on cell proliferation. *J Virol*, **73**:1528-1534.

Greenberg, S., Burridge, K. and Silverstein, S.C. (1990) Colocalization of F-actin and talin during Fc receptor-mediated phagocytosis in mouse macrophages. *J Exp Med*, **172**:1853-1856.

Greenberg, S., Chang, P. and Silverstein, S.C. (1993) Tyrosine phosphorylation is required for Fc receptor-mediated phagocytosis in mouse macrophages. *J Exp Med*, **177**:529-534.

Greenberg, S., Chang, P. and Silverstein, S.C. (1994) Tyrosine phosphorylation of the gamma subunit of Fc gamma receptors, p72syk, and paxillin during Fc receptor-mediated phagocytosis in macrophages. *J Biol Chem*, **269**:3897-3902.

Greenberg, S. (1995) Signal transduction of phagocytosis. *Trends in Cell Biology*, **5**:93-99.

Greenberg, S., Chang, P., Wang, D.C., Xavier, R. and Seed, B. (1996) Clustered syk tyrosine kinase domains trigger phagocytosis. *Proc Natl Acad Sci USA*, **93**:1103-1107.

Greenway, A., Azad, A., Mills, J. and McPhee, D.A. (1996) Human immunodeficiency virus type 1 nef binds directly to Lck and mitogen-activated protein kinase, inhibiting kinase activity. *J Virol*, **70**:6701-6708.

Greenway, A., McPhee, D.A., Grgacic, E., Hewish, M., A, L., Macreadie, I. and Azad, A. (1994) Nef 27, but not the Nef 25 isoform of human immunodeficiency virus type 1 pNL4-3 down regulates surface CD4 and IL-2 R expression in peripheral blood mononuclear cells and transformed T cells. *Virology*, **198**:245-256.

Greenway, A., Dutartre, H., Allen, K., McPhee, D., Olive, D. and Collette, Y. (1999) Simian immunodeficiency virus and human immunodeficiency virus type-1 Nef proteins show distinct patterns and mechanisms of Src kinase activation. *J Virol*, **73**:6152-6158.

Groopman, J.E., Mitsuyasu, R.T., DeLeo, M.J., Oette, D.H. and Golde, D.W. (1987) Effect of recombinant granulocyte-macrophage colony-stimulating factor on myelopoiesis in the Acquired Immunodeficiency Syndrome. *N Engl J Med*, **317**:593-598.

Gruber, M.F., Weih, K.A., Boone, E.J., Smith, P.O. and Clouse, K.A. (1995) Endogenous macrophage CSF production is associated with vital replication in HIV-1 infected human monocyte derived macrophages. *J Immunol*, **154**:5528-5535.

Gruters, R.A., Terpstra, F.G., De Jong, R., Van Noesel, C.J., Van Lier, R.A. and Miedema, F. (1990) Selective loss of T cell functions in different stages of HIV infection. Early loss of anti-CD3-induced T cell proliferation followed by decreased anti-CD3-induced cytotoxic T lymphocyte generation in AIDS-related complex and AIDS. *Eur J Immunol*, **20**:1039-1044.

Gulick, R.M., Mellors, J.W., Havlir, D., Eron, J.J., Gonzalez, C., McMahon, D., Richman, D.D., Valentine, F.T., Jonas, L., Meibohm, A., Emini, E.A. and Chodakewitz, J.A. (1997) Treatment with indinavir, zidovudine and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. *N Engl J Med*, **337**:734-739.

Guyre, P., Morganelli, P. and Miller, R. (1983) Recombinant immune interferon increases immunoglobulin G Fc receptors on cultured human mononuclear phagocytes. *J Clin Invest*, **72**:393-397.

Hackam, D.J., Rotstein, O.D., Schreiber, A., Zhang, W. and Grinstein, S. (1997) Rho is required for the initiation of calcium signalling and phagocytosis by Fcgamma receptors in macrophages. *J Exp Med*, **186:**955-966.

Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science*, **279:**509-514.

Hammer, S.M., Gillis, J.M., Pinkston, P. and Rose, R.M. (1990) Effect of zidovudine and granulocyte-macrophage colony-stimulating factor on human immunodeficiency virus replication in alveolar macrophages. *Blood*, **75:**1215-1219.

Hammer, S.M., Katzenstein, D.A., Hughes, M.D., Gundacker, H., Schooley, R.T., Haubrich, R.H., Henry, W.K., Lederman, M.M., Phair, J.P., Niu, M., Hirsch, M.S. and Merigan, T.C. (1996) A trial comparing nucleoside monotherapy with combination therapy in HIV-infected adults with CD4 cell counts from 200 to 500 per cubic millimeter. AIDS Clinical Trials Group Study 175 Study Team. *N Engl J Med*, **335:**1081-1090.

Hammer, S.M., Squires, K.E., Hughes, M.D., Grimes, J.M., Demeter, L.M., Currier, J.S., Eron, J.J., Feinberg, J.E., Balfour, H.H., Deyton, L.R., Chodakewitz, J.A. and Fischl, M.A. (1997) A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. *N Engl J Med*, **337:**725-733.

Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R.D., Krishna, U.M., Falck, J.R., White, M.A. and Broek, D. (1998) Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. *Science*, **279:**558-560.

Hassaine, G., Courcoul, M., Bessou, G., Barthalay, Y., Picard, C., Olive, D., Collette, Y., Vigne, R. and Decroly, E. (2001) The tyrosine kinase Hck is an inhibitor of HIV-1 replication counteracted by the viral Vif protein. *J Biol Chem 2001 May 18;276(20):*, **276:**16885-16893.

Hattori, N., Michaels, F., Fargnoli, K., Marcon, L., Gallo, R.C. and Franchini, G. (1990) The human immunodeficiency virus type 2 vpr gene is essential for productive infection of human macrophages. *Proc Natl Acad Sci USA*, **87:**8080-8084.

Hawkins, M., Pope, B., Maciver, S.K. and Weeds, A.G. (1993) Human actin depolymerizing factor mediates a pH-sensitive destruction of actin filaments. *Biochemistry*, **32:**9985-9993.

Hayashida, K., Kitamura, T., Gorman, D.M., Arai, K., Yokota, T. and Miyajima, A. (1990) Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): reconstitution of a high affinity GM-CSF receptor. *Proc Natl Acad Sci USA*, **87:**9655-9659.

Hayden, S.M., Miller, P.S., Brauweiler, A. and Bamburg, J.R. (1993) Analysis of the interactions of actin depolymerizing factor with G- and F-actin. *Biochemistry*, **32:**9994-10004.

Hecht, F.M., Grant, R.M., Petropoulos, C.J., Dillon, B., Chesney, M.A., Tian, H., Hellmann, N.S., Bandrapalli, N.I., Digilio, L., Branson, B. and Kahn, J.O. (1998) Sexual transmission of an HIV-1 variant resistant to multiple reverse-transcriptase and protease inhibitors. *N Engl J Med*, **339:**307-311.

Hercus, T., Cambareri, B., Dottore, M., Woodcock, J., Bagley, C., Vadas, M., Shannon, M. and Lopez, A. (1994) Identification of residues in the first and fourth helices of human granulocyte-macrophage colony-stimulating factor involved in biologic activity and in binding to the alpha and beta chains of its receptor. *Blood*, **83:**3500-3508.

Herna Remkema, G. and Saksela, K. (2000) Interactions of HIV-1 Nef with cellular signal transducing proteins. *Front Biosci*, **5:**D268-283.

Hewish, M.J., Meikle, A.M., Hunter, S.D. and Crowe, S.M. (1996) Quantifying phagocytosis of *Mycobacterium avium* complex by human monocytes in whole blood. *Immunol Cell Biol*, **74:**306-312.

Hiregowdara, D., Avraham, H., Fu, Y., London, R. and Avraham, S. (1997) Tyrosine phosphorylation of the related adhesion focal tyrosine kinase in megakaryocytes upon stem cell factor and phorbol myristate acetate stimulation and its association with paxillin. *J Biol Chem*, **272:**10804-10810.

Ho, D., Neumann, A., Perelson, A., Chen, W., Leonard, J. and Markowitz, M. (1995) Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature*, **373:**123-128.

Ho, D.D. (1996) Viral counts count in HIV infection. *Science*, **272:**1124-1125.

Ho, D.D. (1998) Toward HIV eradication or remission: the tasks ahead. *Science*, **280:**1866-1867.

Ho, D.D., Rota, T.R. and Hirsch, M.S. (1986) Infection of monocyte-macrophages by human T lymphotropic virus type III. *J Clin Invest*, **77:**1712-1715.

Hoy, J. (1996) Disseminated *Mycobacterium avium* complex infections and AIDS. Cambridge University Press, Cambridge.

Hsu, N., Young, L. and Bermudez, L. (1995) Response to stimulation with recombinant cytokines and synthesis of cytokines by murine intestinal macrophages infected with the *Mycobacterium avium* complex. *Infect Immun*, **63:**528-533.

Hu, R., Oyaizu, N., Kalyanaraman, V. and Pahwa, S. (1994) HIV-1 gp160 as a modifier of Th1 and Th2 cytokine response: gp160 suppresses interferon-gamma and interleukin-2 production concomitantly with enhanced interleukin-4 production in vitro. *Clin Immunol Immunopathol*, **73:**245-251.

Hufert, F.T., Schmitz, J., Schreiber, M., Schmitz, H., Racz, P. and von-Laer, D.D. (1993) Human Kupffer cells infected with HIV-1 in vivo. *J Acquir Immune Defic Syndr*, **6:**772-777.

Imami, N., Antonopoulos, C., Hardy, G., Gazzard, B. and Gotch, F. (1999) Assessment of type 1 and type 2 cytokines in HIV type-1 infected individuals: impact of highly active antiretroviral therapy. *AIDS Res Hum Retroviruses*, **15:**1499-1508.

Indik, Z.K., Hunter, S., Huang, M.M., Pan, X.Q., Chien, P., Kelly, C., Levinson, A.I., Kimberly, R.P. and Schreiber, A.D. (1994) The high affinity Fc gamma receptor (CD64) induces phagocytosis in the absence of its cytoplasmic domain: the gamma subunit of Fc gamma RIIIA imparts phagocytic function to Fc gamma RI. *Exp Hematol*, **22:**599-606.

