GENERATION OF CD8+ T CELL IMMUNITY
WITH HELP FROM CD4+ T CELLS

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ABSTRACT

Cross-presentation describes the presentation of exogenous antigens into the major histocompatibility complex (MHC) class I restricted pathway as a result of intracellular processing events. This mechanism has been proven to be important in induction of cytotoxic T lymphocyte (CTL) immunity, a process referred to as cross-priming, and in recognition of self-antigen for cross-tolerance. Cross-priming was shown to rely on presentation by bone marrow-derived cells, probably dendritic cells, and CD4⁺ helper T cells. Although cross-tolerance is much less well characterised, it has been speculated to be induced by a distinct type of APC or, alternatively, by a lack of costimulatory signals. To gain further insights into the mechanism for CTL cross-priming, I have examined factors involved in cross-priming versus cross-tolerance, including the nature of the help from CD4⁺ T cells, identification of the cross-presenting APC and the antigenic requirements during cross-presentation.

The helper requirement for induction of CTL-mediated autoimmunity was examined in transgenic RIP-OVA⁺ mice. In this model, diabetes induction was dependent on class I-restricted recognition of OVA on both bone marrow-derived APCs and the islet cells. In addition, transgenic OVA-specific CD8⁺ T cells from OT-I mice required help from in vitro activated OVA-specific CD4⁺ T cells to cause diabetes. Two kinds of CD4⁺ T cell help were observed. One was CD40L-dependent and appeared when low numbers of OT-I cells were transferred with activated CD40L⁺ OVA specific OT-II cells into the recipients. The other was CD40L-independent and was evident when high frequencies of OT-I cells were transferred with CD40L⁻/⁻ OT-II cells. In addition, anti-CD40 Ab was unable to assist naïve OT-I cells to cause diabetes. While, it helped when both naïve OT-I and low numbers of CD40L⁺OT-II cells were provided. Moreover, contrary to previous studies, generation of autoimmunity did not require co-recognition of the same APC by the CD4⁺ and CD8⁺ T cells, which provided the first evidence
indicating that there is a component of CD4⁺ T cell help that does not require linked recognition of the APC. Nonetheless, although the requirement for class II-restricted recognition was not essential, it optimized the efficiency of the CD4⁺ T cell help when available.

Attempts at identifying the cross-priming APC confirmed that CD8⁺ DCs were most likely responsible for the activation of CD8⁺ T cells. However, this DC subpopulation also appeared responsible for activating the same OT-I cells in cross-tolerance. This suggests that the maturation status of an APC rather than a distinct APC subpopulation largely determines the subsequent outcome of cross-presentation. Finally, in examining the antigenic requirement for cross-priming, I observed that cell-associated antigen was much more efficiently presented than soluble antigen when administrated intravenously. Overall, the thesis describes the optimum helper and antigenic requirements for cross-presentation and provides some insight into the mechanisms that select priming versus tolerization.
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The work presented in this thesis was performed at The Walter and Eliza Hall Institute of Medical Research, under the supervision of Dr. William Heath and Dr. Frank Carbone from Melbourne University. Ming Li was supported by Monash International Postgraduate Research Scholarship (IPRS) and Monash Research Scholarship (MRS).

This thesis contains no material that has been previously submitted for the award of any other degree or diploma. The experiments presented in this thesis comprise the candidate's original work, except where due acknowledgment and explanation has been made in the text of the thesis. Therefore, the candidate's overall contribution to the results is estimated to be about 95%. The thesis is less than 100,000 word in length exclusive of tables, graphs and bibliography.

Ming Li
PUBLICATIONS

Part of the work described in this thesis has been published or prepared for publication as follows:


5. Ming Li, Gayle M. Davey, Richard Flavell, Francis R. Carbone and William R. Heath. Effector CTLs require distinct CD4+ T cell help to that need for naïve CTL
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ABBREVIATIONS

Ab  antibody
Ag  antigen
AICD  activation-induced cell death
APCs  antigen-presenting cells
ATP  adenosine triphosphate
β₂m  β₂-microglobulin
BM  bone marrow
B6  C57BL/6
BSA  bovine serum albumin
BSS  balanced salt solution
CD  cluster of differentiation
CD40L  CD40 ligand, or CD154
cDNA  complementary DNA
CFA  complete Freund’s adjuvant
CFSE  5,6-carboxyfluoresceine -diacetate -succinimidyl -ester
Ci  curie
cm  centimetre
CLIP  class II-associated invariant chain peptide
Con A  concanavalin A
CPM  counts per minutes
CTL  cytotoxic T lymphocyte
CTLA-4  cytotoxic T lymphocyte antigen-4
DC  dendritic cell
DMSO  dimethyl sulphoxide
DNA  deoxyribonucleic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis(β-aminoethyl ether)-N,N',N'-tetra-acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated protein kinases</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FSC</td>
<td>forward light scatter</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>G</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>gB</td>
<td>glycoprotein B</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>H-2</td>
<td>histocompatibility-2, mouse major histocompatibility complex</td>
</tr>
<tr>
<td>HEM</td>
<td>HEPES Eagle's medium</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'2-ethane sulfonic acid</td>
</tr>
<tr>
<td>HEV</td>
<td>high endothelial venules</td>
</tr>
<tr>
<td>HLA</td>
<td>human leucocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>heat stable antigen</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>³H-TdR</td>
<td>[³H]-thymidine</td>
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<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ICOS</td>
<td>the inducible co-stimulatory molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>Ii</td>
<td>invariant chain</td>
</tr>
<tr>
<td>IL-</td>
<td>interleukin-</td>
</tr>
<tr>
<td>IL-2R</td>
<td>interleukin-2 receptor</td>
</tr>
<tr>
<td>ITAMs</td>
<td>immunoreceptor tyrosine-based activation motifs</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun amino-terminal kinases</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>l</td>
<td>litre</td>
</tr>
<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LFA</td>
<td>lymphocyte function-associated antigen</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>m</td>
<td>milli (10⁻³)</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>μ</td>
<td>micro (10⁻⁶)</td>
</tr>
<tr>
<td>μm</td>
<td>micrometre</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MLR</td>
<td>mixed lymphocyte reaction</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
</tbody>
</table>
nm  nanometer
nM  nanomolar
*Rag-1*"gBT-I  gBT-I mice, the herpes simplex virus glycoprotein B-specific CD8 TCR transgenic mice
*Rag-1*" OT-I  OT-I mice, OVA-specific CD8 TCR transgenic mice
*Rag-1*"OT-II  OT-II mice, OVA-specific CD4 TCR transgenic mice
OVA  ovalbumin
OCS  OVA-coated spleen cells
PBS  phosphate buffered saline
PE  R-Phycoerythrin
PI  propidium iodide
PMA  phorbol myristate acetate
PTKs  protein tyrosine kinases
*Rag*  the recombination activating genes, *Rag-1* and *Rag-2*
RANKL  receptor activator of NFκB ligand
RCRB  red blood cell removal buffer
RF10  RPMI 1640 containing 10% FCS, 50 μM 2-mercaptoethanol and 2 mM L-glutamine
RIP  rat insulin promoter
RNA  ribonucleic acid
RT  room temperature
SAC  splenic adherent cell
s.c.  subcutaneous
SEA  *Staphylococcus aureus* enterotoxin A
SEB  *Staphylococcus aureus* enterotoxin B
SCID  severe combined immunodeficiency
TAP  transporter associated with antigen processing
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TCR/CD3</td>
<td>complex of T cell receptor and CD3 molecule</td>
</tr>
<tr>
<td>Th0/1/2</td>
<td>T helper 0/T helper 1/T helper 2</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TRANCE</td>
<td>TNF-related activation-induced cytokine</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
</tr>
<tr>
<td>wk</td>
<td>week</td>
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<tr>
<td>w/v</td>
<td>weight/volume</td>
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Chapter 1 Literature Review

1.1 Antigen processing and presentation

To initiate an efficient immune response against an antigen, naive T cells must undergo activation and differentiation in response to peptidic antigens presented on specialized antigen presentation-cells (APC) (Carbone and Bevan, 1990; Germain and Margulies, 1993; Ingulli et al., 1997; Morrison et al., 1986; Sigal et al., 1999; Zinkernagel and Doherty, 1974). This is achieved through delivery of antigenic peptides from different intracellular compartments to the T cells by two distinct major histocompatibility molecules (MHC), namely MHC class-I and class-II. The mechanism that converts the native protein into peptides and leads to their association with MHC molecules is referred to as antigen processing. The process of displaying these peptides on the cell surface with MHC molecules is referred to as antigen presentation (Bevan, 1987; Klein et al., 1993; Neefjes and Momburg, 1993; Rock, 1996; Sigal and Rock, 2000). The MHC class-I restricted presentation pathway is often termed the "endogenous" pathway in reference to the usually intracellular nature of the antigen, whereas the MHC class-II restricted presentation pathway, is referred to as the "exogenous" pathway because of the "extracellular" or "exogenous" source of its antigen. Under some circumstances, exogenous antigens can enter the MHC class I pathway, so that they can be presented to CD8+ T cells (Bevan, 1976b). This process is termed cross-presentation (Carbone et al., 1998; Heath et al., 1998; Huang et al., 1994; Sigal et al., 1999). Elucidation of the mechanisms underlying MHC-restricted antigen presentation may provide us with a better understanding how the adaptive immune response is generated. In this part of the introduction, I will discuss the different pathways for antigen processing and presentation, with particular emphasis on cross-presentation.
1.1.1 Structure of MHC Class I and Class II molecules

MHC class I molecules are heterodimers made up of a membrane-spanning heavy (α) chain of approximately 43 kDa, encoded by a gene located in the MHC, and a smaller non-covalently associated chain, β2-microglobulin (β2m), of approximately 12 kDa, which is not encoded in the MHC. The extra-cellular portion of the class I heavy chain folds into three domains, α1, α2 and α3. The α3 domain and β2m fold into a structure similar to that of the immunoglobulin constant domain. The CD8-binding site is in the α3 domain. The α1 and α2 domains fold together to generate the peptide-binding groove, which consists of an eight-stranded β-pleated sheet bounded by two α helices (Bjorkman et al., 1987a; Bjorkman et al., 1987b; Madden, 1995a). This structure generates a cleft on the surface of the molecule forming the site for peptide binding. This is the contact point for the T cell receptor (TCR) on CD8+ T cells (Bjorkman et al., 1987a). The overall structure of the MHC I molecules is conserved between human and mice (Bjorkman et al., 1987b; Zhang et al., 1992).

MHC Class II molecules are composed of a heterodimer of non-covalently associated transmembrane glycoproteins, α (34 kDa) and β chains (29 kDa). Each chain is composed of two domains, referred to as α1 and α2, and β1 and β2. The two chains form a compact structure with four-domains similar to that of MHC class I molecules. The peptide binding groove is formed from the α1 and β1 domains respectively (Brown et al., 1993; Madden, 1995b). This has a more open groove at the end of the peptide-binding cleft, which allows longer peptides to bind in the MHC II cleft (Chicz et al., 1993; Chicz et al., 1992). The α2 and β2 domains generate a structure, like that of MHC I molecules, that is similar to the structure of the immunoglobulin constant domain. This region
Chapter 1

Literature review

contains the CD4-binding site.

1.1.2 Conventional MHC class I and class II restricted antigen processing pathways

Different processing pathways operate for MHC-restricted antigen presentation. Antigens presented in association with MHC class I molecules are generally derived from intracellularly synthesised proteins. The peptides are generated in the cytosol via degradation by the multicatalytic protease complex, the proteasome (Goldberg and Rock, 1992). They are transported into the endoplasmic reticulum (ER) by ATP-dependent transporters associated with antigen processing (TAPs), where they associate with newly synthesized MHC class I molecules (Germain, 1994). These transport and presentation competent complexes move to the cell surface for presentation to CD8+ T cells (Townsend and Bodmer, 1989).