Indik, Z.K., Park, J.G., Hunter, S., Mantaring, M. and Schreiber, A.D. (1995a) Molecular dissection of Fc gamma receptor-mediated phagocytosis. *Immunol Lett*, **44:**133-138.

Indik, Z.K., Park, J.G., Hunter, S. and Schreiber, A.D. (1995b) The molecular dissection of Fc gamma receptor mediated phagocytosis. *Blood*, **86:**4389-4399.

Indik, Z.K., Park, J.G., Pan, X.Q. and Schreiber, A.D. (1995c) Induction of phagocytosis by a protein tyrosine kinase. *Blood*, **85:**1175-1180.

Iversen, P.O., Lo, L.B. and Lopez, A.F. (1996) Apoptosis of hemopoietic cells by the human granulocyte macrophage colony-stimulating factor mutant E21R. *Proc Natl Acad Sci USA*, **93:**2785-2789.

Iversen, P., Hercus, T., Zacharakis, B., Woodcock, J., Stomski, F., Kumar, S., Nelson, B., Miyajima, A. and Lopez, A. (1997) The apoptosis inducing granulocyte-macrophage colony-stimulating factor (GM-CSF) analog E21R functions through specific regions of the heterodimeric GM-CSF receptor and requires interleukin-1 beta converting enzyme like proteases. *J Biol Chem*, **272:**9877-9883.

Jacks, T., Power, M.D., Masiarz, F.R., Luciw, P.A., Barr, P.J. and Varmus, H.E. (1988) Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. *Nature*, **331:**280-283.

Jamieson, B.D., Aldrovandi, G.M., Planelles, V., Jowett, J.B., Gao, L., Bloch, L.M., Chen, I.S. and Zack, J.A. (1994) Requirement of human immunodeficiency virus type 1 Nef for *in vivo* replication and pathogenicity. *J Virol*, **68:**3478-3485.

Jassoy, C., Harrer, T., Rosenthal, T., Navia, B.A., Worth, J., Johnson, R.P. and Walker, B.D. (1993) Human immunodeficiency virus type 1-specific cytotoxic T lymphocytes release gamma interferon, tumor necrosis factor alpha (TNF-alpha), and TNF-beta when they encounter their target antigens. *J Virol 1993*, **67:**2844-2852.

Johnson, R.T., McArthur, J.C. and Narayan, O. (1988) The neurobiology of human immunodeficiency virus infections. *FASEB J*, **2:**2970-2981.

Joiner, K.A., Fuhrman, S.A., Miettinen, H.M., Kasper, L.H. and Mellman, I. (1990) *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. *Science*, **249:**641-646.

Jordan, C.A., Watkins, B.A., Kufta, C. and Dubois-Dalcq, M. (1991) Infection of brain microglial cells by human immunodeficiency virus type-1 is CD4 dependent. *J Virol*, **65:**736-742.

Jouvin, M.H., Adamczewski, M., Numerof, R., Letourneur, O., Valle, A. and Kinet, J.P. (1994) Differential control of the tyrosine kinases Lyn and Syk by the two

signalling chains of the high affinity immunoglobulin E receptor. *J Biol Chem*, **269:**5918-5925.

Kallenius, G., Koivula, T., Rydgard, K.J., Hoffner, S.E., Valentin, A., Asjo, B., Ljungh, C., Sharma, U. and Svenson, S.B. (1992) Human immunodeficiency virus type-1 enhances intracellular growth of *Mycobacterium avium* in human macrophages. *Infect Immun*, **60:**2453-2458.

Kanakaraj, P., Duckworth, B., Azzoni, L., Kamoun, M., Cantley, L.C. and Perussia, B. (1994) Phosphatidylinositol-3 kinase activation induced upon Fc gamma RIIIA-ligand interaction. *J Exp Med*, **179:**551-558.

Kaplan, G. and Gaudermack, G. (1982) In vitro differentiation of human monocytes. Differences in monocyte phenotypes induced by cultivation on glass or on collagen. *J Exp Med*, **156:**1101-1114.

Kaplan, L., Kahn, J., Crowe, S., Northfelt, D., Neville, P., Grossberg, H., Abrams, D., Tracey, J., Mills, J. and Volberding, P. (1991) Clinical and virologic effects of recombinant human granulocyte-macrophage colony-stimulating factor (rGM-CSF) in patients receiving chemotherapy for HIV associated non-Hodgkin's lymphoma; results of a randomized trial. *J Clin Oncol*, **9:**929-940.

Karczewski, M. and Strebel, K. (1996) Cytoskeleton association and virion incorporation of the human immunodeficiency virus type 1 Vif protein. *J Virol*, **70:**494-507.

Kaslow, R.A., Carrington, M., Apple, R., Park, L., Munoz, A., Saah, A.J., Goedert, J.J., Winkler, C., O'Brien, S.J., Rinaldo, C., Detels, R., Blattner, W., Phair, J., Erlich, H. and Mann, D.L. (1996) Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat Med*, **2:**405-411.

Kazazi, F., Mathijs, J.M., Chang, J., Malafiej, P., Lopez, A., Dowton, D., Sorrell, T.C., Vadas, M.A. and Cunningham, A.L. (1992) Recombinant interleukin-4 stimulates human immunodeficiency virus production by infected monocytes and macrophages. *J Gen Virol*, **73:**941-949.

Kedzierska, K., Maerz, A., Warby, T., Jaworowski, A., Chan, H.T., Mak, J., Sonza, S., Lopez, A. and Crowe, S.M. (2000a) Granulocyte-macrophage colony-stimulating factor inhibits HIV-1 replication in monocyte-derived macrophages. *AIDS*, **14:**1739-1748.

Kedzierska, K., Mak, J., Jaworowski, A., Greenway, A., Violo, A.C., H, Hocking, J., Purcell, D., Sullivan, S., Mills, J. and Crowe, S.M. (2001a) *nef*-deleted HIV-1 inhibits phagocytosis by monocyte-derived macrophages *in vitro* but not by peripheral blood monocytes *in vivo*. *AIDS*, **15**:945-955.

Kedzierska, K., Mak, J., Mijch, A., Cooke, I., Rainbird, M., Roberts, S., Paukovics, G., Jolley, D., Lopez, A. and Crowe, S.M. (2000b) Granulocyte-macrophage colony-stimulating factor augments phagocytosis of *Mycobacterium avium* complex by human immunodeficiency virus -infected monocytes/macrophages *in vitro* and *in vivo*. *J Infect Dis*, **181**:390-394.

Kedzierska, K., Vardaxis, N.J., Jaworowski, A. and Crowe, S.M. (2001b) Fc gamma R-mediated phagocytosis by human macrophages involves Hck, Syk, and Pyk2 and is augmented by GM-CSF. *J Leukoc Biol*, **70**:322-328.

Kedzierska, K. and Crowe, S.M. (2001) Cytokines and HIV-1: interactions and clinical implications. *Antivir Chem Chemother*, **12**:133-145.

Keler, T., Wallace, P.K., Vitale, L.A., Russoniello, C., Sundarapandiyan, K., Graziano, R.F. and Deo, Y.M. (2000) Differential effect of cytokine treatment on Fc alpha receptor I- and Fc gamma receptor I-mediated tumor cytotoxicity by monocyte-derived macrophages. *J Immunol 2000*, **164**:5746-5752.

Kemper, C.A., Bermudez, L.E. and Deresinski, S.C. (1998) Immunomodulatory treatment of *Mycobacterium avium* complex bacteremia in patients with AIDS by use of recombinant granulocyte-macrophage colony-stimulating factor. *J Infect Dis*, **177**:914-920.

Kesson, A.M., Fear, W.R., Kazazi, F., Mathijs, J.M., Chang, J., King, N.J. and Cunningham, A.L. (1993) Human immunodeficiency virus type-1 infection of human placental macrophages in vitro. *J Infect Dis*, **168**:571-579.

Kestler, H.W., Ringler, D.J., Mori, K., Panicali, D.L., Sehgal, P.K., Daniel, M.D. and Desrosiers, R.C. (1991) Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell*, **65**:651-662.

Kiefer, F., Brumell, J., Al-Alawi, N., Latour, S., Cheng, A., Veillette, A., Grinstein, S. and Pawson, T. (1998) The Syk protein tyrosine kinase is essential for Fcgamma receptor signalling in macrophages and neutrophils. *Mol Cell Biol*, **18**:4209-4220.

Kiener, P.A., Rankin, B.M., Burkhardt, A.L., Schieven, G.L., Gilliland, L.K., Rowley, R.B., Bolen, J.B. and Ledbetter, J.A. (1993) Cross-linking of Fc gamma receptor I (Fc gamma RI) and receptor II (Fc gamma RII) on monocytic cells activates a signal transduction pathway common to both Fc receptors that involves the stimulation of p72 Syk protein tyrosine kinase. *J Biol Chem*, **268:**24442-24448.

Kinter, A.L., Bende, S.M., Hardy, E.C., Jackson, R. and Fauci, A.S. (1995) Interleukin-2 induces CD8+ T cell-mediated suppression of human immunodeficiency virus replication in CD4+ T cells and this effect overrides its ability to stimulate virus expression. *Proc Natl Acad Sci USA*, **92:**10985-10989.

Klein, M.R., van der Burg, S.H., Hovenkamp, E., Holwerda, A.M., Drijfhout, J.W., Melief, C.J. and Miedema, F. (1998) Characterization of HLA-B57-restricted human immunodeficiency virus type 1 Gag- and RT-specific cytotoxic T lymphocyte responses. *J Gen Virol*, **79:**2191-2201.

Knight, K.R., Vairo, G. and Hamilton, J.A. (1992) Regulation of pinocytosis in murine macrophages by colony-stimulating factors and other agents. *J Leukoc Biol*, **51:**350-359.

Kornbluth, R.S., Oh, P.S., Munis, J.R., Cleveland, P.H. and Richman, D.D. (1989) Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by human immunodeficiency virus *in vitro*. *J Exp Med*, **169:**1137-1151.

Kornbluth, R.S., Rhodes, J. and Richman, D.D. (1993) Recovery of infectious HIV following the passage of apoptotic cellular debris through phagocytic macrophages. *Chest*, **103:**102S-103S.

Kornbluth, R.S., Kee, K. and Richman, D.D. (1998) CD40 ligand (CD154) stimulation of macrophages to produce HIV-1-suppressive beta-chemokines. *Proc Natl Acad Sci USA*, **95:**5205-5210.