In contrast to class I molecules, the peptides associated with class II molecules are derived from exogenous proteins which have been internalized by APCs and degraded by proteases in acid pH vesicular intracellular compartments. Several proteolytic enzymes were discovered within the endosome/lysosome system, such as cathepsins C, B, H, L etc. (Riese and Chapman, 2000). Class II αβ heterodimers assemble in the ER and rapidly associate with a non-polymorphic invariant chain (Ii), which prevents MHC II molecules from binding to exogenous peptides in the endoplasmic reticulum. Cleavage of Ii allows MHC II to bind incoming peptides and complete dissociation of Ii releases MHC II molecules carrying antigenic peptide (Cresswell, 1994; Roche and Cresswell, 1990b). Cathepsin S or L convert a 10 kDa amino-terminal Ii fragment (Iip10) to CLIP (class-II-associated Ii peptide) (Riese et al., 1996), which continues to stably associate with class II MHC. Release of CLIP and the subsequent loading of antigenic peptide onto the MHC II molecule are facilitated by HLA-DM. This accessory molecule catalyzes the exchange of
peptides bound to MHC class II molecules (Kropshofer et al., 1999; Sloan et al., 1995). Generated peptides are loaded onto MHC class II molecules in the lysosomal compartment and selectively presented to CD4+ T cells (Pierre et al., 1997; Roche and Cresswell, 1990a; Roche and Cresswell, 1991; Teyton et al., 1990). Mature, cell-surface class II MHC molecules can also recycle via internalization into the early endosomes, where they are reloaded with peptides generated from endocytosed proteins (Pinet et al., 1995; Reid and Watts, 1990). Recognition of MHC-peptide-complexes by the T cell receptor on antigen specific T cells generates the first signal for initiation of T cell activation.

1.1.3 Cross-presentation: the MHC class I-restricted exogenous antigen presenting pathway

MHC class I-restricted antigen presentation is usually associated with the processing of endogenous antigens (Monaco, 1992; Townsend and Bodmer, 1989). Under some circumstances, however, exogenous antigens access the class I-restricted pathway and initiate CTL responses - first revealed 26 years ago by Bevan (Bevan, 1976b; Bevan, 1976a). He found that immunization with a MHC-mismatched grafts, lacking host MHC-alleles but bearing foreign minor histocompatibility antigens, led to host MHC-allele restricted CTL immunity against graft minor histocompatibility antigens which he termed cross-priming (Bevan, 1976b; Bevan, 1976a). Bevan proposed that the induction of primary CD8+ T cell responses to tissue-restricted antigens might occur via cross-priming APCs that capture antigens, migrate into the secondary lymphoid compartments, and present these exogenous antigens in an MHC class I restricted manner to CTL (Bevan, 1987). Since induction of CTL responses through this pathway has been referred to as cross-priming, the antigen processing and presentation, was subsequently described as cross-presentation (Carbone et al., 1998; Heath et al., 1998; Huang et al., 1994; Sigal et al., 1999). In this process, antigens are transferred from the endocytic compartment into the cytosol where they are degraded into oligopeptides. This mechanism is thought to require TAP to ferry peptides to class I molecules in the ER. The peptides are then
presented on the cell surface in the context of MHC class I molecules for the recognition by CD8\(^+\) T cells (Huang et al., 1996; Kovacssovics-Bankowski and Rock, 1995).

1.1.3.1 Evidence of cross-presentation of exogenous self-antigens

Cross-presentation describes the uptake and presentation of class I-restricted antigens derived from outside the APC (Carbone et al., 1998; Huang et al., 1994; Sigal et al., 1999). A number of protein antigens have been found to be cross-presented to CD8\(^+\) T cells and initiate CTL immunity. For example, virus antigens (Lenz et al., 2000; Sigal and Rock, 2000), bacterial antigens (Pfeifer et al., 1993; Svensson et al., 1997a; Svensson et al., 1997b), tumor antigens (Chiodoni et al., 1999; Gooding and Edwards, 1980; Huang et al., 1994; Ronchetti et al., 1999a; Wolfers et al., 2001), minor histocompatibility antigens (mHC) (Bevan, 1976b; Bevan, 1976a) and some other antigens such as ovalbumin (OVA) (Carbone and Bevan, 1990) and β-galactosidase (Carbone and Bevan, 1990) can all cross-prime CTL responses.

In addition to cross-priming, MHC-class I restricted cross-presentation can be involved in the induction of cross-tolerance. Several studies conducted using transgenic mice indicated that transgenically expressed peripheral antigens, such as ovalbumin (Kurts et al., 1996) were constitutively cross-presented to specific TCR bearing CD8\(^+\) T cells. Activation of naïve CD8 T cells recognising cross-presented self-antigens can be visualized by labelling adoptively transferred CD8 T cells with 5-(and-6)- carboxyfluorescein diacetate succinimidyl ester (CFSE). Despite activation, effective CTL priming does not take place in such recipients, and transferred CD8 T cells are deleted, leading to the induction of cross-tolerance. Priming can, however, be facilitated by provision of CD4\(^+\) T cell help or anti-CD40 stimulation (Ingulli et al., 1997; Ridge et al., 1998; van Kooten and Banchereau, 2000), the latter of which substitutes for CD4 T cell help (Bennett et al., 1998; Schoenberger et al., 1998). This clearly reveals that one of the approaches to
generate peripheral self-tolerance is through cross-presentation (Forster and Lieberam, 1996; Adler et al., 1998a; Kurts et al., 1997a; Morgan et al., 1999a; Morgan et al., 1999b). This process is largely mediated by deletion of self-reactive CTL (Kurts et al., 1997b; Morgan et al., 1999a; Adler et al., 1998b). Therefore, cross-presentation as a general mechanism for the activation of specific T cells against exogenous antigens plays an important role in the surveillance of peripheral tissues for pathogen invasion or tumors and in the maintenance of self-tolerance to peripheral antigens.

1.1.3.2 Capture of exogenous antigens by cross-priming APCs

Professional APCs use distinct mechanisms to capture exogenous antigens. Ingestion of infectious agents, or cellular debris by phagocytosis is the primary function of macrophages and has also been observed for immature DCs (Brown, 1995; Fossum and Rolstad, 1986; Inaba et al., 1993; Matsuno et al., 1996; Reis e Sousa et al., 1993; Sallusto et al., 1995; Svensson et al., 1997b). Phagocytosis is initiated by the interaction of specific cell surface receptors on the phagocyte, e.g. mannose receptors (Jiang et al., 1995; Sallusto et al., 1995), Fc receptors, and complement receptors (Fanger et al., 1997; Sallusto and Lanzavecchia, 1994) with ligands on the antigenic/opsonised particle. Inflammation or infection can augment this process (Collins and Bancroft, 1992; Regnault et al., 1999; Sallusto et al., 1995; Sampson et al., 1991). Macropinocytosis, another pathway utilized by both macrophages and DCs to take up fluid-phase antigens (Cella et al., 1997; Sallusto et al., 1995; Swanson and Watts, 1995; Watts, 1997) is inefficient in B cells (Watts, 1997). Binding to antigen-Ig complexes mediates internalization of the complexes. This triggers APC maturation and promotes efficient MHC class-I and II restricted presentation of peptides from exogenous antigens (Banchereau and Steinman, 1998; Ravetch, 1994; Regnault et al., 1999). Characteristics, such as phagocytosis and cell surface receptors for specific forms of antigen endow APCs with the capacity to process antigens with great efficiency.
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An increasing number of observations indicate that apoptotic bodies are a favorite target for cross-priming APCs (Albert et al., 1998b; Arrode et al., 2000; Bellone et al., 1997; Steinman et al., 2000; Subklewe et al., 2001; Yrlid and Wick, 2000). DCs can capture apoptotic cells and induce either class-I restricted CTL responses (Albert et al., 1998b) or tolerance to self antigens in vivo (Huang et al., 2000; Steinman et al., 2000). Macrophages are also capable of engulfing apoptotic bodies and can then be targeted by specific CTL (Bellone et al., 1997). Current reports suggest that immature DCs phagocytose apoptotic bodies by means of αvß5 and CD36, which links DCs to the apoptotic bodies and increases the efficiency of antigen targeting (Albert et al., 1998a). In addition to apoptotic cells, necrotic cells lines (Sauter et al., 2000; Subklewe et al., 2001) and even healthy cells (Harshyne et al., 2001) were also shown to be capable of being captured by DCs and inducing DC maturation. It appears that APCs can utilize more than one type of cellular antigen source for processing into the MHC-I pathway.

1.1.3.3 Exogenous antigens enter the MHC class-I restricted antigen-processing pathway

Although the routes for exogenous antigen entry into the MHC class I pathway have been extensively reviewed (Bevan, 1995; Heath and Carbone, 2001; Yewdell et al., 1999), knowledge of which pathway operates in vivo during cross-presentation is still very limited. Despite the fact that there is no well established mechanism(s) by which a macrophage or dendritic cells can translocate extracellular antigens into class I MHC presentation pathway, there have been several proposed (see below). The importance of each pathway in cross-presentation in vivo remains to be clarified.

1.1.3.3.1 A phagosome to cytosol pathway

Several groups have observed that MHC-I restricted cross-presentation of exogenous antigens is TAP-dependent (Brossart and Bevan, 1997; den Haan et al., 2000;
Kovacsovics-Bankowski and Rock, 1995; Norbury et al., 1997). This process could be abrogated when various peptide aldehyde inhibitors of the proteasome were applied (Brossart and Bevan, 1997; Kovacsovics-Bankowski and Rock, 1995; Norbury et al., 1997; Oh et al., 1997). These observations reveal that this pathway requires a translocation of peptides from the cytosol to MHC-I molecules and relies on intact proteasomes. In addition, cross-presentation is blocked in the presence of protein synthesis inhibitors (Kovacsovics-Bankowski and Rock, 1995; Norbury et al., 1997; Norbury et al., 1995; Reis e Sousa and Germain, 1995) demonstrating its dependence on nascent MHC class I molecule synthesis. These data imply that some antigens, ingested by phagocytes, may exit the phagosome to the cytoplasm where they enter the conventional MHC class I restricted antigen-presenting pathway (Peppelenbosch et al., 2000).

1.1.3.3.2 "Regurgitation" pathway
Phagocytes may digest ingested material in their lysosomes and regurgitate antigenic peptides onto MHC class I molecules on their cell surface (Harding and Song, 1994; Pfeifer et al., 1993; Schirmbeck et al., 1995a) or load recycled class I MHC molecules in the endosomes (Bachmann et al., 1995; Schirmbeck et al., 1995a; Schirmbeck et al., 1995b). This pathway is TAP-independent (Bachmann et al., 1995; Song and Harding, 1996; Zhou et al., 1993) and resistant to proteasome inhibitors (Harding and Song, 1994; Pfeifer et al., 1993; Song and Harding, 1996; Zhou et al., 1993), which indicates that it does not rely on translocation of antigen from phagosome to cytosol. Rather, MHC class I-restricted epitopes might be generated by vacuolar proteolysis. This is supported by the observation that cell surface MHC class I molecules enter endosomal compartments for recycling (Hochman et al., 1991; Reid and Watts, 1990; Tse and Pernis, 1984).

1.1.3.3.3 Heat shock protein (HSP) chaperone pathway
Heat shock proteins (HSP) have been shown to induce specific CTL responses against those cells from which they were derived (Srivastava, 1994). For example, HSPs have been
HSP-peptide complexes have been reported to access the MHC I pathway by receptor-mediated endocytosis. For example, CD91 functions as the receptor for gp96 on DC (Arnold-Schild et al., 1999). Stimulation through pg96-CD91 leads to DC maturation as well as secretion of IL-12 and TNF-α (Binder et al., 2000; Singh-Jasuja et al., 2001). Similarly, the route of endocytosis of HSC70 is also found to be mediated through a receptor (Arnold-Schild et al., 1999). Therefore, the high efficiency of antigen presentation is thought to be derived from the presence of an HSP receptors on the surface of APCs (Arnold-Schild et al., 1999; Binder et al., 2001; Fujihara et al., 1999; Singh-Jasuja et al., 2000).

1.1.3.3.4 Exosome pathway

Exosomes are membrane vesicles secreted by hematopoietic cells like DC upon fusion of late multivesicular endosomes with the plasma membrane (Pan et al., 1985). Upon exposure to tumor peptide, DC-derived exosomes induce potent antitumor immune responses to tumor cells (Srivastava et al., 1998) or virus-infected cells (Blachere et al., 1993). Meth A sarcoma derived HSP70, a cytosolic HSP (Udono and Srivastava, 1993), and HSP69, an abundant HSP of lumen of the endoplasmic reticulum (Li and Srivastava, 1993), were shown to elicit tumor-specific immunity. Interestingly, the gene sequences of HSPs were found highly conserved in different tumor cells and between tumors and normal tissue (Srivastava and Udono, 1994), but each tumor generally induced an immune response specifically towards itself and not other tumors (Srivastava and Old, 1988). This suggested that the specificity of the immune response initiated by HSPs is based on the associated antigen peptides, and not the HSP itself (Nieland et al., 1996; Udono and Srivastava, 1993). This was further supported by the observation that depletion of peptide from HSP70 abrogated its immunogenicity (Udono and Srivastava, 1993).
responses *in vitro* (Wolfers et al., 2001) and in mice (Zitvogel et al., 1998), resulting in the regression of established tumors (Zitvogel et al., 1998). Exosomes contain several cytosolic proteins, such as heat shock cognate protein hsc73, a cytosolic heat shock protein (hsp). Hsc73 was shown to induce antitumor immune responses *in vivo* (Thery et al., 1999). Exosomes also contain different integral or peripherally associated membrane proteins, which may be involved in the selective accumulation of the hsc73 and targeting of antigen peptides to APCs (Thery et al., 1999). Exosome production is downregulated upon DC maturation, indicating that *in vivo*, exosomes are produced by immature DCs in peripheral tissues (Thery et al., 1999). Despite our poor understanding of how exosomes are generated and function, they appear to represent a novel approach for shuttling exogenous antigen into MHC class I restricted antigen presentation pathway.

### 1.1.3.3.5 Endocytic pathway

Both DCs and macrophages process extracellular fluid by forming large pinocytic vesicles. This is termed macropinocytosis (Sallusto et al., 1995). They may also phagocytose glycosylated proteins, particles or microbes by receptor-mediated endocytosis (Inaba et al., 1993; Reis e Sousa et al., 1993; Svensson et al., 1997b). Receptor-mediated endocytosis, phagocytosis, and macropinocytosis all contribute to the ingestion of antigen by antigen-presenting cells. These antigens may then be processed by antigen processing mechanisms. A recent study (Peppelenbosch et al., 2000) suggested that antigens ingested through macropinocytosis were preferentially presented via MHC I molecules. In contrast, receptor-mediated endocytosis of antigens might mediate MHC-II restricted presentation. It is proposed that in the macrophage, the mode of antigen capture might play a decisive role in determining which MHC molecule presents the antigen.

### 1.1.3.4 Cross-priming APCs

Naïve T lymphocytes are programmed to recirculate from the blood to the secondary lymphoid tissues, and back to the blood again. To encounter antigens present in the
peripheral tissues, CD8 T cells require a special vehicle that transfers peripheral antigens to the lymph nodes and then presents them in a class I-restricted manner. This is achieved by the cross-priming APC, which can present MHC-I bound peptides to naive CD8+ T cells in the context of adhesion receptors and potent co-stimulatory molecules essential for CTL priming (Bevan, 1976b; Bevan, 1976a; Cella et al., 1997; Rock, 1996).

Two distinct functional phases have been described for APCs (Cella et al., 1997; Friedl and Gunzer, 2001; Lanzavecchia and Sallusto, 2001; Randolph et al., 1999; Steinman, 2001). The first phase involves antigen uptake and transportation; the second phase involves T cell activation. These phases are regulated by inflammatory stimuli such as tumor necrosis factor-α (TNF-α), IL-1 and bacterial lipopolysaccharide (LPS). The ability to capture and present antigens on MHC class I molecules is largely limited to dendritic cells (DC) and microphages. Several groups observed that different cells were able to cross-present antigen in vitro. These include macrophages (Norbury et al., 1997; Norbury et al., 1995), DCs (Albert et al., 1998b; Ronchetti et al., 1999b) and even B cells (Ke and Kapp, 1996). However, recent reports indicate that DCs are the most efficient cell type for cross-presenting exogenous antigen to CD8+ T cells (Regnault et al., 1999; Rodriguez et al., 1999; Yrlid and Wick, 2000).

1.1.3.4.1 Subpopulations of mouse dendritic cells
DCs are the most potent APCs, with the unique capability of initiating adaptive immunity. Over the years, great progress has been made in understanding the origin, maturation and antigen presenting function of DCs (Banchereau et al., 2000; Cella et al., 1997; Friedl and Gunzer, 2001; Kamath et al., 2000; Shortman and Wu, 2001; Steinman, 2001; Steinman and Cohn, 1973). Three distinct populations of DCs have been identified in the mouse spleen, distinguished primarily by their surface expression of CD4 and CD8α. These populations are further defined as CD4+8αDEC-205+ CD11b+ (CD4+), CD4+8αDEC-205−
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CD11b^+ (CD8^+) and CD4^+ 8α^+ DEC-205^+ CD11b^+ DCs (Vremec et al., 2000). In addition, all three subsets express high levels of CD11c and MHC class II molecules. Histologic analysis indicated that different types of DCs reside in different areas of the spleen. CD11b^+ DCs are found in the T cell areas of the periarteriolar lymphatic sheaths (PALS), whereas CD4^+ and CD4^+ CD8^+ DCs (both CD4^+ 8α^+ and CD4^+ 8α^+ DCs are referred to as CD8α^+ DCs) localize in the marginal zone of a mouse spleen (Steinman et al., 1997). According to the original description of mouse DC development, CD8α^+ DCs were termed lymphoid DCs (Ardavin et al., 1993; Wu et al., 1996), whereas CD8α^+ DCs were referred to as myeloid DCs (Inaba et al., 1992; Scheicher et al., 1992; Steinman and Inaba, 1999). Recently, in studies examining DC turnover rates, Shortman and colleagues demonstrated that the above three splenic DC subpopulations are derived from three independent developmental streams, with different kinetics of development from bone marrow precursors (Kamath et al., 2000; Shortman and Wu, 2001). Traver et al found that a bone marrow derived precursor produced both CD8^+ and CD8^+ DCs (Traver et al., 2000), indicating that CD8^+ DC were not necessarily of lymphoid origin.

DC populations present in the lymph nodes (LN) were also examined (Henri et al., 2001; Steinman et al., 1997). The same three populations of splenic DCs were identified in mouse LNs, but two additional populations were characterized by their relatively low expression of CD8 and moderate or high expression of DEC-205. One of these populations was identified as the mature form of the epidermal langerhans cell (LC) and was found in LN draining the skin (Henri et al., 2001).

In addition to their different origin and localization, DCs have been described as having two distinct developmental stages. DCs residing outside the lymphoid organs show an "immature" phenotype characterized by a high propensity for antigen capture and
processing, but limited ability to stimulate naïve T cells (Cella et al., 1997). All three splenic DC subsets retain the ability to capture antigen in vivo, even after further maturation initiated by administration of LPS (Karnath et al., 2000), suggesting that the ability to capture antigen is not only limited to immature stages. Upon exposure to signals associated with tissue damage, DCs migrate to regional lymphoid organs and undergo a maturation process characterized by the capacity to activate naïve T cells (Hermans et al., 2000). Recently, in vivo antigen loaded splenic DC subpopulations were shown to initiate proliferation of naïve antigen specific T cells after capturing cell associated or soluble antigen. Importantly, only the splenic CD8α+ DC subpopulation activated CD8+ T cells (den Haan et al., 2000; Pooley et al., 2001), whereas all DC subsets were capable of activating CD4+ T cells. These observations, establish for the first time, a potential role for CD8α+ DCs as the cross-priming APC. At the same time, other in vivo studies showed that both CD8α+ and CD8α- DCs can prime antigen specific CD4+ T cells efficiently (Maldonado-Lopez et al., 1999; Pulendran et al., 1999).

Although all three populations of splenic DCs are able to initiate T cell activation in vivo and in vitro, different DC subtype exerts distinct functions when they interact with T cell subsets. A notable difference is that CD8α+ DCs have the potential to produce large amount of IL-12 and initiate Th1 responses upon stimulation with microbes and cytokines GM-CSF or IFN-γ (Hochrein et al., 2001), while CD8- DCs play an integral role in Th2 responses (MacDonald et al., 2001). This raised the possibility that the fate of activated T cells may be polarized by different DC subpopulations (MacDonald et al., 2001; Maldonado-Lopez et al., 2001; Maldonado-Lopez and Moser, 2001; Pulendran et al., 2001). In addition to this, CD8α+ DCs were reported to manipulate the T cell response by initiating Fas-mediated apoptosis on CD4+ T cells and by limiting IL-2 production by CD8+
T cells (Kronin et al., 1996; Suss and Shortman, 1996). In contrast, CD8α+ DCs were suggested as strong stimulators of primary T cell responses (Suss and Shortman, 1996). This further suggested that different subpopulations of DCs might generate different types of T cell responses; for example, one population may induce tolerance while another induces T cell priming (den Haan et al., 2000; Kronin et al., 1996; Maldonado-Lopez et al., 1999; Pulendran et al., 1999).

1.1.3.4.2 Other antigen-presenting cells

In addition to a large number of studies indicating DCs are important antigen presenting cells for cross-presentation, a few other cell types have been shown to have this ability. Macrophages were the first cell reported to present exogenous antigen via the class I pathway (Kovacsovics-Bankowski et al., 1993). They have also been shown to process engulfed apoptotic bodies and yield T cell epitopes, which enabled these cells to be targeted by antigen-specific CTL (Bcllone et al., 1997). Pulaski and colleagues showed that “macrophage-like cells” isolated from the tumor tissue transfected with genes of IL-3 and ovalbumin were able to activate OVA specific CD8+ T cells (Pulaski et al., 1996). However, so far, no in vivo studies have provided evidence for the role of macrophages in cross-priming.

B cells normally do not present exogenous antigens by MHC class I molecules. It was found, however, that B cells can take up exogenous antigen through receptor-mediated endocytosis, and present the antigen via the MHC class I pathway (Ke and Kapp, 1996). Ligation of CD40 molecules on B cells up-regulates expression of co-stimulatory molecules (Kennedy et al., 1994; Shinde et al., 1996), and initiates a series of signals that enable these cells to induce proliferation of CD4+ T cells, supporting the idea that B cells might be capable of cross-priming. However, no requirement for B cells was found for cross-priming CTL to tumor antigens in B6 µMT mice, which lack mature B cells.
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(Schoenberger et al., 1998).

1.2 T lymphocytes

T cells are derived from bone marrow stem cells. T cell progenitors migrate from the bone marrow to the thymus where they undergo two selective processes known as positive selection and negative selection (Korngold et al., 1989; Sprent et al., 1989; von Boehmer et al., 1989c; Zinkernagel et al., 1978b). During this process, they undergo gene rearrangement, and express a unique antigen receptor (Adkins et al., 1987; Blackman et al., 1986; Nikolic-Zugic, 1991). Mature T cells enter the peripheral lymphoid compartment, where they are responsible for generating cellular immune responses against infectious agents and tumour cells. According to the expression of their surface molecules, T lymphocytes can be divided into two subpopulations: CD8+ and CD4+ (Kaye et al., 1989; von Boehmer et al., 1989b). CD8+ T cells are referred to as cytolytic T lymphocytes (CTL), characterized by developing into killers capable of destroying targets cells in an antigen-specific manner. Their activity may be used to enable the killing of intracellular pathogens such as viruses and some bacterial, or even to destroy tumor cells. CD4+ T cells function as "helpers" in immune responses. They assist cytotoxic T cell differentiation and expansion (Husmann and Bevan, 1988; Keene and Forman, 1982b), promote B cell proliferation and immunoglobulin production, and they are able to activate APC through a CD40-CD40L signal and via other molecular interactions (Bennett et al., 1998; Clarke, 2000; Ridge et al., 1998; Schoenberger et al., 1998).

Based on distinct differentiation states, T cells are termed naive, effector or memory T cells, and function differently at each stage. Each mature lymphocyte bears a single type of receptor of a unique specificity, termed the T cell receptor (TCR). Unlike B cells, which directly bind antigen through their B cell receptor, T cells recognise antigen through the TCR binding to a small peptide fragment displayed at a cell surface in the context of MHC
molecules (Owen, 1988; von Boehmer et al., 1989a; von Boehmer et al., 1989b). When naïve T cells encounter an antigen in the lymph node or spleen, they become activated, undergo further clonal expansion and differentiate into effector and memory T cells. T cells, together with other immune cells, such as B cell, dendritic cells, macrophage, NK cells, neutrophil cells, mast cells etc. generate adaptive specific immunity leading to long-lasting protection against invaders (Mackay, 1993; Xie et al., 1999; Young et al., 1993).

1.2.1 T cell development

The thymus consists of cortical and medullary epithelia, together with connective tissue which form the thymic stroma, providing a specialised microenvironment for the development of T cells (Anderson et al., 2000; van Ewijk, 1991; von Boehmer, 1992a). The earliest T cell progenitors in the thymus do not express CD4 or CD8 molecules, nor T-cell receptors, and are termed double negative cells (for CD4 and CD8 negative). After vigorous proliferation and TCR gene rearrangement, progenitors develops into two lineages of T cells, γ:δ and α:β (Hedrick and Sharp, 1998; Kang et al., 2001; von Boehmer, 1992a; Winoto and Baltimore, 1989). Maturation of α:β T cells occurs through the "double-positive" stage when thymocytes express both CD8 and CD4 molecules and a low level of TCR. At this stage, they undergo positive selection for self-MHC restriction (Fink and Bevan, 1978; Kisielow et al., 1988; Zinkernagel et al., 1978b). Cells that fail positive selection die in the thymus via programmed cell death mediated by apoptosis (Huesmann et al., 1991; Shortman et al., 1991; von Boehmer, 1992b). Double positive cells must also undergo negative selection whereby self-reactive T cells are eliminated in the thymus through apoptosis (Jenkinson et al., 1989; Kappler et al., 1987; Surh and Sprent, 1994). Thymic deletion generates central tolerance to self-antigens expressed within the thymus. Because of the constraints of both positive and negative selection, only a small proportion of thymocytes are successfully selected to mature into single-positive
thymocytes (either CD4$^+$ or CD8$^+$) (Crispe et al., 1987; Sprent and Webb, 1987; von Boehmer et al., 1989b).