Koup, R.A., Safrit, J.T., Cao, Y., Andrews, C.A., McLeod, G., Borkowsky, W., Farthing, C. and Ho, D. (1994) Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol*, **68:**4650-4655.

Koyanagi, Y., O'Brien, W.A., Zhao, J.Q., Golde, D.W., Gasson, J.C. and Chen, I.S.Y. (1988) Cytokines alter production of HIV-1 from primary mononuclear phagocytes. *Science*, **241:**1673-1675.

Kreutz, M., Krause, S.W., Hennemann, B., Rehm, A. and Andreesen, R. (1992) Macrophage heterogeneity and differentiation: defined serum-free culture conditions induce different types of macrophages *in vitro*. *Res Immunol*, **143:**107-115.

Krown, S.E., Paredes, J., Bundow, D., Polsky, B., Gold, J.W. and Flomenberg, N. (1992) Interferon-alpha, zidovudine, and granulocyte-macrophage colony-stimulating factor: a phase I AIDS Clinical Trials Group study in patients with Kaposi's sarcoma associated with AIDS. *J Clin Oncol*, **10:**1344-1351.

Kusner, D.J., Hall, C.F. and Jackson, S. (1999) Fc gamma receptor-mediated activation of phospholipase D regulates macrophage phagocytosis of IgG-opsonized particles. *J Immunol*, **162:**2266-2274.

Kutza, J., Crim, L., Feldman, S., Hayes, M., Gruber, M., Beeler, J. and Clouse, K. (2000) Macrophage colony-stimulating factor antagonists inhibit replication of HIV-1 in human macrophages. *J Immunol*, **164:**4955-4960.

Lafeuillade, A., Poggi, C. and Tamalet, C. (1996) GM-CSF increases HIV-1 load. *Lancet*, **347:**1123-1124.

Lahdevirta, J., Maury, C., Teppo, A. and Repo, H. (1988) Elevated levels of circulating cachectin/tumor necrosis factor in patients with acquired immunodeficiency syndrome. *Am J Med*, **85:**289-291.

Landau, N.R., Warton, M. and Littman, D.R. (1988) The envelope glycoprotein of the human immunodeficiency virus binds to the immunoglobulin like domain of CD4. *Nature*, **334:**159-162.

Landor, M. (1998) Principles of current adult HIV pharmacotherapy. *Ann Allergy Asthma Immunol*, **80:**363-367.

Lassmann, H., Schmied, M., Vass, K. and Hickey, W.F. (1993) Bone marrow derived elements and resident microglia in brain inflammation. *Glia*, **7:**19-24.

Laursen, A., Moller, B., Rungby, J., Petersen, C. and Andersen, P. (1994) *Pneumocystis carinii* induced activation of the respiratory burst in human monocytes and macrophages. *Clin Exp Immunol*, **98:**196-202.

Le Gall, S., Prevost, M.C., Heard, J.M. and Schwartz, O. (1997) Human immunodeficiency virus type I Nef independently affects virion incorporation of major histocompatibility complex class I molecules and virus infectivity. *Virology*, **229**:295-301.

Learmont, J., Tindall, B., Evans, L., Cunningham, A., Wells, J., Penny, R., Kaldor, J. and Cooper, D.A. (1992) Long term symptomless HIV-1 infection in recipients of blood products from a single donor. *Lancet*, **340**:863-867.

Learmont, J., Geczy, A., Mills, J., Ashton, L., Raynes-Greenow, C., Garsia, R., Dyer, W., McIntyre, L., Oelrichs, R., Rhodes, D., Deacon, N. and Sullivan, J. (1999) Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1. A report from the Sydney Blood Bank Cohort. *N Engl J Med*, **340**:1715-1722.

Lee, C.H., Saksela, K., Mirza, U.A., Chait, B.T. and Kuriyan, J. (1996) Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. *Cell*, **85**:931-942.

Lee, D.J., Cox, D., Li, J. and Greenberg, S. (2000) Rac1 and Cdc42 are required for phagocytosis, but not NF-kappaB dependent gene expression, in macrophages challenged with *Pseudomonas aeruginosa*. *J Biol Chem*, **275**:141-146.

Lee, T., Sunzeri, F., Tobler, L., Williams, B. and Busch, M. (1991) Quantitative assessment of HIV-1 DNA load by coamplification of HIV-1 gag and HLA-DQ-alpha genes. *AIDS*, **5**:683-691.

Levy, J. (1993a) Pathogenesis of human immunodeficiency virus infection. *Microbiol Rev*, **57**:183-289.

Levy, J.A. (1993b) HIV pathogenesis and long-term survival. *AIDS*, **7**:1401-1410.

Levy, J.A., Hoffman, A.D., Kramer, S.M., Landis, J.A., Shimabukuro, J.M. and Oshiro, L.S. (1984) Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science*, **255**:840-842.

Lewin, S.R., Sonza, S., Irving, L.B., McDonald, C.F., Mills, J. and Crowe, S.M. (1996) Surface CD4 is critical to *in vitro* HIV infection of human alveolar macrophages. *AIDS Res Hum Retroviruses*, **12**:877-883.

Lewin, S.R., Kirihara, J., Sonza, S., Irving, L., Mills, J. and Crowe, S.M. (1998) HIV-1 DNA and mRNA concentrations are similar to peripheral blood monocytes and alveolar macrophages in HIV-1 infected individuals. *AIDS*, **12:**719-727.

Lewin, S.R., Vesanen, M., Kostrikis, L., Hurley, A., Duran, M., Zhang, L., Ho, D. and Markowitz, M. (1999) Use of real-time PCR and molecular beacons to detect virus replication in human immunodeficiency virus type 1-infected individuals on prolonged effective antiretroviral therapy. *J Virol*, **73:**6099-6103.

Li, X., Hunter, D., Morris, J., Haskill, J.S. and Earp, H.S. (1998) A calcium-dependent tyrosine kinase splice variant in human monocytes. Activation by a two-stage process involving adherence and a subsequent intracellular signal. *J Biol Chem 1998*, **273:**9361-9364.

Liuzzi, G., Chirianni, A., Clementi, M., Bagnarelli, P., Valenza, A., Cataldo, P.T. and Piazza, M. (1996) Analysis of HIV-1 load in blood, semen and saliva: evidence for different viral compartments in a cross-sectional and longitudinal study. *AIDS*, **10:**F51-F56.

Lopez, A., Shannon, M., Hercus, T., Nicola, N., Cambareri, B., Dottore, M., Layton, M., Eglington, L. and Vadas, M. (1992) Residue 21 of human granulocyte-macrophage colony-stimulating factor is critical for biological activity and for high but not low affinity binding. *EMBO J*, **11:**909-916.

Lowell, C.A., Soriano, P. and Varmus, H.E. (1994) Functional overlap in the src gene family: inactivation of hck and fgr impairs natural immunity. *Genes Dev*, **8:**387-398.

Lu, X., Wu, X., Plemenitas, A., Yu, H., Sawai, E.T., Abo, A. and Peterlin, B.M. (1996) CDC42 and Rac1 are implicated in the activation of the Nef-associated kinase and replication of HIV-1. *Curr Biol*, **6:**1677-1684.

Lucey, D.R., Pinto, L.A., Bethke, F.R., Rusnak, J., Melcher, G.P., Hashemi, F.N., Landay, A.L., Kessler, H.A., Paxton, R.J., Grabstein, K. and Shearer, G.M. (1997) *In vitro* immunologic and virologic effects of interleukin-15 on peripheral blood mononuclear cells from normal donors and human immunodeficiency virus type 1-infected patients. *Clin Diagn Lab Immunol*, **4:**43-48.

Lydyard, P. and Grossi, C. (1993) Development of the immune system. Mosby-Year Book Europe Ltd, London.

Maciaszek, J.W., Parada, N.A., Cruikshank, W.W., Center, D.M., Kornfeld, H. and Viglianti, G.A. (1997) IL-16 represses HIV-1 promoter activity. *J Immunol*, **158**:5-8.

MacMicking, J., Xie, Q.W. and Nathan, C. (1997) Nitric oxide and macrophage function. *Annu Rev Immunol*, **15**:323-350.

Macreadie, I., Castelli, L., Hewish, D., Kirkpatrick, A., Ward, A. and Azad, A. (1995) A domain of human immunodeficiency virus type 1 Vpr containing repeated H(S/F)RIG amino acid motifs causes cell growth arrest and structural defects. *Proc Natl Acad Sci USA*, **92**:2770-2774.

Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K. and Narumiya, S. (1999) Signalling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science*, **285**:895-898.

Maggi, E., Mazzetti, M., Ravina, A., Annunziato, F., de Carli, M., Piccinni, M.P., Manetti, R., Carbonari, M., Pesce, A.M. and del Prete, G. (1994) Ability of HIV to promote a TH1 to TH0 shift and to replicate preferentially in TH2 and TH0 cells. *Science*, **265**:244-248.

Mak, J., Jiang, M., Hammarskjold, M.L., Rekosh, D., Wainberg, M.A. and Kleiman, L. (1994) Role of Pr160gag-pol in mediating the selective incorporation of tRNAlys into human immunodeficiency virus type 1 particles. *J Virol*, **68**:2065-2072.

Mak, J. and Kleiman, L. (1997) Primer tRNAs for reverse transcription. *J Virol*, **71**:8087-8095.

Manser, E., Huang, H., Lou, T., Chen, X., Dong, J., Leung, T. and Lim, L. (1997) Expression of constitutively active PAK reveals effects on the kinase on actin and focal complexes. *Mol Cell Biol*, **17**:1129-1143.

Mansfield, P.J., Shayman, J.A. and Boxer, L.A. (2000) Regulation of polymorphonuclear leukocyte phagocytosis by myosin light chain kinase after activation of mitogen-activated protein kinase. *Blood*, **95**:2407-2412.

Marfaing-Koka, A., Aubin, J.T., Grangeot-Keros, L., Portier, A., Benattar, C., Merrien, D., Agut, H., Aucouturier, P., Autran, B., Wijdenes, J. (1996) *In vivo*

role of IL-6 on the viral load and on immunological abnormalities of HIV-infected patients. *J Acquir Immune Defic Syndr Hum Retrovircl*, **11**:59-68.