The importance of the thymus in T-cell development was first demonstrated by Miller (Miller, 1961; Miller, 1962). Further support for this view was provide by latter observations that T cells do not develop in mutant mice such as nude mice, which have defective generation of thymic epithelium (Pritchard and Micklem, 1973). While scid and Rag mutant mice are unable to generate T cells because of a defect in receptor-gene recombination, their thymic stroma is normal (Fulop and Phillips, 1989). Interestingly, thymic epithelial cells from scid mice can induce the maturation of T cells in nude mice, and nude bone marrow precursors develop normally in a scid thymus. This indicates that both gene rearrangement and the microenvironment are essential for maturation of bone marrow derived cells into T cells.

**1.2.2 Naive T cell recirculation, migration and homing**

Mature T cells leave the thymus and most recirculate continuously from the bloodstream to tissues and back to the blood again. This process enables lymphocytes to scan antigens at all possible sites (Picker, 1993). Naive T cells are programmed to selectively home to the peripheral lymphoid compartments, such as lymph nodes, spleen, Peyer's patches and lymphatics etc. until they encounter antigen on APC. Only a few naive T cells are specific for any one antigen, and when they encounter it on a professional antigen presenting cell, they are activated, proliferate and then differentiate into effector cells (Heath and Carbone, 2001; Picker, 1993; Rock, 1996; Young et al., 1993). In response to an antigenic stimulation, effector and memory cells home preferentially to non-lymphoid tissues and localize to the gut, skin and other tissues (Mackay, 1993; Picker, 1994). If naive T cells enter the lymph node but do not encounter an APC bearing their specific antigen, they leave via the efferent lymphatics and enter the blood where they again exit to lymph nodes or re-circulate through the spleen. Re-circulation continues until they are either activated
by antigen or die by homeostatic mechanism, the latter of which regulate the size of a particular lymphocyte pool (Butcher and Picker, 1996).

The physiological process of lymphocyte migration is complex and dynamic. Naïve T cell recirculation is restricted by specific homing receptors targeting T cells to the peripheral lymph nodes and the gut mucosal tissue respectively (Mackay, 1993; Picker, 1994). The ligands for these receptors are expressed at high levels on endothelial cells in the specialised blood vessels of the lymph nodes such as high endothelial venules (HEV) (Girard and Springer, 1995). Naïve T cells use CD62L for rolling on vessel walls, and CCR7 chemokine receptors for integrin activation and extravasation from the blood to the lymph nodes (Bradley and Watson, 1996; Butcher and Picker, 1996; Springer, 1994). The interaction of a homing receptor with its specific ligand promotes sequential events including primary adhesion and lymphocyte rolling along vascular endothelium followed by rapid lymphocyte activation, activation-dependent arrest, and transmigration (Butcher and Picker, 1996).

1.2.3 T cell activation

Cytotoxic T lymphocytes (CTL) are responsible for lysing target cells presenting the appropriate peptide on MHC class I molecules. Naïve CTL, when primed, become effector CTL, capable of target cell lysis (Carbone et al., 1989; Carbone et al., 1988; Germain, 1994; Heath and Carbone, 1999). Priming is regarded to depend on the provision of both antigenic and co-stimulatory signals by professional APCs (Chambers and Allison, 1997; Sperling et al., 1996), which express molecules such as B7 and CD40, and secrete cytokines such as IL-12 (Hochrein et al., 2001; Maldonado-Lopez et al., 2001). Activation of cytotoxic T cells is triggered by a series of signals both inside and between cells, and some responses may relay on CD4+ T cell help.
1.2.3.1 T cell receptor complex

T cells recognize antigens and transmit antigen receptor-derived signals through the antigen specific T cell receptor/CD3 complex (TCR/CD3). This multimetric protein complex comprises ligand-binding (TCR) and signal-transducing subunits (CD3). TCR are heterodimers composed of two disulfide-linked transmembrane glycoproteins. The external portion of each chain folds into two Ig-like domains, one having a relatively invariant structure, the other exhibiting a high degree of variability (Chothia et al., 1988; Claverie et al., 1989; Novotny et al., 1986; Patten et al., 1984). Two types of T cells can be distinguished by the type of TCR they express. More than 90% of peripheral T cells express a TCR composed of an α and β chain. These are the αβ T cells and are regarded as the main class of T cells (von Boehmer et al., 1989b). Another type of TCR, composed of a γ and δ chain (Haas et al., 1993, Raulet, 1989 #996) is expressed by 1-5% of CD3+ T cells in human peripheral blood. Such cells are, however, more prominent in the intestinal epithelium. The role γδ T cells in immunity is still under investigation.

TCRs are expressed in association with the invariant proteins of the CD3 complex. CD3 consists of several distinct proteins, ε, δ, γ and ζ. In their cytoplasmic domains are located sequences associated with cytosolic protein tyrosine kinases (PTKs). Upon TCR recognition of antigen, signals are transferred into the interior of the T cells through the CD3 complex, informing the cell of the binding of antigen. Antigen stimulation induces TCR-mediated activation of the src-family kinases, Lck and Fyn (Straus and Weiss, 1992; Watts et al., 1992) resulting in phosphorylation of tyrosine residues within specific immunoreceptor tyrosine-based activation motifs (ITAMs) in CD3 cytoplasmic tail and ζ-chain components of TCR complex (Cambier, 1995; Wange and Samelson, 1996). This subsequently leads to the activation of another family of PTKs, the SyK/ZAP-70 family (Chan et al., 1994; Hatada et al., 1995; Isakov et al., 1995; Wange and Samelson, 1996).
and ultimately results in recruitment and activation of different signal transduction pathways (Davis, 1990; Marx et al., 1995; Qian et al., 1997). Downstream cascade signals, such as some early secondary messengers like calcium mobilization and inositol phospholipid metabolism are crucial for the initiation of cellular immune responses (van Oers et al., 1998).

CD4 and CD8 molecules, used as markers for distinct T cell subpopulations, are required for efficient signalling through the TCR complex (Janeway, 1992; Singer et al., 1999). The cytoplasmic tails of the CD4 and CD8 molecules associate with a protein tyrosine kinase, lck, whereas their extracellular portions associate with invariant parts of MHC class II and class I molecules respectively. This synergizes with the TCR in signalling, increasing the strength of the T cell response to antigen.

1.2.3.2 B7/CD28/CTLA-4 molecules and co-stimulation
Two signals are considered essential for the activation of naïve T cells (Allison, 1994; Croft and Dubey, 1997; Sabzevari et al., 2001). The first signal is antigen specific, triggered by engagement of peptide-MHC complexes on APCs with TCR molecules on T cells. This tells the T cell that antigen has been encountered. However, ligation of the TCR alone does not allow naïve T cells to proliferate and differentiate into effectors, instead, it leads to tolerance (Gill et al., 1996; Schwartz, 1996). There is a requirement for the second signal, a nonspecific signal, mediated by a number of distinct receptor-ligand engagements termed the co-stimulatory signal. This signal is delivered by the same APC that presents the antigen to the T cells and has been found to be essential for activation of naïve T cells. Different accessory molecules have been identified, such as B7/CD28, 4-1BB/4-1BBL, OX40/OX40L, ICOS/B7 and their ligands. CD40/CD40L and TRANCE are sometimes considered as co-stimulatory signals (Lederman, 2001; Whitmire et al., 1999) because of their importance in inducing co-stimulatory events on APCs.
B7 family members, B7.1 or CD80 and B7.2 or CD86, form homodimers and are members of the immunoglobulin superfamily (Boussiotis et al., 1996; Chambers and Allison, 1997; Croft and Dubey, 1997; Lenschow et al., 1996; Sperling et al., 1996). B7 molecules are exclusively expressed on the surface of antigen presenting cells such as on DCs, macrophages, B cells and T cells. Both B7.1 and B7.2 have been found to be up-regulated upon a variety of stimuli (Hathcock et al., 1994; Inaba et al., 1994; Lenschow et al., 1996). Signals from the engagement of B7 with its receptors CD28/CTLA-4 family have been extensively investigated, displaying complex pathways of T cell signalling that profoundly modulate their responses.

CD28 is the most widely studied co-stimulatory molecule. It is constitutively expressed on all murine resting T cells (Gross et al., 1992; Gross et al., 1990) and most human resting T cells (Lenschow et al., 1996). PMA treatment rapidly up-regulates CD28, and anti-CD28 signalling synergizes with anti-CD3 to enhance T cell proliferation (Turka et al., 1990). Interruption of this signalling pathway with CD28 antagonists not only results in suppression of the immune response, but also in some cases it induces antigen-specific tolerance (Abbas et al., 1999; Lenschow et al., 1996; Radvanyi et al., 1996; Sperling et al., 1996), which might result from T cell apoptosis following activation in the absence of a CD28 signal (Radvanyi et al., 1996). Prevention of apoptosis by CD28 co-stimulation was associated with increased expression of Bcl-xL (Radvanyi et al., 1996; Sperling et al., 1996). In addition, in CD28 deficient mice, pathogenic T cell trafficking into the inflammatory sites was apparently decreased in autoimmune mouse models (Girvin et al., 2000). A similar result was obtained in another autoimmunity model by administration of anti-B7.1 (but not anti-B7.2) (Liu et al., 2001). This prevented the wasting disease associated with colitis, abrogated leukocyte infiltration, and reduced production of pro-inflammatory cytokines IL-2 and IFN-γ by lamina propria CD4+ cells (Liu et al., 2001), revealing the involvement of C28/B7 co-stimulatory pathway in regulating cell migration.
These data show that signals via the engagement of CD28/B7 are critical for T cell expansion, cytokine production, and for induction of effector T cells. Currently, the role of CD28 in the Th2 differentiation has also been demonstrated in a number of in vitro experimental models and in autoimmune disease settings in which the differentiation of naïve CD4+ T cells towards Th2 is strictly dependent on a CD28/B7 co-stimulatory signal (Falcone et al., 1999; Kubo et al., 1999; Rogers and Croft, 2000; Rulifson et al., 1997; Stack et al., 1998). Importantly, the CD28/B7 signalling pathway is not only critical for stimulating T cell responses, but is also involved in regulating the amount of immunoregulatory CD25+CD4+ T cells. In either CD28 or B7 deficient mice, the population of CD25+CD4+ T cells was severely depleted, which appears to contribute to the diabetes occurrence (Salomon et al., 2000). Together, CD28/B7 signals result in multiple effects on the immune response. So far no other cell surface receptor has been found considered fully redundant with CD28.

Cytotoxic T lymphocyte antigen-4 (CTLA-4) or CD152, another receptor for B7, is a homologue of CD28 (Freeman et al., 1993). CTLA-4 is expressed on a proportion of human and murine CD4+ and CD8+ T cells at a very low level (Linsley et al., 1994; Salomon and Bluestone, 2001). Expression is inducible and enhanced on activated T cells (Salomon and Bluestone, 2001). It competes with CD28 for binding B7 and binds with a higher affinity as a counter-regulatory receptor participating in the negative regulation of T cell responses (Bluestone, 1997; Chambers et al., 1997; Salomon and Bluestone, 2001; Tivol et al., 1996; Walunas et al., 1996; Waterhouse et al., 1996). In vitro experiments indicate that CTLA-4 cross-linking inhibits T cell proliferation, IL-2 secretion and anti-CD3-induced cyclins (Krummel and Allison, 1996; Walunas et al., 1994). In contrast, CTLA-4-deficient mice develop a lymphoproliferative disorder resulting from over-expansion of polyclonal CD4+ T cells (Chambers et al., 1997; Tivol et al., 1995). The negative regulation of T cell function was found to be associated with inhibition of TCR signal transduction (Calvo et al., 1997; Lee et al., 1998). Recently, it has been reported that CD25+CD4+ cells from CD28− mice, like those from the wild-type mice, efficiently
prevent intestinal mucosal inflammation induced by wild-type CD45RB (high) CD4+ T cells (Liu et al., 2001), and the inhibitory function of these regulatory T cells could be blocked by anti-CTLA-4. Therefore, the CTLA-4 signal appears essential for the function of CD25+CD4+ T cells in the regulation of inflammatory responses and the control of tolerance induction (Bluestone, 1997; Read et al., 2000; Salomon and Bluestone, 2001; Takahashi et al., 2000). It appears that negative regulation by CTLA-4/B7, which is an active process that occurs through the transduction of inhibitory signals, is a major regulator of peripheral tolerance and normal T cell responses.

Overall, CD28/B7 co-stimulation promotes T cell activation and differentiation, whereas CTLA-4 /B7 engagement delivers a negative signal to down-regulate the progress of immune responses. The integration of co-stimulatory and inhibitory signals mediated by interaction between B7 and CD28 or CTLA-4, respectively, contributes an important mechanism among multiple pathways in the modulation of T cell responses initiated by TCR ligation.

1.2.3.3 Other co-stimulatory molecules
As well as the critical role of CD28/B7 mediated co-stimulation in T cell activation (Chambers and Allison, 1997; Lenschow et al., 1996), other molecules have been identified that are responsible for CD28-independent activation of T cells (Gause et al., 1997; Shahinian et al., 1993). In CD28− mice, blockade of CTLA-4 triggered graft rejection (Yamada et al., 2001), suggesting that additional B7 receptor(s) other than CD28 or CTLA-4 may provide a co-stimulatory signal. In addition to CD28 and CTLA-4, many other molecules have been found to act as alternative co-stimulatory factors, such as CD2/CD58 (LFA-3), LFA-1(CD18)/ICAM-1 (CD54), and currently identified new cell surface molecules ICOS/B7h (Salomon and Bluestone, 2001; Waliiin et al., 2001), OX40/OX40L (Bansal-Pakala et al., 2001; Ndhllovu et al., 2001) and 4-1BB/4-1BBL
(Bansal-Pakala et al., 2001). Those molecules that participate in CD28/B7-independent co-stimulation, function at different stages of T cell activation and differentiation. Here I will give a brief introduction of those newly discovered "costimulatory molecules".

1.2.3.3.1 ICOS

The inducible co-stimulatory molecule (ICOS), another member of CD28/CTLA-4 family was identified during screening for T cell activation antigens (Hutloff et al., 1999; Yoshinaga et al., 1999). ICOS is only expressed on activated T cells. Its ligand, B7H/B7RP-1, or ICOSL, a homologue of B7.1 and B7.2, is found on B cells, macrophages, DCs and non-immune tissues after LPS stimulation (Swallow et al., 1999; Yoshinaga et al., 1999). Ligation of ICOS with its ligand results in T cell proliferation and T cell-dependent Ab responses (Dong et al., 2001; McAdam et al., 2001; Wallin et al., 2001). Enhanced production of the cytokines IL-4, IL-5, GM-CSF, TNF-α, IFN-γ and IL-10 is also seen for CD4 T cells after co-stimulation via ICOS (Hutloff et al., 1999). In ICOS<sup>−/−</sup> mice, ICOS<sup>−/−</sup> T cell activation and proliferation are defective, and they fail to produce IL-4 and IL-13 in an in vitro differentiation assay (Dong et al., 2001). Profound deficits in isotype switching were also observed in these mice. However, the impaired response could be restored by stimulation via CD40 (McAdam et al., 2001). The latter suggests that CD40-CD40L participate in the ICOS/B7RP-1 mediated collaboration between T cells and APCs. In examining the role of ICOS in autoimmune disease, a severe susceptibility to experimental autoimmune encephalomyelitis (EAE) was induced in ICOS<sup>−/−</sup> mice, revealing that ICOS has a role in the negative regulation of inflammatory diseases (Dong et al., 2001). Possibly, this results from a deficiency of IL-13 in ICOS<sup>−/−</sup> mice. Despite the fact that the function of ICOS/B7PR-1 has not been fully characterised, both in vivo and in vitro assays indicate that like CD28, they act as a co-stimulatory receptors, participating in the both T cell and B cell activation and function.
1.2.3.3.2 OX40L/OX40

OX40L is a member of the TNF family, and is expressed on APCs, such as DCs and B cells (Ohshima et al., 1997; Weinberg et al., 1998). Its receptor OX40 (CD134) is expressed on activated T cells (Flynn et al., 1998). Signalling of OX40 on T cells promotes Th2 responses via increasing IL-4, IL-5 and IL-13 production by CD4 T cells (Ohshima et al., 1998; Rogers and Croft, 2000). This further inhibits IFN-γ expression by CD8 T cells and by IL-12-stimulated CD4 T cells (Flynn et al., 1998). However, in OX40L−/− mice, T cell responses to antigen were impaired, and in transgenic mice that expressed OX40 constitutively in T cells, T cell responses to protein antigens were greatly increased (Ndhlovu et al., 2001). In addition, experimental autoimmune encephalomyelitis (EAE) showed a greater severity in the same OX40 transgenic mice (Ndhlovu et al., 2001). This suggests that OX40/OX40L interactions are not only involved in the regulation of Th2 responses (Flynn et al., 1998; Ohshima et al., 1998), but may also improve Th1 responses (Rogers and Croft, 2000). Signalling through OX40 was found to increase expression of Bcl-xL and Bcl-2, which promotes CD4 T cell survival and suppresses apoptosis following antigen stimulation (Rogers et al., 2001).

1.2.3.3.3 4-1BBL

4-1BB (CD137) and 4-1BBL, are TNFR/TNF family members, and were found to be involved in both CD4+ and CD8+ T cell activation (Cannons et al., 2001). Like OX40, 4-1BB, a co-stimulatory receptor is expressed on activated CD8 and CD4 T cells (Vinay and Kwon, 1998), while 4-1BB ligand is identified at a low level on mature DC, activated B cell and activated macrophages (DeBenedette et al., 1997; Vinay and Kwon, 1998). 4-1BB is one of few co-stimulatory receptors capable of stimulating resting T cells upon strong TCR signal. In the absence of the CD28 molecules, signalling via 4-1BB is able to stimulate a high level of IL-2 production by resting T cells (DeBenedette et al., 1997; Saoulli et al., 1998). Administration of stimulatory anti-4-1BB Ab initiated stronger CD8+
T cell proliferation than that of CD4$^+$ T cells (Shuford et al., 1997), and in vivo analysis showed that anti-4-1BB increased the CTL response in graft-versus-host disease and improved the rejection of the allografts (Shuford et al., 1997). It is suggested that 4-1BB plays a role at the later stage of a response after CD28 downregulation (Watts et al., 1999).

1.3 CD4$^+$ T cell help in CTL cross-priming

The ability of CD4$^+$ T-helper cells to augment the activity of CTL has been extensively characterised (Husmann and Bevan, 1988; Jenkins et al., 2001; Keene and Forman, 1982; Ossendorp et al., 1998; Riberdy et al., 2000; Zinkernagel et al., 1978a). Several studies demonstrate that induction and maintenance of in vivo primary CD8$^+$ T cell responses are dependent on the participation of CD4$^+$ T-helper cells for effective cross-priming (Guerder and Matzinger, 1992; Husmann and Bevan, 1988; Jennings et al., 1991; Keene and Forman, 1982). This research can be traced back to early observation showing a requirement for CD4$^+$ T cell help to trigger cytotoxic T cells against virus and self-antigens (Zinkernagel et al., 1978a). Von Boehmer and Haans then discovered that CD4$^+$ T cell-dependent CTL responses were Ir gene restricted (von Boehmer and Haas, 1979). More recent experiments provided direct evidence to indicate that the absence of CD4$^+$ T cell help reduced or completely prevented the priming of CTL responses (Bennett et al., 1997; Husmann and Bevan, 1988). These observations revealed the important role of CD4$^+$ T cell help in CTL induction. This dependence on CD4$^+$ T cell help was suggested to reflect the avidity of the CD8$^+$ T cell-APC interaction: a low avidity interaction leading to helper-dependent responses whereas a high avidity interaction leading to helper-independent responses (Sprent and Schaefer, 1990). Other factors may also affect the dependence on CD4 helper, for example, the type of pathogens (Borrow et al., 1998; Liu and Mullbacher, 1989) and the APC activation state (Ridge et al., 1998).
Although the nature of help provided by CD4+ T cells has not been completely identified, it is possible that it is mediated through several distinct pathways. When the avidity of the CD8+ T cell-APC interaction is low, CD8+ T cell activation may rely on CD4+ T cell-secreted cytokines to provide additional stimulation to directly activate CTL precursor (CTL-P) in the proximity (Keene and Forman, 1982a; Kirberg et al., 1993). Alternatively, since accessory molecule interactions between T cell and APC can co-stimulate T cell activity and promote endogenous IL-2 production, it is possible that CD4+ T cells may assist CD8+ T cell responses by improving APC co-stimulatory activity (Guerder and Matzinger, 1992).

1.3.1 Activation of CD4 T cells through MHC class II-peptide complexes
CD4+ T cells recognize antigen peptides presented by MHC class II molecules (Pierre et al., 1997). As shown in the generation of ovalbumin (OVA)-specific CTL by cross-priming, deficiency in MHC class II molecule led to a failure in CTL induction because of the inability of the APC to present antigen to specific CD4+ T cells (Bennett et al., 1997). The same phenomena was also observed in mice deficient in CD4+ T cells where OVA specific CTL could not be induced by cross-priming (Bennett et al., 1997). It is important to note that the presence of CD4+ T cells was found to be important only at the in vivo priming stage, since restimulation of the response in vitro could not be prevented by depletion of CD4+ T cells at this stage (Bennett et al., 1997). This suggests the delivery of CD4+ T cell help is essential at the early stage of cross-priming a CTL response.

1.3.2 CD4 help modifies the activity of the cross-priming APC
The requirement for CD4 T cell help at the early stage of cross-priming CTL suggested a role of the helper T cells in modification of APC function. This was supported by the observations that both CD4+ and CD8+ T cells must recognize the antigen on the same APC in order to generate CTL immunity (Bennett et al., 1997; Cassell and Forman, 1988). It is possible that CD4+ T cells provide help via delivery of short range cytokines such as IL-2.
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(Cassell and Forman, 1988; Keene and Forman, 1982a; Morgan et al., 1999a) and IFN-γ (Maraskovsky et al., 1989; Simon et al., 1986). However, the results also suggested a role for accessory molecules that might function through cell-cell interactions to activate the APCs into a status capable of cross-priming CTL. This process is thought to consist of specific physical engagements of antigen-specific T cells and APCs, involving signal transduction between the cells, which eventually results in activation of antigen specific CTL.

1.3.2.1 CD40 dependent CD4 help

Ridge et al first observed that cross-linking CD40 on APCs could bypass the need for CD4⁺ T cells in the differentiation of naïve CD8⁺ T cells into effector CTLs (Ridge et al., 1998). At the same time, evidence from in vivo cross-priming models demonstrated that signalling through CD40 could replace CD4⁺ helper T cells in priming of helper-dependent CD8⁺ CTL responses (Bennett et al., 1998; Schoenberger et al., 1998). Similar conclusions were drawn from virus infection models where primary CTL responses against adenovirus were found to be CD40-dependent (Yang et al., 1996), and CTL memory responses to LCMV were defective in CD40L-deficient mice (Borrow et al., 1996; Borrow et al., 1998). All these observations provided evidence that CD4-dependent CTL responses require a signal through CD40. It is still unclear how signalling through this molecule leads to CTL priming, although, it has been shown that CD40 signalling can upregulate B7 molecules and induce the secretion of IL-12 (Cella et al., 1996; Shu et al., 1995; Yang and Wilson, 1996). More recently, other CD40-independent pathways of APC activation have been described (Lu et al., 2000; Ruedl et al., 1999), but how important each of these is to CTL induction remains unknown.
1.3.2.2 CD40-CD40L signalling

CD40 is a cell surface molecule that belong to the tumor necrosis factor receptor (TNFR) family (Grewal and Flavell, 1996; Stout and Suttles, 1996; van Kooten and Banchereau, 2000; van Kooten and Banchereau, 1997b). Constitutive expression of CD40 has been identified on professional and non-professional APCs, such as B cells, dendritic cells (DCs), follicular dendritic cells, monocytes/macrophages, mast cells, fibroblasts, epithelial cells and endothelial cells, and also on haematopoietic progenitors. CD40 ligand (CD40L), also called CD154 or gp39, is predominantly expressed transiently on activated CD4+ T cells (Lane et al., 1992; Roy et al., 1993; Spriggs et al., 1992). Weak expression has also been detected on a small proportion of CD8+ T cells when stimulated in vitro (Alderson et al., 1993; Lane et al., 1992). In addition, CD40L has been identified on mast cells, basophils, eosinophils, activated B cells and blood DCs (Grewal and Flavell, 1996; Schonbeck and Libby, 2001; Stout and Suttles, 1996; van Kooten and Banchereau, 1997a).