Marshall, J.D., Chehimi, J., Gri, G., Kostman, J.R., Montaner, L.J. and Trinchieri, G. (1999) The interleukin-12-mediated pathway of immune events is dysfunctional in human immunodeficiency virus-infected individuals. *Blood*, **94**:1003-1011.

Massol, P., Montcourrier, P., Guillemot, J.C. and Chavrier, P. (1998) Fc receptor-mediated phagocytosis requires CDC42 and Rac1. *EMBO J*, **17**:6219-6229.

Masur, H., Michelis, M.A., Greene, J.B., Onorato, I., Stouwe, R.A., Holzman, R.S., Wormser, G., Brettman, L., Lange, M., Murray, H.W. and Cunningham-Rundles, S. (1981) An outbreak of community-acquired *Pneumocystis carinii* pneumonia: initial manifestation of cellular immune dysfunction. *N Engl J Med*, **305**:1431-1438.

Matsuda, M., Park, J.G., Wang, D.C., Hunter, S., Chien, P. and Schreiber, A.D. (1996) Abrogation of the Fc gamma receptor IIA-mediated phagocytic signal by stem-loop Syk antisense oligonucleotides. *Mol Biol Cell*, **7**:1095-1106.

Matsuda, S., Akagawa, K., Honda, M., Yokota, Y., Takebe, Y. and Takemon, T. (1995) Suppression of HIV replication in human monocyte-derived macrophages induced by granulocyte-macrophage colony-stimulating factor. *AIDS Res Hum Retroviruses*, **11**:1031-1038.

Matsumoto, T., Miike, T., Nelson, R., Trudeau, W., Lockey, R. and Yodoi, J. (1993) Elevated serum levels of IL-8 in patients with HIV infection. *Clin Exp Immunol*, **93**:149-151.

Mazzarella, G., Petillo, O., Margarucci, S., Calabrese, C. and Peluso, G. (1998) Role of monocyte/macrophage population in immune response. *Monaldi Arch Chest Dis*, **53**:92-96.

McCullough, M.J., Firth, N.A. and Reade, P.C. (1997) Human immunodeficiency virus infection: a review of the mode of infection, pathogenesis, disease course, and the general and clinical manifestations. *Aust Dent J*, **42**:30-37.

McElrath, M.J., Pruett, J.E. and Cohn, Z.A. (1989) Mononuclear phagocytes of blood and bone marrow; comparative roles as viral reservoirs in human immunodeficiency virus type 1 infections. *Proc Natl Acad Sci USA*, **86**:675-679.

McIlroy, D., Autran, B., Cheynier, R., Clauvel, J.P., Oksenhendler, E., Debre, P. and Hosmalin, A. (1996) Low infection frequency of macrophages in the spleens of HIV+ patients. *Res Virol*, **147:**115-121.

McKinney, D.M., Lewinsohn, D.A., Riddell, S.R., Greenberg, P.D. and Mosier, D.E. (1999) The antiviral activity of HIV-specific CD8+ CTL clones is limited by elimination due to encounter with HIV-infected targets. *J Immunol*, **163:**861-867.

Medzhitov, R. and Janeway, C.J. (1997) Innate immunity: impact on the adaptive immune response. *Curr Opin Immunol*, **9:**4-9.

Medzhitov, R., Preston-Hurlburt, P. and Janeway, C.A.J. (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*, **388:**394-397.

Mellors, J.W., Rinaldo, C.R., Gupta, P., White, R.M., Todd, J.A. and Kingsley, L.A. (1996) Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science*, **272:**1167-1170.

Meltzer, M.S., Skillman, D.R., Gomatos, P.J., Kalter, D.C. and Gendelman, H.E. (1990) Role of mononuclear phagocytes in the pathogenesis of human immunodeficiency virus infection. *Annu Rev Immunol*, **8:**169-194.

Metcalf, D. (1985) The granulocyte-macrophage colony-stimulating factors. *Science*, **229:**16-21.

Meyaard, L., Hovenkamp, E., Keet, I., Hooibrink, B., de Jong, I., Otto, S. and Miedema, F. (1996) Single cell analysis of IL-4 and IFN-gamma production by T cells from HIV-infected individuals: decreased IFN-gamma in the presence of preserved IL-4 production. *J Immunol*, **157:**2712-2718.

Meylan, P.R., Baumgartner, M., Ciuffi, A., Munoz, M. and Sahli, R. (1998) The nef gene controls syncytium formation in primary human lymphocytes and macrophages infected by HIV type 1. *AIDS Res Hum Retroviruses*, **14:**1531-1542.

Miller, M.D., Warmerdam, M.T., Gaston, I., Greene, W.C. and Feinberg, M.B. (1994) The human immunodeficiency virus 1 nef gene product, a positive factor for viral infection and replication in primary lymphocytes and macrophages. *J Exp Med*, **179:**101-113.

Miller, M.D., Farnet, C.M. and Bushman, F.D. (1997) Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. *J Virol*, **71**:5382-5390.

Montaner, L.J., Doyle, A.G., Collin, M., Herbein, G., Illei, P., James, W., Minty, A., Caput, D., Ferrara, P. and Gordon, S. (1993) Interleukin 13 inhibits human immunodeficiency virus type 1 production in primary blood derived human macrophages *in vitro*. *J Exp Med*, **178**:743-747.

Montefiori, D., Baba, T., Li, A., Bilska, M. and Ruprecht, R. (1996) Neutralizing and infection-enhancing antibody responses do not correlate with the differential pathogenicity of SIVmac239delta3 in adult and infant rhesus monkeys. *J Immunol*, **157**:5528-5535.

Moore, K., de Waal Malefyt, R., Coffman, R. and O'Garra, A. (2001) Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*, **19**:683-765.

Moorjani, H., Craddock, B.P., Morrison, S.A. and Steibigel, R.T. (1996) Impairment of phagosome-lysosome fusion in HIV-1 infected macrophages. *J Acquir Immune Defic Syndr Hum Retrovirol*, **13**:18-22.

Moran, P.A., Diegel, M.L., Sias, J.C., Ledbetter, J.A. and Zarling, J.M. (1993) Regulation of HIV production by blood mononuclear cells from HIV- infected donors: I. Lack of correlation between HIV-1 production and T cell activation. *AIDS Res Hum Retroviruses*, **9**:455-464.

Mui, A.-F., Wakao, H., O'Farrel, A.-M., Harada, N. and Miyajima, A. (1995) Interleukin-1, granulocyte-macrophage colony-stimulating factor and interleukin-5 transduce signal through two STAT5 homologs. *EMBO J*, **14**:1166-1175.

Munoz, A., Kirby, A.J., He, Y.D., Margolick, J.B., Visscher, B.R., Rinaldo, C.R., Kaslow, R.A. and Phair, J.P. (1995) Long-term survivors with HIV-1 infection: incubation period and longitudinal patterns of CD4+ lymphocytes. *J Acquir Immune Defic Syndr Hum Retrovirol*, **8**:496-505.

Munoz-Fernandez, M., Navarro, J., Garcia, A., Punzon, C., Fernandez-Cruz, E. and Fresno, M. (1997) Replication of human immunodeficiency virus-1 in primary human T cells is dependent on the autocrine secretion of tumor necrosis factor through the control of nuclear factor-kappa B activation. *J Allergy Clin Immunol*, **100**:838-845.

Musher, D.M., Watson, D.A., Nickeson, D., Gyorkey, F., Lahart, C. and Rossen, R.D. (1990) The effect of HIV infection on phagocytosis and killing of *Staphylococcus aureus* by human pulmonary alveolar macrophages. *Am J Med Sci*, **299**:158-163.

Musson, R.A. (1983) Human serum induces maturation of human monocytes in vitro. Changes in cytolytic activity, intracellular lysosomal enzymes, and non-specific esterase activity. *Am J Pathol*, **111**:331-340.

Myones, B.L., Dalzell, J.G., Hogg, N. and Ross, G.D. (1988) Neutrophil and monocyte cell surface p150,95 has iC3b-receptor (CR4) activity resembling CR3. *J Clin Invest*, **82**:640-651.

Naif, H., Ho-Shon, M., Chang, J. and Cunningham, A.L. (1994) Molecular mechanisms of IL-4 effect on HIV expression in promonocytic cell lines and primary human monocytes. *J Leukoc Biol*, **56**:335-339.

Naif, H.M., Li, S., Ho-Shon, M., Mathijs, J.M., Willliamson, P. and Cunningham, A.L. (1997) The state of maturation of monocytes into macrophages determines the effects of IL-4 and IL-13 on HIV replication. *J Immunol*, **158**:501-511.

Naif, H.M., Li, S., Alali, M., Sloane, A., Wu, L., Kelly, M., Lynch, G., Lloyd, A. and Cunningham, A.L. (1998) CCR5 expression correlates with susceptibility of maturing monocytes to human immunodeficiency virus type-1 infection. *J Virol*, **72**:830-836.

Nathan, C.F., Prendergast, T.J., Wiebe, M.E., Stanley, E.R., Platzer, E., Remold, H.G., Welte, K., Rubin, B.Y. and Murray, H.W. (1984) Activation of human macrophages. Comparison of other cytokines with interferon gamma. *J Exp Med*, **160**:600-605.

Nemunaitis, J., Singer, J.W. and Buckner, C.D. (1988) Use of recombinant human granulocyte-macrophage colony-stimulating factor in autologous marrow transplantation for lymphoid malignancies. *Blood*, **72**:834-836.

Newman, G.W., Kelley, T.G., Gan, H., Kandil, O., Newman, M.J., Pinkston, P., Rose, R.M. and Remold, H.G. (1993) Concurrent infection of human macrophages with HIV-1 and *Mycobacterium avium* results in decreased cell viability, increased *M. avium* multiplication and altered cytokine production. *J Immunol*, **151**:2261-2272.

Newman, S.L., Mikus, L.K. and Tucci, M.A. (1991) Differential requirements for cellular cytoskeleton in human macrophage complement receptor- and Fc receptor-mediated phagocytosis. *J Immunol*, **146:**967-974.

Newman, S.L. and Gootee, L. (1992) Colony-stimulating factors activate human macrophages to inhibit intracellular growth of *Histoplasma capsulatum* yeasts. *Infect Immun*, **60:**4593-4597.

Nicholson, S., Bonecini-Almeida, M.D., Lapa e Silva, J.R., Nathan, C., Xie, Q.W., Mumford, R., Weidner, J.R., Calaycay, J., Geng, J. and Boechat, N. (1996) Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J Exp Med*, **183:**2293-2302.