The function of CD40 has primarily been characterised on B cells, where the interaction of CD40 and CD40L was found to be involved in both clonal expansion and deletion of B cells in vivo (Berberich et al., 1994; Foy et al., 1993; Hermann et al., 1995; Noelle et al., 1992; Rathmell et al., 1996; van Kooten and Banchereau, 1997b). This established an essential role of CD40 in the control of humoral immunity (Clark et al., 1996; Foy et al., 1996). In further studies, it was found that CD40 expression on DC and macrophages was also critically important in the generation of T cell immnunity (Clarke, 2000; Grewal and Flavell, 1998; Grewal and Flavell, 1996; Miga et al., 2000). Upon TCR stimulation, CD40L expression is up-regulated on activated T cells, which triggers APCs to upregulate accessory molecules such as B7, ICAM-1, LFA-3, CD44H and MHC class I and class II molecules (Caux et al., 1994; Cella et al., 1997; Grewal and Flavell, 1996; Guo et al., 1996; Kiener et al., 1995; Shinde et al., 1996; Yang and Wilson, 1996). Signals through
CD40 on DCs and monocytes stimulate the secretion of cytokines, such as TNF-α, IL-1β, MIP-1α, IL-6, IL-8, IL-10, and IL-12 (Alderson et al., 1993; Caux et al., 1994; Cella et al., 1996; Koch et al., 1996; Mosca et al., 2000; Schulz et al., 2000; Shu et al., 1995; Wagner et al., 1994). These distinctive cytokines affect migration of activated T cells from the secondary lymphoid compartments to the target organs through regulation of the adhesion molecules produced in endothelia (Grewal and Flavell, 1998) and drive Th1/Th2 responses in different ways. For example, stimulation via CD40 induces IL-12-dependent priming of Th1 cell responses (Kelsall et al., 1996). Recently, signalling through CD40 was also found to enhance the development of autoimmune responses (Frossard et al., 2001; Garza et al., 2000a; Mach et al., 1998; Schonbeck et al., 2000) and graft rejection (Seung et al., 2000). In these cases, CTL responses resulted from the induction of co-stimulatory activity on APCs through CD40 signalling. The absence of CD40L results in a lack of help by activated CD4 T cells which are unable to efficiently activate APC (Caux et al., 1994; Ranheim and Kipps, 1993). These studies highlight the importance of CD40-CD40L interaction in generating the regulation of both humeral and cellular immune responses.

1.3.2.3 CD40-independent CD4 help

Although ligation of CD40 with CD40L has been shown to be important in both induction of APC maturation and CTL effector function, CD40 independent activation of CTL has also been reported (Bachmann et al., 1999; Lu et al., 2000; Ruedl et al., 1999). In these reports, antiviral CD8+ T cells are able to induce the maturation of DCs in the absence of CD40. It is possible that other molecules deliver the signals for induction of T cell responses through activation of APC, e.g. TRANCE/TRANCER (Anderson et al., 1997; Josien et al., 1999; Wong et al., 1997b).
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TRANCE (TNF-related activation-induced cytokine), also called RANKL (receptor activator of NFκB ligand) and its receptor (TRANCE-R, or RANK) are recently described novel members of TNF/TNFR superfamilies emerging as critical regulators of immune responses and bone development (Anderson et al., 1997; Green and Flavell, 1999; Josien et al., 1999; Wong et al., 1997a; Wong et al., 1997b). Upon stimulation through TCR/CD3, TRANCE mRNA and surface molecules are rapidly induced/up-regulated on T cells, and this is further enhanced through CD28-mediated co-stimulation (Josien et al., 1999; Wong et al., 1999b). Unlike CD40 molecules, high levels of TRANCE receptor expression were identified on mature DCs and low levels on activated T cell and B cells, but not on freshly isolated B cells, T cells or macrophages (Josien et al., 1999). TRANCE induces an up-regulation of Bcl-xL, functionally inhibiting apoptosis of DC in vivo and improving DC survival, which subsequently promotes DC-mediated T cell proliferation (Wong et al., 1997a). The signal from TRANCE activates anti-apoptotic molecules through a signalling complex involving TNFR-associated factor 6 (TRAF 6) and c-Src (Arron et al., 2001; Wong et al., 1999a). A deficiency in c-Src or addition of Src family kinase inhibitors blocks TRANCE-mediated PKB activation in osteoclasts (Wong et al., 1999a). In addition to mediating activation and maturation of DCs, TRANCE also stimulates DCs to produce cytokines such as IL-6, IL-1, IL-12, and IL-15 (Josien et al., 1999; Wong et al., 1999b).

1.3.2.4 CD4 help impairs peripheral tolerance

Negative selection of autoreactive T cells in the thymus has been regarded as the major mechanism for self-tolerance (von Boehmer, 1990; von Boehmer, 1992a). Nevertheless, many observations over the past decade have revealed that peripheral mechanisms are also important in the maintenance of tolerance and the control of autoimmunity (Bertolino et al., 1995; Chen et al., 1995; Jones et al., 1990; Kurts et al., 1996; Miller and Morahan, 1992; Rocha and von Boehmer, 1991). Presentation of self-antigen from non-lymphoid tissues
was observed in several experimental models, in which the failure in control of self-tolerance resulted in autoimmune induction (Garza et al., 2000; Kurts et al., 1997a; Morgan et al., 1996; Wong and Janeway, 1999). It appears that the availability of the CD4+ T cell help is an important control of the CD8+ T cell response (Guerder and Matzinger, 1992; Mora et al., 1999). The help seems to prevent the deletion of self-reactive CD8+ T cells, rather than prevent their activation. A well characterised example of this effect involves the transgenic RIP-mOVA mouse, in which CD4 help impairs cross-tolerance resulting from class I-restricted cross-presentation of self-antigen. This model uses the neo-autoantigen, OVA expressed in the pancreatic islets and the kidney proximal tubular cells (Kurts et al., 1996). Adoptive transfer of OVA-specific CD8 T cells into the mice led to tolerance induction mediated by deletion of CD8+ T cells (Kurts et al., 1996; Kurts et al., 1997a). If, however, OVA-specific CD4 T cells were co-transferred into the mice, CD8+ T cell deletion was impaired resulting in the onset of diabetes in a large proportion of recipients (Kurts et al., 1997a). The CD4+ T cells were later found to improve survival of CD8+ T cells but did not increase their proliferation (Kurts et al., 1997a). This is consistent with previous reports that CD4+ T cell help could prevent the deletion of autoreactive CD8+ T cell (Kirberg et al., 1993).

1.4 Thesis aims
This chapter summarizes the factors regulating the induction of T cell immunity and tolerance. However, the cell-cell interaction between CD8 T cells, CD4 T cells and APCs in cross-priming is still not fully defined. Therefore, one of the areas this thesis aims to investigate is the basis of CD4+ T cell help in the induction of CD8+ T cell immunity. As we know, generation of effective CD8+ T cell immunity appears to rely not only on the activation and differentiation of naive antigen-specific CD8+ T cells into effector CTLs, but also on the induction of long-lasting CD8+ T cell memory. This process relies, in part, on MHC class I restricted cross-presentation of exogenous antigens.
The mechanisms involved in class I MHC-restricted presentation of exogenous antigen are poorly defined. While the identify of the cross-priming APCs has just begun to be revealed after a 25 year search, it must be further characterized. In addition to mediating cross-priming, cross-presentation is also involved in tolerance induction. Like cross-priming, this requires TCR/CD3 engagement, but may lack appropriate co-stimulatory signals resulting in the deletion of antigen-specific T cells. Tolerance can be induced at different levels, such as T cell deletion, which is usually preceded by a period of activation; or anergy, a state of T cell unresponsiveness that may result from a failure to stimulate IL-2 production in the absence of co-stimulation. So far, the APC responsible for inducing cross-tolerance has not been identified. However, knowledge of the nature of the cross-priming APC is likely to speed the discovery of the tolerogenic APC.

1.4.1 Description of experimental systems

Two types of models have been used in my studies. Both models utilize OVA as the experimental antigen and C57BL/6 (B6) mice as the host animals. The advantage of examining OVA-specific responses in B6 mice is that both class I and class II MHC-restricted epitopes of the antigen are defined. The CD4⁺ T cell response to OVA is specific for the OVA\textsubscript{323-339} peptide restricted by the class II MHC molecule, I-A\textsuperscript{b}, whereas the CD8⁺ T cell response is specific for the OVA\textsubscript{257-264} peptide restricted to the class I MHC molecule, H-2K\textsuperscript{b}. These studies take advantage of the congenic bm1 murine line that differs from B6 mice only at the H-2K locus. The bm1 mice are unable to generate OVA-specific CTL because the K\textsuperscript{bm1} molecule, unlike K\textsuperscript{b}, is unable to present OVA to CD8⁺ T cells (Nikolic-Zugic and Carbone, 1990). Another advantage of using OVA in B6 mice is that I had access to the H-2K\textsuperscript{b}-restricted OVA\textsubscript{257-264} specific TCR transgenic line, OT-I and I-A\textsuperscript{b}-restricted OVA\textsubscript{323-336} specific TCR transgenic line, OT-II.
For cross-priming studies, I primarily used a model where OVA-coated irradiated spleen cells were injected intravenously into B6 mice. This has been shown to prime OVA-specific CTL (Carbone and Bevan, 1990). For cross-tolerance studies I used transgenic lines that express OVA in the pancreatic islet β cells. These lines include RIP-mOVA mice, which has membrane bound OVA in the pancreatic islets and the kidney proximal tubular cells, and RIP-OVAhi mice, which has a high level of soluble OVA in the pancreatic islets compared with RIP-OVAint and RIP-OVAlo lines. These models allowed me to investigate the interplay between CD4+ T cells, CD8+ T cells and DCs during induction of autoimmunity and to search for the identity of the cross-tolerance APC.

1.4.2 Specific aims

1.4.2.1 Cross-priming CTL and the role of CD4+ T cell help

The first aim of this thesis is to examine the importance of class I MHC-restricted cross-presentation in the generation of primary CD8+ T cell responses in vivo. The emphasis is placed on the requirement for CD4+ T cell help during CTL cross-priming and the nature of this help. In particular, I examine how CD4+ T cells modify APC activity and whether the interaction between CD40-CD40L can replace this T cell help.

1.4.2.2 Identification of cross-priming and cross-tolerizing APCs

The second aim of this thesis is to identify the APC subsets responsible for cross-priming and cross-tolerance. These studies generally focus on the role of DCs in cross-presentation, since this cell type is critical for stimulating naïve T cells. Both in vivo and in vitro models were used. For analysis of the cross-priming APC, I have chosen to examine a cross-priming model where B6 mice are immunized with OVA coated irradiated spleen cells. The priming sites (spleen and/or lymph nodes) were examined and I attempt to isolate the cross-priming APC from this site. To search for the cross-tolerizing APC, I used transgenic mice such as the RIP-mOVA and RIP-OVAhi mice, which express
different forms of OVA antigen in their pancreas. This antigen is cross-presented in the pancreatic draining lymph node, from where I attempt to isolate or deplete the cross-tolerizing APC.

1.4.2.3 Examination of antigenic requirements for cross-presentation
To investigate antigen requirements for cross-presentation, different forms of OVA antigen were used. The responses of CD4 and CD8 T cells to cell-associated OVA (on both living cells and irradiated cells), soluble OVA and OVA peptides were examined in B6 mice. In addition, the effect of altering the route of antigen administration was also examined, comparing cross-presentation after intravenous versus subcutaneous injection. These studies provide a basic understanding of the best forms of antigen and the most appropriate route of delivery to induce responses by cross-presentation.
Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Medium, solutions and reagents

The following solutions marked with the * symbol were prepared by the Media Department at The Walter and Eliza Hall Institute. Those solutions were sterilised by filtration through a 0.22 μm membrane filter with compressed nitrogen and were stored in the dark at 4°C.

4% acetic acid solution was prepared by mixing 40 ml glacial acetic acid (BDH Chemicals, Victoria, Australia) with 960 ml double distilled water (DDW). This solution was not sterilised.

Bouin's Fluid was prepared by mixing 75 ml saturated aqueous picric acid, 25 ml 40% Formaldehyde and 5 ml glacial acetic acid. This solution was provided by The Histology laboratory of the Walter and Eliza Hall Institute.

0.1% bovine serum albumin (BSA)-PBS was prepared by dissolving 0.1 g BSA (>98% purity, standard grade, Hämosan, Austria, Batch No.BSS 91102) in 100 ml PBS. The solution was sterilised by filtration through a 0.45 μm sterile syringe filter.

*BSS (Mouse Tonicity, HEPES Buffered, Balanced Salt Solution, pH 7.2) was prepared by mixing the following stock solutions: 17,052 ml of Milli Q water, 1,694 ml of 1.68 M NaCl (98.2 g per litre of Milli Q water), 42 ml of 1.68 M KCl (125 g
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per litre of Milli Q water), 42 ml of 1.12 M CaCl$_2$ (165 g CaCl$_2$.2H$_2$O per litre of Milli Q water), 14 ml of 1.68 M MgSO$_4$ (414 g MgSO$_4$.7H$_2$O per litre of Milli Q water), 28 ml of potassium phosphate buffer (prepared by mixing 1.68 M KH$_2$PO$_4$ (229 g per litre of Milli Q water) with 1.12 M K$_2$HPO$_4$ (226 g K$_2$HPO$_4$.3H$_2$O or 195 g K$_2$HPO$_4$ per litre of Milli Q water), 168 ml of HEPES (N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid, Gibco BRL, Grand Island, NY, USA) buffer (1.68 M HEPES pH to 7.22 at 20°C with NaOH). Finally, adjust pH of BSS to 7.2.

BSS-FCS buffer for flow cytometry assay consists of BSS containing 2.5% FCS and 0.02% NaN$_3$.

5,6-Carboxyfluoresceine-diacetate-succinimidyl-ester (CFSE) (Molecular Probes, Eugene, OR, USA) was kept as a stock solution (5 mM in DMSO) and stored at -20°C.

Dimethyl sulfoxide (DMSO) 99.5%, was purchased from BDH, AnalaR®, MERCK Pty. Ltd., Kilsyth, Vic., Australia. Lots No. 015635.

*EDTA-BSS mouse tonicity, buffered, contains NaCl and KCl in a 40:1 ratio, no Ca$^{++}$ or Mg$^{++}$, pH 7.2. HEPES buffer and 5 mM EDTA, final osmolarity 308m. EDTA-BSS was prepared by mixing the stock solution for BSS and EDTA stock solutions: 484 ml 1.68 M NaCl, 12 ml 1.68 M KCl, 8 ml PO$_4$ buffer, 48 ml 1.68 M HEPES, 280 ml 0.099 M EDTA (For 100 ml: 3.68 g EDTA disodium salt. 2H$_2$O
make up to 50 ml in 0.1 M HaOH, neutralise to pH 7.2 with NaOH and then make up to 100 ml), 4.712 ml DDW.

Add all contents to a 10 litre pressure tank and bubble gently with nitrogen gas. Bubble continuously as each volume is added. Adjust the pH to 7.2 and check osmolalety.

**Fetal Calf Serum (FCS)** (CSL Ltd., Parkville, Vic., Australia) was heat-inactivated by immersing in a 56°C water bath for 30 min. The heat-inactivated FCS was stored at -20°C. Each batch was tested to ensure it supported a primary *in vitro* allogeneic proliferative response, as well as the *in vitro* induction of antigen-specific CTL from unprimed antigen-specific TCR transgenic CD8\(^+\) T cells.

*HEPES Eagle’s Medium (HEM)* was prepared by mixing a 1 x 10 litre packet of Minimum Essential Medium (Cat. No. 41500-018, Gibco BRL, Grand Island, NY, USA), 160 ml of a 1 M HEPES solution, 1.0 g penicillin and 1.0 g streptomycin in Milli Q water to a final volume of 8.75 litres. pH was adjusted to 7.2 with NaOH.

**HF2.5** consisted of HEPES Eagle’s Medium (HEM) containing 2.5% FCS.

2-mercaptopoethanol (BDH, MERCK Pty. Ltd., Kilsyth, Vic., Australia) 0.29 ml of 2-mercaptopoethanol (14.3 M) was diluted in 400 ml pure water, sterilised by filtration through a 0.45 μm sterile syringe filter and stored at -20°C.
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Ovalbumin (OVA) (grade V, Sigma Chemical Co., St Louis, MO, USA. Lot No 78H7007) was freshly made in 10mg/ml or to an indicated concentration.

OVA_{257-264} and OVA_{323-339} peptides were synthesized using an Applied Biosystems model 431A synthesizer (ABI, FosterCity, CA) and provided by Dr. J. Fecondo, Swinburne University of Technology, Hawthorn, VIC, Australia.

**Phosphate Buffered Saline (PBS)** (Mouse Tonicity), pH 7.3, (0.02 M PO_4) was prepared as a 10 × concentrate by dissolving 570 g of Na_2HPO_4.2H_2O (0.016 M HPO_4), 125 g of NaH_2PO_4.2H_2O (0.004 M PO_4) and 1,740 g of NaCl (0.149 M) in a total of 20 litres Milli Q water. 1 × concentrate was prepared by diluting 100 ml of 10 x concentrate in 900 ml Milli Q water.

Propidium Iodide (Calbiochem, La Jolla, CA, USA) was made up as a 100 μg/ml solution in Normal Saline and stored away from light at 4°C. It was used at a final concentration of 1 μg/ml to label dead cells.

Rabbit Complement (C-six Diagnostics Inc., Wisconsin, USA) Each batch was tested to ensure it was capable of killing ^51Cr-labelled BALB/c lymph node cells in the presence of a 1:4 dilution of anti-CD4 (RL172) antibody supernatant.

**Red blood cell removal buffer (RCRB)** was prepared by dissolving 15.58 g NH_4Cl (0.146 M), 0.074 g ethylenediaminetetra-acetic acid (EDTA)-disodium salt,
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2.0 g NaHCO₃ in 2 litres of Milli Q water. Final pH = 7.3. RCRB was usually pre-warmed to 37°C before use.

RF10 consisted of RPMI-1640 containing 10% FCS, 50 μM 2-mercaptoethanol and 2 mM L-glutamine (Gibco BRL, Grand Island, NY, USA). RF10 was usually pre-warmed to 37°C before use.

*RPMI 1640* (Mouse Tonicity) was prepared by dissolving a 1 × 10 litre packet of RPMI 1640 (Cat. No. 50-020-PB-R, TRACE Biosciences Pty. Ltd., Australia), 9.0 g NaCl, 20 g NaHCO₃, 1.1 g sodium pyruvate, 1.0 g penicillin and 1.0 g streptomycin in 10 litres of DDW.

*RPMI HEPES* was prepared by mixing a 1 × 10 litre packet of RPMI 1640, 59.4 g HEPES, 500 mg hypoxanthine and 6.25 ml gentomycin (25 mg/ml) with 9.6 litres of Milli Q water. The solution was made to pH 6.73 at 23°C with NaOH.

**Scintillation solution** (Microscint-O scintillation fluid, Packard, Meriden, USA).

5% *Triton X-100 solution* (Calbiochem-Behring, La Jolla, CA, USA) was prepared by mixing 5 ml Triton X-100 with 95 ml Milli Q water. This solution was not sterilised.

**Trypan Blue Solution** 0.4%, sterile, Sigma Chemical Co., St Louis, USA. Lot No. 91K2305.
2.1.2 Reagents for isolation of DCs

All these reagents were provided by Professor Ken Shortman.

**Collagenase** (type II, Worthington Biochemical, USA. Freehold, NJ; verified as free of trypsin-like protease activity) was pre-weigh (7 mg/tube), stored at -70°C, dissolved just before use and made up of 1 mg/ml as the final concentration.

**DNAse** (Grade II bovine pancreatic DNase I, Boehringer-Mannheim, Mannheim, Germany). 0.1% DNAse was made in 2% FCS-RPMI 1640, aliquoted at 1 ml/tube and kept at -20°C. 7 mg of collagenase powder and 1 ml aliquots of DNAse were used for digesting 8 spleens.

**EDTA** (isoosmotic) Ethylenediamine tetraacetic acid, final concentration 0.1M for mouse osmolarity, pH 7.2. Normally made from the disodium salt, neutralized to the correct pH with NaOH.

**EDTA-FCS** consists of 1 ml of 0.1M EDTA (isoosmotic) and 9 ml FCS.

**EDTA-BSS-FCS** BSS containing 5 mM EDTA and 2.5% EDTA-FCS.

**KDS-RPMI-FCS** RPMI-1640 (308 m osmolar) buffered with HEPES to pH 7.2 and supplemented with 2% FCS.

**Immuno-magnetic beads** Sheep anti-rat Ig coated M450 Dynabeads (Dynal, Oslo, Norway).
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Density separation medium  Isoosmotic Nycodenz (Nycomed Pharma AS, Oslo, Norway) medium was made as a stock of 0.372 M (30.55g/100 ml), close to 308m osmolar (adjust if not) and had a density about 1.16g/cm³ at 4°C. Stored frozen in sealed plastic container and protect from light. The stock was further diluted with EDTA-BSS to a density of 1.077g/cm³ at 4°C for splenic DC isolation, or 1.082g/cm³ at 4°C for lymph node DC isolation.

2.1.3 Monoclonal Antibodies

R-Phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAb). Anti-CD4-PE (CT-CD4), anti-CD8-PE (CT-CD8α), anti-CD4-FITC (CT-CD4), anti-CD8-FITC (CT-CD8α), Streptavidin-PE and Streptavidin-Tri-Color were purchased from CALTAG Laboratories, Burlingame, CA, USA. Anti-Kβ-biotin (5F1), anti-Kβ-biotin (K9-178), anti-Vα2-biotin (B20.1), anti-Vα2-FITC (B20.1) and anti-Vβ5-biotin (MR9-40) were made from hybridomas available from the Walter and Eliza Hall Institute. Anti-CD11c-FITC (N418), anti-H-2A-Alexa 594 (M5/114), anti-I-Aβ-FITC (25-5-16) and anti-CD8-cy5 (YTS 169.4) were kindly provided by Dr. K. Shortman. Anti-CD4 (H129), anti-CD8 (53.6.72), goat-anti-ratIgG-HRP, guinea pig (gp)-anti-insulin and anti-gp-HRP were kindly provided by Dr. J. Allison. for immunohistology assays.

Before use, all PE or FITC-conjugated mAb were titrated on 10⁶ spleen cells or lymph node cells to determine the optimal staining concentration for detection by flow cytometry.
Supernatants from anti-CD4 (RL172), anti-CD8 (3.168), anti-Thy-1 (J1j), anti-heat stable antigen (HSA) (J11d) and anti-CD40 (FGK45) hybridomas were sterilised by filtration through a 0.22 μm filter unit (Nalge Co. Rochester, NY, USA). The FGK45 hybridoma was the gift of Dr T. Rolink (Rolink et al., 1996).

**Depletion antibody cocktail for DC isolation** consisted of anti-CD3 (KT3-1.1), anti-Thy1 (T24/31.7, a pan Thy-1), anti-B220 (RA3-6B2), anti-Gr-1 (RB6-8C5), and anti-erythrocyte (TER-119) monoclonal antibodies. These were pre-titrated and used at near-saturation, except anti-Thy-1 which was used at one third of the near-saturation amount to avoid removing any DC that had absorbed low levels of Thy-1.

**Ascites** Anti-Thy-1 (T24), anti-CD40L (FGK45), anti-CD8 (YTS 169.4) and anti-CD4 (GK1.5) ascites were grown in BALB/c nude mice. The ascites were collected and centrifuged for 5 min at 450 g and 4°C. The supernatant was stored at -20°C.

**2.1.4 Cell line**

The tumour cell line used in this thesis was the H-2b, B6-derived thymoma, EL4. EL4 cells were grown in RF10 media at 37°C and 5% CO₂.

**2.1.5 Mice**

All mice were bred and maintained under specific pathogen-free conditions at The Walter and Eliza Hall Institute animal breeding facility. Unless otherwise specified, male and female mice between 6 and 12 weeks of age were used for all experiments and transgenic mice were generated on or backcrossed to B6 mice.
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1. Inbred mice used include: C57BL/6J (B6) (Kb, I-Ab), bm 1(Kbm1, I-Ab), (B6 × bm1) F1 and (B6.Kathy × bm1) F1 mice.

2. Transgenic mice expressing different amounts of OVA under the control of the rat insulin promoter (RIP) include:
   1) RIP-mOVA: expressing a membrane-bound form of OVA protein, approximately 2 μg/mg of islet cell protein.
   2) RIP-OVA[^1]: expressing soluble OVA protein, 1 μg/mg of islet cell protein.
   3) RIP-OVA[^2]: expressing soluble OVA protein, 0.04 μg/mg of islet cell protein.
   4) RIP-OVA[^3]: expressing soluble OVA protein, less than 0.03 μg/mg of islet cell protein.

3. Other OVA transgenic mice:
   I-mOVA and II-mOVA mice, which express a membrane-bound form of OVA under the control of the MHC class I and class II promoters respectively.