Nielsen, H., Kharazmi, A. and Faber, V. (1986) Blood monocyte and neutrophil functions in the acquired immune deficiency syndrome. *Scand J Immunol*, **24:**291-196.

Ninomiya, N., Hazeki, K., Fukui, Y., Seya, T., Okada, T., Hazeki, O. and Ui, M. (1994) Involvement of phosphatidylinositol 3-kinase in Fc gamma receptor signalling. *J Biol Chem*, **269:**22732-22737.

Nokta, M. and Pollard, R. (1991) Human immunodeficiency virus infection: association with altered intracellular levels of cAMP and cGMP in MT-4 cells. *Virology*, **181:**211-217.

Nottet, H.S.L.M., de Graaf, L., de Vos, N.M., Bakker, L.J., van Strijp, J.A.G., Visser, M.R. and Verhoef, J. (1993) Phagocytic function of monocyte-derived macrophages is not affected by human immunodeficiency virus type 1 infection. *J Infect Dis*, **168:**84-91.

Nunn, M.F. and Marsh, J.W. (1996) Human immunodeficiency virus type 1 Nef associates with a member of the p21-activated kinase family. *J Virol*, **70:**6157-6161.

Oberlin, E., Amara, A., Bachelerie, F.M., Bussia, C., Virelizier, J.L., Schwartz, O., Arenzana-Seisdedos, F., Schwartz, O., Heard, J.M., Clark-Lewis, I., Legler, D.F., Loetsecher, M., Baggiolini, M. and Moser, B. (1998) The CXC chemokine SDF-1 is the ligand for LESTR/fusinn and prevents infection T-cell-line-adapted lab. *Nature*, **382:**833-835.

Ohashi, T., Arai, M., Kato, H., Kubo, M., Fuji, M., Yamamoto, N., Iwamoto, A. and Kannagi, M. (1998) High SDF-1 expression in HIV-1 carriers does not correlate with CD8+ T-cell-mediated suppression of viral replication. *Virology*, **244**:467-472.

Ohno, M., Fornerod, M. and Mattaj, I.W. (1998) Nucleocytoplasmic transport: the last 200 nanometers. *Cell*, **92**:327-336.

Olafsson, K., Smith, M.S., Marshburn, P., Carter, S.G. and Haskill, S. (1991) Variation of HIV infectibility of macrophages as a function of donor, stage of differentiation, and site of origin. *J Acquir Immune Defic Syndr*, **4**:154-164.

Orenstein, J.M., Fox, C. and Wahl, S.M. (1997) Macrophages as a source of HIV during opportunistic infections. *Science*, **276**:1857-1861.

Palella, F.J.J., Delaney, K.M., Moorman, A.C., Loveless, M.O., Fuhrer, J., Satten, G.A., Aschman, D.J. and Holmberg, S.D. (1998) Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med*, **338**:853-860.

Pan, X.Q., Darby, C., Indik, Z.K. and Schreiber, A.D. (1999) Activation of three classes of nonreceptor tyrosine kinases following Fc gamma receptor crosslinking in human monocytes. *Clin Immunol*, **90**:55-64.

Pantaleo, G., Graziosi, C. and Fauci, A.S. (1993) The immunopathogenesis of human immunodeficiency virus infection. *N Engl J Med*, **328**:327-335.

Pantalone, R.M. and Page, R.C. (1975) Lymphokine-induced production and release of lysosomal enzymes by macrophages. *Proc Natl Acad Sci USA*, **72**:2091-2094.

Parhar, R.S., Ernst, P., Sheth, K.V. and Sedairy, S.T. (1992) Anti-tumor cytotoxic potential and effect on human bone marrow GM-CFU of human LAK cells generated in response to various cytokines. *Eur Cytok Net*, **3**:299-306.

Park, J.G., Murray, R.K., Chien, P., Darby, C. and Schreiber, A.D. (1993) Conserved cytoplasmic tyrosine residues of the gamma subunit are required for a phagocytic signal mediated by Fc gamma RIIIA. *J Clin Invest*, **92**:2073-2079.

Park, J.G. and Schreiber, A.D. (1995) Determinants of the phagocytic signal mediated by the type IIIA Fc gamma receptor, Fc gamma RIIIA: sequence requirements

and interaction with protein-tyrosine kinases. *Proc Natl Acad Sci USA*, **92:**7381-7385.

Pautrat, G., Suzan, M., Salaun, D., Corbeau, P., Allasia, C., Morel, G. and Filippi, P. (1990) Human immunodeficiency virus type 1 infection of U937 cells promotes cell differentiation and a new pathway of viral assembly. *Virology*, **179:**749-758.

Pauza, C.D. (1990) Two bases are deleted from the termini of HIV-1 linear DNA during integrative recombination. *Virology*, **179:**886-889.

Peck, R. (1991) Gamma interferon induces monocyte killing of *Listeria monocytogenes* by an oxygen-dependent pathway; alpha- or beta-interferons by oxygen-independent pathways. *J Leukoc Biol*, **46:**434-440.

Perelson, A.S., Essunger, P., Cao, Y., Vesanen, M., Hurley, A., Saksela, K., Markowitz, M. and Ho, D.D. (1997) Decay characteristics of HIV-1 infected compartments during combination therapy. *Nature*, **387:**188-191.

Perno, C.F., Yarchoan, R., Cooney, D.A., Hartmann, J., Webb, D.S., Hao, Z., Mitsuya, H., Johns, D.G. and Broder, S. (1989) Replication of human immunodeficiency virus in monocytes. Granulocyte-macrophage colony-stimulating factor (GM-CSF) potentiates viral production yet enhances the antiviral effect mediated by 3'-azido-2'3'-dideoxythymidine (AZT) and other dideoxynucleoside congeners of thymidine. *J Exp Med*, **169:**933-951.

Perno, C.F., Cooney, D.A., Gao, W.Y., Hao, Z., Johns, D.G., Foli, A., Hartman, N.R., Calio, R., Broder, S. and Yarchoan, R. (1992) Effects of bone marrow stimulatory cytokines on human immunodeficiency virus replication and the antiviral activity of dideoxynucleosides in cultures of monocytes/macrophages. *Blood*, **80:**995-1003.

Perno, C.F., Aquaro, S., Rosenwirth, B., Balestra, E., Peichl, P., Billich, A., Villani, N. and Calio, R. (1994) In vitro activity of inhibitors of late stages of the replication of HIV in chronically infected macrophages. *J Leukoc Biol*, **56:**381-386.

Petit, A.J., Tersmette, M., Terpstra, F.G., de Goede, R.E., van Lier, R.A. and Miedema, F. (1988) Decreased accessory cell function by human monocytic cells after infection with HIV. *J Immunol*, **140:**1485-1489.

Pfeifer, J.D., Wick, M.J., Roberts, R.L., Findlay, K., Normark, S.J. and Harding, C.V. (1993) Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature 1993*, **361**:359-362.

Pierson, T., McArthur, J. and Siliciano, R.F. (2000) Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. *Annu Rev Immunol*, **18**:665-708.

Pittis, M.G., Prada, F., Mangano, A., Perez, L., Sternik, G., Redondo, J., Bolongna, R. and Sen, L. (1997) Monocyte phagolysosomal fusion in children born to human immunodeficiency virus infected mothers. *Pediatr Infect Dis J*, **16**:24-28.

Pittis, M.G., Sternik, G., Sen, L., Diez, R.A., Planes, N., Pirola, D. and Estevez, M.E. (1993) Impaired phagolysosomal fusion of peripheral blood monocytes from HIV infected subjects. *Scand J Immunol*, **38**:423-427.

Plata, F., Autran, B., Martins, L.P., Wain-Hobson, S., Raphael, M., Mayaud, C., Denis, M., Guillon, J.M. and Debre, P. (1987) AIDS virus specific cytotoxic T lymphocytes in lung disorders. *Nature*, **328**:348-351.

Poli, G., Orenstein, J.M., Kinter, A., Folks, T.M. and Fauci, A.S. (1989) Interferon alpha but not AZT suppresses HIV expression in chronically infected cell lines. *Science*, **244**:575-577.

Poli, G., Bressler, P., Kinter, A., Duh, E., Timmer, W.C., Rabson, A., Justement, J.S., Stanley, S. and Fauci, A.S. (1990) Interleukin-6 induces human immunodeficiency virus expression in infected monocytic cells alone and in synergy with tumor necrosis factor alpha by transcriptional and post-transcriptional mechanisms. *J Exp Med*, **172**:151-158.

Poli, G. and Fauci, A.S. (1992) The effect of cytokines and pharmacologic agents on chronic HIV infection. *AIDS Res Hum Retroviruses*, **8**:191-197.

Poli, G., Kinter, A. and Fauci, A. (1994) Interleukin-1 induces expression of the human immunodeficiency virus alone and in synergy with interleukin-6 in chronically infected U1 cells: inhibition of inductive effects by the interleukin-1 receptor antagonist. *Proc Natl Acad Sci USA*, **91**:108-112.

Pollard, V.W. and Malim, M.H. (1998) The HIV-1 Rev protein. *Annu Rev Microbiol*, **52**:491-532.

Pomerantz, R.J., Feinberg, M.B., Trono, D. and Baltimore, D. (1990) Lipopolysaccharide is a potent monocyte/macrophage specific stimulator of human immunodeficiency virus type 1 expression. *J Exp Med*, **172:**253-261.

Pope, M., Betjes, M.G., Romani, N., Hirmand, H., Cameron, P.U., Hoffman, L., Gezelter, S., Schuler, G. and Steinman, R.M. (1994) Conjugates of dendritic cells and memory T lymphocytes from skin facilitate productive infection with HIV-1. *Cell*, **78:**389-398.

Pope, M., Gezelter, S., Gallo, N., Hoffman, L. and Steinman, R.M. (1995) Low levels of HIV-1 infection in cutaneous dendritic cells promote extensive viral replication upon binding to memory CD4+ T cells. *J Exp Med*, **182:**2045-2056.

Popovic, M., Sarngadharan, M.G., Read, E. and Gallo, R.C. (1984) Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science*, **224:**497-500.

Price, R.W., Brew, B., Sidtis, J., Rosenblum, M., Scheck, A.C. and Cleary, P. (1988) The brain in AIDS; central nervous system HIV-1 infection and AIDS dementia complex. *Science*, **239:**586-592.

Quaranta, M., Camponeschi, B., Straface, E., Malorni, W. and Viora, M. (1999) Induction of interleukin-15 production by HIV-1 Nef protein: a role in the proliferation of uninfected cells. *Exp Cell Res*, **250:**112-121.

Ralph, P. (1989) Colony stimulating factors. Human Monocytes. *Eds: Zembala, M Asherson, GL. Academic Press*227-247.

Re, M., Zauli, G., Furlini, G., Ranieri, S. and La Placa, M. (1992) Progressive and selective impairment of IL-3 and IL-4 production by peripheral blood CD4+ T-lymphocytes during the course of HIV-1 infection. *Viral Immunol*, **5:**185-194.

Reece, J.C., Vardaxis, N.J., Marshall, J.A., Crowe, S.M. and Cameron, P.U. (2001) Uptake of HIV and latex particles by fresh and cultured dendritic cells and monocytes. *Immunol Cell Biol*, **79:**255-263.

Reed, L. and Muench, H. (1938) A simple method for estimating 50 percent endpoint. *Am J Hyg*, **27:**493-497.

Reed, S.G., Nathan, C.F., Pihl, D.L., Rodricks, P., Shanebeck, K., Conlon, P.J. and Grabstein, K.H. (1987) Recombinant granulocyte-macrophage colony-stimulating factor activates macrophages to inhibit *Trypanosoma cruzi* and

release hydrogen peroxide. Comparison with interferon gamma. *J Exp Med*, **166:**1734-1746.

Rhee, S.S. and Marsh, J.W. (1994) Human immunodeficiency virus type-1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. *J Virol*, **68:**5156-5163.

Rich, E.A., Chen, I.S.Y., Zack , J.A., Leonard, M.L. and O'Brien, W.A. (1992) Increased susceptibility of differentiated mononuclear phagocytes to productive infection with human immunodeficiency virus-1 (HIV-1). *J Clin Invest*, **89:**176-183.

Rinaldo, C., Huang, X.L., Fan, Z.F., Ding, M., Beltz, L., Logar, A., Panicali, D., Mazzara, G., Liebmann, J., Cottrill, M. and Gupta, P. (1995) High levels of anti-human immunodeficiency virus type-1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. *J Virol*, **69:**5838-5842.

Roilides, E. and Pizzo, P. (1992) Modulation of host defenses by cytokines; evolving adjuncts in prevention and treatment of serious infections in immunocompromised hosts. *Clin Infect Dis*, **15:**508-524.

Roilides, E., Holmes, A., Blake, C., Pizzo, P.A. and Walsh, T.J. (1993) Defective antifungal activity of monocyte-derived macrophages from human immunodeficiency virus infected children against *Aspergillus fumigatus*. *J Infect Dis*, **168:**1562-1565.

Roilides, E., Blake, C., Holmes, A., Pizzo, P.A. and Walsh, T.J. (1996) Granulocyte-macrophage colony-stimulating factor and interferon-gamma prevent dexamethasone-induced immunosuppression of antifungal monocyte activity against *Aspergillus fumigatus* hyphae. *J Med Vet Mycol*, **34:**63-66.

Rossman, M.D., Ruiz, P., Comber, P., Gomez, F., Rottem, M. and Schreiber, A.D. (1993) Modulation of macrophage Fc gamma receptors by rGM-CSF. *Exp Hematol*, **21:**177-183.

Ruco, L.P. and Meltzer, M.S. (1978) Macrophage activation for tumor cytotoxicity: increased lymphokine responsiveness of peritoneal macrophages during acute inflammation. *J Immunol*, **120:**1054-1062.

Ruprecht, R., Baba, T., Liska, V., Bronson, R., Penninck, D. and Greene, M. (1996) "Attenuated" simian immunodeficiency virus in macaque neonates. *AIDS Res Hum Retroviruses*, **12:**159-460.

Rutherford, M.S., Witsell, A. and Schook, L.B. (1993) Mechanisms generating functionally heterogeneous macrophages: chaos revisited. *J Leukoc Biol*, **53:**602-618.

Saag, M.S. and Kilby, J.M. (1999) HIV-1 and HAART: a time to cure, a time to kill. *Nat Med*, **5:**609-611.

Sadeghi, H., Weiss, L., Kazatchkine, M. and Haeffner-Cavaillon, N. (1995) Antiretroviral therapy suppresses the constitutive production of interleukin-1 associated with human immunodeficiency virus infection. *J Infect Dis*, **172:**547-550.

Saksela, K., Cheng, G. and Baltimore, D. (1995) Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. *EMBO J*, **14:**484-491.

Salahuddin, S.Z., Rose, R.M., Groopman, J.E., Markham, P.D. and Gallo, R.C. (1986) Human T lymphotropic virus type III infection of human alveolar macrophages. *Blood*, **68:**281-284.

Samson, M., Libert, F., Doranz, B., Bucker, J., Liesnard, C., Farber, C., Saragosti, S., Lapoumeroulie, C., Cognaux, J., Forceille, C., Muyldermans, G., Verhofstede, C., Burtonboy, G., Georges, M., Imui, T., Rana, S., Yi, Y., Smyth, R., Collman, R., Doms, R., Vassart, G. and Parmentier, M. (1996) Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR5 chemokine receptor gene. *Nature*, **382:**722-725.

Sarmay, G., Pecht, I. and Gergely, J. (1994) Protein-tyrosine kinase activity tightly associated with human type II Fc gamma receptors. *Proc Natl Acad Sci USA*, **91:**4140-4144.

Sasaki, H., Nagura, K., Ishino, M., Tobioka, H., Kotani, K. and Sasaki, T. (1995) Cloning and characterization of cell adhesion kinase beta, a novel protein-tyrosine kinase of the focal adhesion kinase subfamily. *J Biol Chem*, **270:**21206-21219.

Schibeci, S.D., Clegg, A.O., Biti, R.A., Sagawa, K., Stewart, G.J. and Williamson, P. (2000) HIV-Nef enhances interleukin-2 production and phosphatidylinositol 3-kinase activity in a human T cell line. *AIDS*, **14:**1701-1707.

Schlesinger, L.S. and Horwitz, M.A. (1991) Phagocytosis of *Mycobacterium leprae* by human monocyte-derived macrophages is mediated by complement receptors CR1 (CD35), CR3 (CD11b/CD18) and CR4(CD11c/CD18) and IFN-gamma activation inhibits complement receptor function and phagocytosis of the bacterium. *J Immunol*, **147:**1983-1993.

Schmidt, A. and Hall, M.N. (1998) Signalling to the actin cytoskeleton. *Annu Rev Cell Dev Biol*, **14:**305-338.

Schmidtmayerova, H., Nottet, H., Nuovo, G., Raabe, T., Flanagan, C., Dubrovsky, L., Gendelman, H., Cerami, A., Bukrinsky, M. and Sherry, B. (1996) Human immunodeficiency virus type 1 infection alters chemokine beta peptide expression in human monocytes: implications for recruitment of leukocytes into brain and lymph nodes. *Proc Natl Acad Sci USA*, **93:**700-704.

Schmitt, M.P., Steffan, A.M., Gendrault, J.L., Jaeck, D., Royer, C., Schweitzer, C., Beyer, C., Schmit, C., Auberti, A.M. and Kim, A. (1990) Multiplication of human immunodeficiency virus in primary cultures of human Kupffer cells-possible role of liver macrophage infection in the physiopathology of AIDS. *Res Virol*, **141:**143-152.

Schnittman, S.M., Vogel, S., Baseler, M., Lane, H.C. and Davey, R.T., Jr. (1994) A phase I study of interferon-alpha 2b in combination with interleukin- 2 in patients with human immunodeficiency virus infection. *J Infect Dis*, **169:**981-989.

Schuitemaker, H., Kootstra, N.A., van Oers, M.H., van Lambalgen, R., Tersmette, M. and Miedema, F. (1990) Induction of monocyte proliferation and HIV expression by IL-3 does not interfere with anti-viral activity of zidovudine. *Blood*, **76:**1490-1493.

Schuitemaker, H., Kootstra, N.A., de Goede, R.E., de Wolf, F., Miedema, F. and Tersmette, M. (1991) Monocytotropic human immunodeficiency virus type 1 (HIV-1) variants detectable in all stages of HIV-1 infection lack T cell line tropism and syncytium inducing ability in primary T cell culture. *J Virol*, **65:**356-363.

Schuitemaker, H., Kootstra, N.A., Groenink, M., De Goede, R.E., Miedema, F. and Tersmette, M. (1992a) Differential tropism of clinical HIV-1 isolates for primary monocytes and promonocytic cell lines. *AIDS Res Hum Retovir*, **8:**1679-1682.

Schuitemaker, H., Kootstra, N.A., Koppelman, M.H., Bruisten, S.M., Huisman, H.G., Tersmette, M. and Miedema, F. (1992b) Proliferation-dependent HIV-1 infection of monocytes occurs during differentiation into macrophages. *J Clin Invest*, **89:**1154-1160.

Schuitemaker, H., Kootstra, N.A., Fouchier, R.A., B, H. and Miedema, F. (1994) Productive HIV-1 infection of macrophages restricted to the cell fraction with proliferative capacity. *EMBO*, **13:**5929-5936.

Schwartz, O., Marechal, V., Danos, O. and Heard, J.M. (1995) Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell. *J Virol*, **69:**4053-4059.

Schwartz, O., Marechal, V., Le Gall, S., Lemonnier, F. and Heard, J.M. (1996) Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nat Med*, **2:**338-342.

Segura, I., Delmelle-Vibaut, C., Janssens, M., Cleuter, Y., van den Broeke, A., Kettmann, R. and Willard-Gallo, K. (1999) Human immunodeficiency virus type 2 produces a defect in CD3-gamma gene transcripts similar to that observed for human immunodeficiency virus type-1. *J Virol*, **73:**5207-5213.

Shankarappa, R., Margolick, J.B., Gange, S.J., Rodrigo, A.G., Upchurch, D., Farzadegan, H., Gupta, P., Rinaldo, C.R., Learn, G.H., He, X., Huang, X.L. and Mullins, J.I. (1999) Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type-1 infection. *J Virol*, **73:**10489-10502.

Shapiro, L., Puren, A.J., Barton, H.A., Novick, D., Peskind, R.L., Shenkar, R., Gu, Y., Su, M.S. and Dinarello, C.A. (1998) Interleukin-18 stimulates HIV type-1 in monocytic cells. *Proc Natl Acad Sci USA*, **95:**12550-12555.

Shearer, G.M. and Clerici, M. (1991) Early T-helper cell defects in HIV infection. *AIDS*, **5:**245-253.

Shehu-Xhilaga, M., Crowe, S. and Mak, J. (2001) Maintenance of the Gag/Gag-Pol ratio is important for human immunodeficiency virus type-1 RNA dimerization and viral infectivity. *J Virol*, **75**:1834-1841.

Shiratsuchi, H., Johnson, J.L., Toossi, Z. and Ellner, J.J. (1994) Modulation of the effector function of human monocytes for *Mycobacterium avium* by human immunodeficiency virus-1 envelope glycoprotein gp120. *J Clin Invest*, **93**:885-891.

Siegal, F.P., Lopez, C., Hammer, G.S., Brown, A.E., Kornfeld, S.J., Gold, J., Hassett, J., Hirschman, S.Z., Cunningham-Rundles, C. and Adelsberg, B.R. (1981) Severe acquired immunodeficiency in male homosexuals, manifested by chronic perianal ulcerative herpes simplex lesions. *N Engl J Med*, **305**:1439-1444.

Skowron, G., Stein, D., Drusano, G., Melbourne, K., Bilello, J., Mikolich, D., Rana, K., Agosti, J., Mongillo, A., Whitmore, J. and Gilbert, M. (1999) The safety and efficacy of granulocyte-macrophage colony-stimulating factor (Sargramostim) added to indinavir- or ritonavir-based antiretroviral therapy: a randomized double-blind, placebo-controlled trial. *J Infect Dis*, **180**:1064-1071.

Smith, P., Lamerson, C., Banks, S., Saini, S., Wahl, L., Calderone, B. and Wahl, S. (1990) Granulocyte-macrophage colony-stimulating factor augments human monocyte fungicidal activity for *Candida albicans*. *J Infect Dis*, **161**:999-1005.

Smithgall, M.D., Wong, J.G., Critchett, K.E. and Haffar, O.K. (1996) IL-7 up-regulates HIV-1 replication in naturally infected peripheral blood mononuclear cells. *J Immunol*, **156**:2324-2330.

Sodroski, J., Goh, W.C., Rosen, C. and al, e. (1986) Role of HTLV-III/LAV envelope in syncytium formation and cytopathicity. *Nature*, **322**:470.

Sonza, S., Maerz, A., Uren, S., Violo, A., Hunter, S., Boyle, W. and Crowe, S. (1995) Susceptibility of human monocytes to HIV type 1 infection *in vitro* is not dependent on their level of CD4 expression. *AIDS Res Hum Retroviruses*, **11**:769-776.

Sonza, S., Maerz, A., Deacon, N., Meanger, J., Mills, J. and Crowe, S. (1996) Human immunodeficiency virus type-1 replication is blocked prior to reverse transcription and integration in freshly isolated peripheral blood monocytes. *J Virol*, **70**:3863-3869.

Sonza, S., Mutimer, H.P., Oelrichs, R., Jardine, D., Harvey, K., Dunne, A., Purcell, D.F., Birch, C. and Crowe, S.M. (2001) Monocytes harbour replication-competent, non-latent HIV-1 in patients on highly active antiretroviral therapy. *AIDS 2001*, **15**:17-22.

Stahl, P.D. and Ezekowitz, R.A. (1998) The mannose receptor is a pattern recognition receptor involved in host defense. *Curr Opin Immunol*, **10**:50-55.

Stefanova, I., Saville, M., Peters, C., Cleghorn, F., Schwartz, D., Venzon, D., Weinhold, K., Jack, N., Bartholomew, C., Blattner, W., Yarchoan, R., Bolen, J. and Horak, I. (1996) HIV infection-induced posttranslational modification of T cell signalling molecules associated with disease progression. *J Clin Invest*, **98**:1290-1297.

Stent, G., Irving, L., Lewin, S. and Crowe, S. (1995) The kinetics of surface expression of CD11/CD18 integrins and CD54 on monocytes and macrophages. *Clin Exp Immunol*, **100**:366-376.

Stevenson, M., Meier, C., Mann, A.M., Chapman, N. and Wasiak, A. (1988) Envelope glycoprotein of HIV induces interference and cytolysis resistance in CD4+ cells; mechanism for persistence in AIDS. *Cell*, **53**:483-496.

Stevenson, M., Stanwick, T.L., Dempsey, M.P. and Lamonica, C.A. (1990) HIV-1 replication is controlled at the level of T cell activation and proviral integration. *EMBO J*, **9**:1551-1560.

Stoler, M.H., Eskin, T.A., Benn, S., Angerer, R.C. and Angerer, L.M. (1986) Human T-cell lymphotropic virus type III infection of the central nervous system. A preliminary in situ analysis. *J Am Med Assoc*, **256**:2360-2364.

Strzelecka, A., Pyrzynska, B., Kwiatkowska, K. and Sobota, A. (1997) Syk kinase, tyrosine-phosphorylated proteins and actin filaments accumulate at forming phagosomes during Fc gamma receptor-mediated phagocytosis. *Cell Motil Cytoskeleton*, **38**:287-296.

Sundstrom, C. and Nilsson, K. (1976) Establishment and characterization of a human histiocytic lymphoma cell line (U937). *Int J Cancer*, **17**:565-577.

Suzuki, K., Lee, W., Hashimoto, T., Tanaka, E., Murayama, T., Amitani, R., Yamamoto, K. and Kuze, F. (1994) Recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) or tumour necrosis factor alpha (TNF-

alpha) activate human alveolar macrophages to inhibit growth of *Mycobacterium avium* complex. *Clin Exp Immunol*, **98:**169-173.

Swanson, J.A., Johnson, M.T., Beningo, K., Post, P., Mooseker, M. and Araki, N. (1999) A contractile activity that closes phagosomes in macrophages. *J Cell Sci*, **112:**307-316.

Swingler, S., Mann, A., Jacque, J., Brichacek, B., Sasseville, V., Williams, K., Lackner, A., Janoff, E., Wang, R., Fisher, D. and Stevenson, M. (1999) HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. *Nat Med*, **9:**997-1003.

Takai, T., Li, M., Sylvestre, D., Clynes, R. and Ravetch, J. (1994) FcR gamma chain deletion results in pleiotrophic effector cell defects. *Cell 1994*, **76:**519-529.

Takeshita, S., Breen, E.C., Ivashchenko, M., Nishanian, P.G., Kishimoto, T., Vredevoe, D.L. and Martinez-Maza, O. (1995) Induction of IL-6 and IL-10 production by recombinant HIV-1 envelope glycoprotein 41 (gp41) in the THP-1 human monocytic cell line. *Cell Immunol*, **165:**234-242.

Tas, M., Drexhage, H.A. and Goudsmit, J. (1988) A monocyte chemotaxis inhibiting factor in serum of HIV-infected men shares epitopes with HIV transmembrane protein gp41. *Clin Exp Immunol*, **71:**13-18.

Temin, H.M. and Mizutani, S. (1970) RNA dependent DNA polymerase in virions of Rous sarcoma virus. *Nature*, **226:**1211-1213.

Thomas, C.A., Weinberger, O.K., Ziegler, B.L., Greenberg, S., Schieren, I., Silverstein, S.C. and El Khoury, J. (1997) Human immunodeficiency virus-1 env impairs Fc receptor-mediated phagocytosis via a cyclic adenosine monophosphate-dependent mechanism. *Blood*, **90:**3760-3765.

Thomas, C.A., Li, Y., Kodama, T., Suzuki, H., Silverstein, S.C. and El Khoury, J. (2000) Protection form lethal gram-positive infection by macrophage scavenger receptor-dependent phagocytosis. *J Exp Med*, **191:**147-156.

Thomas, S.A. and Segal, M.B. (1998) The transport of the anti-HIV drug, 2'3'-didehydro-3'-deoxythymidine (d4T), across the blood-brain and blood cerebrospinal fluid barrier. *Brit J Pharm*, **125:**49-54.

Tornatore, C., Nath, A., Amemiya, K. and Major, E.D. (1991) Persistent human immunodeficiency virus type-1 infection in human fetal glial cells reactivated by

T cell factor(s) or by the cytokines tumor necrosis factor alpha and interleukin-1 beta. *J Virol*, **65**:6094-6100.

Trimble, L.A. and Lieberman, J. (1998) Circulating CD8 T lymphocytes in human immunodeficiency virus-infected individuals have impaired function and downmodulate CD3 zeta, the signalling chain of the T-cell receptor complex. *Blood*, **91**:585-594.

Trkola, A., Dragic, T., Arthos, J., Binley, J.M., Olson, W.C., Allaway, G.P., Cheng-Mayer, C., Robinson, J., Maddon, P.J. and Moore, J.P. (1996) CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature*, **384**:184-187.

Turner, B.G. and Summers, M.F. (1999) Structural biology of HIV. *J Mol Biol*, **285**:1-32.

Turner, C.E. and Burridge, K. (1991) Transmembrane molecular assemblies in cell-extracellular matrix interactions. *Curr Opin Cell Biol*, **3**:849-853.

Turner, C.E. (1998) Paxillin. *Int J Biochem Cell Biol*, **30**:955-959.

Turner, C., Brown, M., Perrotta, J., Riedy, M., Nikolopoulos, S., McDonald, A., Bagrodia, S., Thomas, S. and Leventhal, P. (1999) Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: A role in cytoskeletal remodelling. *J Cell Biol*, **145**:851-863.

Tuttle, D.L., Harrison, J.K., Anders, C., Sleasman, J.W. and Goodenow, M.M. (1998) Expression of CCR5 increases during monocyte differentiation and directly mediates macrophage susceptibility to infection by human immunodeficiency virus type 1. *J Virol*, **72**:4962-4969.

Vadas, M.A., Nicola, N.A. and Metcalf, D. (1983) Activation of antibody-dependent cell-mediated cytotoxicity of human neutrophils and eosinophils by separate colony-stimulating factors. *J Immunol*, **130**:795-799.

van Furth, R., Raeburn, J.A. and van Zwet, T.L. (1979) Characteristics of human mononuclear phagocytes. *Blood*, **54**:485-500.

van Vugt, M., Heijnen, A., Capel, P., Park, S., Ra, C., Saito, T., Verbeek, J. and van de Winkel, J. (1996) FcR gamma-chain is essential for both surface expression and function of human Fc gamma RI (CD64) *in vivo*. *Blood*, **87**:3593-3599.

Vazeux, R., Brousse, N., Jarry, A., Henin, D., Marche, C., Vedrenne, C., Mikol, J., Wolff, M., Michon, C and Rozenbaum, W. (1987) AIDS subacute encephalitis. Identification of HIV-infected cells. *Am J Pathol*, **126:**403-410.

Vazquez, J.A., Gupta, S. and Villanueva, A. (1998) Potential utility of recombinant human GM-CSF as adjunctive treatment of refractory oropharyngeal candidiasis in AIDS patients. *Eur J Clin Microbiol Infect Dis*, **17:**781-783.

Vodicka, M.A., Koepp, D.M., Silver, P.A. and Emerman, M. (1998) HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. *Genes Dev*, **12:**175-185.

Vyakarnam, A., McKeating, J., Meager, A. and Beverley, P.C. (1990) Tumour necrosis factors (alpha, beta) induced by HIV-1 in peripheral blood mononuclear cells potentiate virus replication. *AIDS*, **4:**21-27.

Wagner, R.P., Levitz, S.M., Tabuni, A. and Kornfeld, H. (1992) HIV-1 envelope protein (gp120) inhibits the activity of human bronchoalveolar macrophages against *Cryptococcus neoformans*. *Am Rev Respir Dis*, **146:**1434-1438.

Wahl, S.M., Allen, J.B., Gartner, S., Orenstein, J.M., Popovic, M., Chenoweth, D.E., Arthur, L.O., Farrar, W.L. and Wahl, L.M. (1989) HIV-1 and its envelope glycoprotein down regulate chemotactic ligand receptors and chemotactic function of peripheral blood monocytes. *J Immunol*, **142:**3553-3559.

Wang, A., Scholl, P. and Geha, R. (1994) Physical and functional association of the high affinity immunoglobulin G receptor (Fc gamma RI) with the kinases Hck and Lyn. *J Exp Med*, **180:**1165-1170.

Wang, J., Roderiquez, G., Oravecz, T. and Norcross, A. (1998) Cytokine regulation of human immunodeficiency virus type-1 entry and replication in human monocytes/macrophages through modulation of CCR5 expression. *J Virol*, **72:**7642-7647.

Wang, W.K., Chen, M.Y., Chuang, C.Y., Jeang, K.T. and Huang, L.M. (2000) Molecular biology of human immunodeficiency virus type 1. *J Microbiol Immunol Infect*, **33:**131-140.

Watkins, B.A., Dorn, H.H., Kelly, W.B., Armstrong, R.C., Potts, B.J., Michaels, F., Kuffa, C.V. and Dubois-Dalcq, M. (1990) Specific tropism of HIV-1 for microglial cells in primary human brain cultures. *Science*, **249:**549-553.

Watts, N., Sackett, D., Ward, R., Miller, M., Wingfield, P., Stahl, S. and Steven, A. (2000) HIV-1 Rev depolymerizes microtubules to form stable bilayered rings. *J Cell Biol*, **150:**349-360.

Wehle, K., Schirmer, M., Dunnebacke-Hinz, J., Kupper, T. and Pfitzer, P. (1993) Quantitative differences in phagocytosis and degradation of *Pneumocystis carinii* by alveolar macrophages in AIDS and non-HIV patients *in vivo*. *Cytopathology*, **4:**231-236.

Wei, P., Garber, M.E., Fang, S.M., Fischer, W.H. and Jones, K.A. (1998) A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell*, **92:**451-462.

Wei, X., Ghosh, S., Taylor, M., Johnson, V., Emini, E., Deutsch, P., Lifson, J.D., Bonhoeffer, S., Nowak, M., Hahn, B., Saag, M. and Shaw, G. (1995) Viral dynamics in human immunodeficiency virus type-1 infection. *Nature*, **373:**117-122.

Weissman, D., Poli, G. and Fauci, A.S. (1994) Interleukin-10 blocks HIV replication in macrophages by inhibiting the autocrine loop of tumor necrosis factor alpha and interleukin-6 induction of virus. *AIDS Res Hum Retrovir*, **10:**1199-1206.

Weissman, D., Poli, G. and Fauci, A.S. (1995) IL-10 synergizes with multiple cytokines in enhancing HIV production in cells of monocytic lineage. *J Acquir Immune Defic Syndr Hum Retrovirol*, **9:**442-449.

Weissman, D., Daucher, J., Barker, T., Adelsberger, J., Baseler, M. and Fauci, A.S. (1996) Cytokine regulation of HIV replication induced by dendritic cell-CD4-positive T cell interactions. *AIDS Res Hum Retroviruses*, **12:**759-767.

Wells, A.L., Lin, A.W., Chen, L.Q., Safer, D., Cain, S.M., Hasson, T., Carragher, B.O., Milligan, R.A. and Sweeney, H.L. (1999) Myosin VI is an actin-based motor that moves backwards. *Nature*, **401:**505-508.

Wesselingh, S.L., Glass, J., McArthur, J.C., Griffin, J.W. and Griffin, D.E. (1994) Cytokine dysregulation in HIV-associated neurological disease. *Adv Neuroimmunol*, **4:**199-206.

Wesselingh, S.L. and Thompson, K.A. (2001) Immunopathogenesis of HIV-associated dementia. *Curr Opin Neurol*, **14:**375-379.

Wiley, C.A., Schrier, R.D., Nelson, J.A., Lampert, P.W. and Oldstone, M.B. (1986) Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. *Proc Natl Acad Sci USA*, **83:**7089-7093.

Williams, M.A., Newland, A.C. and Kelsey, S.M. (1997) Monocyte mediated killing of human leukaemia is enhanced by administration of granulocyte-macrophage colony-stimulating factor following chemotherapy. *Brit J Haematol*, **98:**960-968.

Wiltschke, C., Krainer, M., Wagner, A., Linkesch, W. and Zielinski, C.C. (1995) Influence of in vivo administration of GM-CSF and G-CSF on monocyte cytotoxicity. *Exp Hematol*, **23:**402.

Wing, E.J., Magee, M., Whiteside, T.L., Kaplan, S.S. and Shadduck, R.K. (1989) Recombinant human granulocyte-macrophage colony-stimulating factor enhances monocyte cytotoxicity and secretion of tumor necrosis factor alpha and interferon in cancer patients. *Blood*, **73:**643.

Wong, J.K., Hezareh, M., Gunthard, H.F., Havlir, D.V., Ignacio, C.C., Spina, C.A. and Richman, D.D. (1997) Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science*, **278:**1291-1295.

Wright, S.D., Levin, S.M., Jong, M.T., Chad, Z. and Kabbash, L.G. (1989) CR3 (CD11b/CD18) express one binding site for Arg-Gly-Asp-containing peptides and a second site for bacterial lipopolysoccharide. *J Exp Med*, **169:**175-183.

Wright, S.D., Rao, P.E., Van Voorhis, W.C., Craigmle, L.S., Lida, K., Talle, M.A., Westberg, E.F., Goldstein, G. and Silverstein, S.C. (1983) Identification of the C3bi receptor of human monocytes and macrophages by using monoclonal antibodies. *Proc Natl Acad Sci USA*, **80:**5699-5703.

Wu, J., Zhao, Q., Kurosaki, T. and Weiss, A. (1997) The Vav binding site (Y315) in ZAP-70 is critical for antigen receptor- mediated signal transduction. *J Exp Med*, **185:**1877-1882.

Wyatt, R. and Sodroski, J. (1998) The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science*, **280:**1884-1888.

Xu, X. and Chong, A.S. (1996) Vav in natural killer cells is tyrosine phosphorylated upon cross- linking of Fc gamma RIIIA and is constitutively associated with a serine/threonine kinase. *Biochem J*, **318:**527-532.

Yakrus, M. and Good, R. (1990) Geographic distribution, frequency and specimen source of Mycobacterium avium complex serotypes isolated from patients with acquired immunodeficiency syndrome. *J Clin Microbiol*, **28:**926.

Yaku, H., Sasaki, T. and Takai, Y. (1994) The Dbl oncogene product as a GDP/GTP exchange protein for the Rho family: its properties in comparison with those of Smg GDS. *Biochem Biophys Res Commun*, **198:**811-817.

Yarchoan, R., Pluda, J.M., Perno, C.F., Mitsuya, H., Thomas, R.V., Wyvill, K.M. and Broder, S. (1990) Initial clinical experience with dideoxynucleosides as single agents and in combination therapy. *Ann NY Acad Sci*, **616:**328-343.

Yoshioka, M., Bradley, W.G., Shapshak, P., Nagano, I., Stewart, R.V., Xin, K.Q., Srivastava, A.K. and Nakamura, S. (1995) Role of immune activation and cytokine expression in HIV-1 associated neurologic diseases. *Adv Neuroimmunol*, **5:**335-358.

Zagury, D., Bernard, J., Leonard, R., Cheynier, R., Feldman, M., Sarin, P.S. and Gallo, R.C. (1986) Long term cultures of HTLV-III infected T cells; a model of cytopathology of T cell depletion in AIDS. *Science*, **231:**850-853.

Zerlauth, G., Maier, E., Chehadeh, H., Zimmermann, K., Eibl, M. and Mannhalter, J. (1995) Interaction with human immunodeficiency virus type-1 modulates innate effector functions of human monocytes. *J Infect Dis*, **172:**1598-1601.

Zhang, L., Ramratnam, B., Tenner-Racz, K., He, Y., Vesanen, M., Lewin, S., Talal, A., Racz, P., Perelson, A.S., Korber, B.T., Markowitz, M. and Ho, D. (1999) Quantifying residual HIV-1 replication in patients receiving combination antiretroviral therapy. *N Engl J Med*, **340:**1605-1613.

Zheleznyak, A. and Brown, E.J. (1992) Immunoglobulin-mediated phagocytosis by human monocytes requires protein kinase C activation. Evidence for protein kinase C translocation to phagosomes. *J Biol Chem*, **267:**12042-12048.

Ziegler-Heitbrock, H.W.L. and Ulevitch, R.J. (1993) CD14: cell surface receptor and differentiation marker. *Immunol Today*, **14:**121-124.