4. Antigen specific TCR transgenic mice:
   1) Rag-1[^4]OT-I mice (OT-I mice): CD8^+ T cell TCR specific for OVA_{257-264}; Rag-1[^4] mice were the gift of Dr L. Corcoran.
   2) Rag-1[^5]OT-II mice (OT-II mice): CD4^+ T cell TCR specific for OVA_{323-339}, because OT-II mice possess very few T cells when crossed to Rag-1[^4] mice, these mice were used on a Rag-1-wild-type background.
   3) Rag-1[^6]gBT-I mice (gBT-I mice): CD8^+ T cell TCR specific for gB_{498-505}, of the herpes simplex virus glycoprotein B.
5. Other transgenic and knockout mice:

1) MHC-II- B6 mice: mice lacking MHC class II molecules (A<sup>–/–</sup> A<sup>–/–</sup> mice) were the gift of Dr D. Matthis (Cosgrove et al., 1991).

2) GK5 mice: mice expressing an anti-CD4 antibody under the control of RIP and CMV promoters were provided by Dr. A. Lew (Han et al., 2000).

3) CD40L-<sup>–</sup> mice were the kind gift of Dr R. Flavell.

2.2 Methods

2.2.1 Lymphocyte preparation procedures

2.2.1.1 Isolation of murine cells from spleen and lymph nodes

Mice were sacrificed by cervical dislocation or CO<sub>2</sub> asphyxiation. The spleen or lymph nodes were removed and placed into 10 ml HF2.5 and kept on ice. Lymph nodes (LN) including the superficial cervicals, axillary, brachial, inguinal and mesenteric lymph nodes were analysed either individually or pooled. Single cell suspensions were obtained by gently pushing the organs through stainless steel mesh sieves or from individual LNs by scraping the LN on diamond-head forceps. Cells were then centrifuged and resuspended in the appropriate medium.

2.2.1.2 OVA-coating irradiated spleen cells

Spleens were removed from donor mice, usually bm1 mice unless specified, and a single cell suspension was prepared in HF2.5. The cells were irradiated with a dose of 10 Gy, centrifuged to wash cells and then underlaid with 1 ml FCS for 7 minutes (min) at room temperature (RT). After removal of FCS underlay, cells were again centrifuged and the supernatant discarded. For OVA coating, the cells were then
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resuspended in HEM containing 10 mg/ml OVA protein (0.5 ml per spleen). This solution was made freshly from ovalbumin powder. After being incubated at 37°C for 10 min, the cells were washed twice and counted. For immunizing mice, $2 \times 10^7$ cells in 0.5 ml HF2.5 was given i.v. to each mouse.

2.2.1.3 Peptide-pulsing irradiated B6 spleen cells

For peptide coating, B6 cells were irradiated with a dose of 15 Gy, washed once, FCS underlaid and centrifuged. To prepare stimulator for Bulk Culture in CTL assays, $10^8$ cells were resuspended in 2 ml of HF2.5 containing 0.2 μg/ml of OVA$_{257}$-264. To prepare DC stimulators, DCs were resuspended in EDTA-BSS-FCS containing 1 μM OVA$_{257}$-264 or gB$_{498}$-505 and incubated at 37°C for 30 min followed by 2 washes with HF2.5 and then counted. Peptid-pulsed cells were suspended in specified media for different experiments.

2.2.1.4 Purification of naive OT-I and OT-II T cells

Naive OT-I cells were obtained from the spleens and lymph nodes of OT-I mice (Kurts et al., 1997a) by gently pushing the organs through stainless steel mesh sieves to obtain a single cell suspension. These cells were then resuspended in HF2.5 containing a 1/3 dilution of anti-CD4 (RL172) and anti-HSA (J11d) antibody supernatant (2 mL per mouse) and incubated on ice for 30 min. The cells were centrifuged, resuspended to 2 ml in 10% FCS-HEM containing a 1/8 dilution of rabbit complement and incubated at 37°C for 30 min. The cells were washed twice in HF2.5, underlaid with 1 ml FCS to remove dead cell aggregates, centrifuged and counted. OT-II cells were prepared from the lymph nodes of OT-II mice and the
cells were treated with 3.168 (anti-CD8) and J11d antibody supernatant followed by complement as described for OT-I cell preparations. An aliquot of up to 10^6 cells was taken and analysed by flow cytometry using anti-V_α2-FITC & anti-CD8-PE for OT-I or anti-V_α2-FITC & anti-CD4-PE for OT-II cells. The proportion of OT-I cells in semi-purified cell populations was 70-85% (CD8^+ V_α2^+) and OT-II cells, 60-70% (CD4^+ V_α2^+). Specified numbers of CD8^+ V_α2^+ OT-I cells alone or together with naive or activated OT-II T cells were then injected i.v. into the mice or cultured in vitro.

2.2.1.5 Preparation of responding T cells for in vitro culturing with DCs
Naive OT-I and gBT-I cells were harvested from the spleens and lymph nodes of OT-I mice or gBT-I mice. These cells were resuspended in an Ab cocktail including 0.5 ml anti-CD4 (GK1.5), 0.5 ml anti-MHC II (TIB 120) and 1 ml RP10 for each mouse. After incubation at 4°C for 30 min, the cells were washed once and resuspended in 6 ml RF10, 400 µl anti-rat Dynabeads (Dynabeads, Dynal, Oslo, Norway) and 400 µl sheep-anti-mouse Dynabeads. The beads and cells in a 6:1 ratio were mixed and incubated at 4°C for 40-50 min with continuous slow rotation in the cold room. The unwanted cells with attached beads were removed magnetically. After this stage, the T cells were 70-92% pure.

2.2.1.6 CFSE-labelling of T cells
CFSE-labelling was performed according to the method of Lyons and Parish (Lyons and Parish, 1994). After the above enrichment steps (section 2.2.1.4), the semi-purified T cells (OT-I or OT-II cells) were centrifuged and resuspended in PBS.
containing 0.1% BSA, filtered through sterile 100 μm nylon mesh (Australian Filter Services, BCNY 013-100-102) to remove aggregates, centrifuged and resuspended in PBS containing 0.1% BSA for counting. Cells were then resuspended at $10^7$ cells/ml in 0.1% BSA PBS. For every ml of cell suspension, 1 μl of a CFSE stock solution (5 mM in DMSO) was added. The cell suspension was immediately vortexed and incubated for 10 min at 37°C. The cells were washed twice in HF2.5, resuspended in 10 ml of HF2.5, underlaid with 1 ml FCS to remove aggregates, centrifuged and counted. An aliquot of up to $10^6$ cells was taken prior to CFSE labelling and analysed by flow cytometry using anti-CD8/CD4-FITC and anti-Vα2-biotin followed by Streptavidin-PE. $2 \times 10^6$ CD8$^+$ Vα2$^+$ CFSE-labelled OT-I cells or CD4$^+$ Vα2$^+$ CFSE-labelled OT-II were injected i.v. into recipient mice in 0.5 ml HF2.5.

2.2.1.7 In vitro activation of OT-II cells

OT-II cell suspension was prepared as described above. The cells were cultured at $2 \times 10^6$ cells/ml for 4-5 days at 37°C and 5% CO$_2$ in RF10 stimulated with 1mg/ml of OVA protein. Before transferring into recipients, the cells were centrifuged, resuspended in 2 ml HF2.5, and enriched by complement depletion using the same protocol as described for the preparation of naive OT-II cells (section 2.2.1.4). After analysis of the percentage of OT-II T cells by flow cytometry, indicated numbers of CD4$^+$ Vα2$^+$ OT-II cells alone or together with naive OT-I T cells were then injected i.v. into the mice.
2.2.1.8 Elimination of dead spleen cells

Spleens were harvested from mice, sieved, centrifuged and resuspended in the pre-cold Nycodenz medium (density=1.091g/cm³ thawed at 4°C). This cell suspension was overlayed on the top of the same volume of Nycodenz medium and then 2 ml FCS was added on top. The samples were centrifuged at 2900 rpm, 10 min with low brake at 4°C. The cells above the lower interface were collected, washed twice and counted. Viability was about 98%-99%.

2.2.2 T cell proliferation

OT-I and OT-II cell proliferation in response to OVA peptides was examined by [³H]-thymidine incorporation. Preparation of OT-I and OT-II cell suspensions is described in the section 2.2.1.3. The proportion of OT-I or OT-II cells was determined by flow cytometric analysis before addition to culture. 2.5 × 10⁴ OT-I cells/well or 5 × 10⁴ OT-II cells/well were cultured in RF10 in triplicate in 96 well plates stimulated with a serial titration of peptides: OVA₂₅₇₋₂₆₄ for OT-I, and OVA₃₂₃₋₃₉₉ for OT-II cells. At the indicated culture time (day 3, 4 and 5), 1 μCi [³H]-thymidine (Amersham International, U.K) was added to each well and incubation continued for the last 18 h. The plates were then harvested using a Micro 96 Harvester (type IH-110, Bartelt Instruments Pty. Ltd. INOTECH, Switzerland) onto glass fibre filters (type G-7, 95 × 125 mm, IH-201 A, INOTECH). The filters were dried and the incorporated radioactivity measured using a liquid scintillation β-counter (Packard, Instrument Co., Meriden, CT). 35 μl scintillation solution was added to each well. [³H]-thymidine incorporation was expressed as a mean of counts per minute (CPM).
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T cell proliferation in response to OVA-presenting-DCs The ability of DCs to stimulate T cell proliferation was examined by a \[^3\text{H}\]-thymidine incorporation assay. DCs were either derived from the spleens of the mice immunised with OVA-coated bm1 cells or isolated from the lymph nodes of RIP-mOVA mice. Indicated numbers of irradiated (15 Gy) DCs were incubated with $2.5 \times 10^4$ OT-I cells in a final volume of 200 $\mu$L of RF10 in 96 well flat-bottomed plates. After culturing for 48 h, 1 $\mu$Ci \[^3\text{H}\]-thymidine/well was added and culture continued for another 18 h. The cells were harvested and \[^3\text{H}\]-thymidine incorporation was measured as described above. As a negative control, gBT-I cells were seeded in the same way as OT-I cells. Positive controls were prepared by coating B6 splenic DCs (15 Gy) with 1 $\mu$M OVA\(_{257-264}\) peptide or gB\(_{498-503}\) peptide for 30 min at 37°C, followed by three washes in 2% FCS-EDTA-BSS. Indicated numbers of peptide pulsed DCs were then cultured with OT-I cells or gBT-I cells.

2.2.3 Generation of bone marrow chimeras

The hind leg femur and tibia of donor mice were removed and the bone marrow cells were flushed from the bones into 10 ml HF2.5. To deplete T cells, the bone marrow cells were centrifuged, resuspended at $5 \times 10^7$ cells/ml in HF2.5 containing a 1:5 dilution of anti-Thy-1.2 (J1j), a 1:5 dilution of anti-CD8 (3.168) and a 1:5 dilution of anti-CD4 (RL172) antibody supernatants and incubated for 30 min on ice. The cells were then centrifuged, resuspended at $5 \times 10^7$ cells/ml in a 1:8 dilution of rabbit complement in HEM containing 10% FCS and incubated for 30 min at 37°C. The cells were washed twice in HF2.5, FCS underlaid, counted, centrifuged and resuspended at $10^7$ cells/ml in HF2.5. $5 \times 10^6$ T cell depleted bone marrow cells
were injected i.v. into recipient mice, 8-12 weeks of age, that had been lethally irradiated with a dose of 9 Gy. The next day, mice were injected intraperitoneally with 0.1 ml of T24 (anti-Thy-1) ascites to deplete radio-resistant T cells. These mice were left 8 weeks for reconstitution of the bone marrow before being used in experiments.

2.2.4 **Histology**

Pancreas and spleen were dissected from mice and placed into Bouin’s fixative solution for 2 h at RT, washed with 70% alcohol twice, then left in 70% alcohol overnight. 5 μm paraffin-embedded sections were cut and stained with hemotoxylin and eosin. Tissue sectioning and staining with H&E were performed by the histology laboratory at The Walter and Eliza Hall Institute of Medical Research.

2.2.5 **Immunohistochemical assay**

Pancreas and spleen were dissected, snap-frozen in OCT (Tissue Tek®, SAKURA, U.S.A) in an iso-pentane/liquid nitrogen bath, and stored at -70°C. Serial 5 μm cryostat sections were cut, fixed in acetone, air dried and stored at -20°C. Immunohistochemical staining was performed using an immunoperoxidase technique. To decrease non-specific staining, the sections were blocked in 10% FCS for 15 min at RT followed by one wash in PBS. The tissue sections were incubated with anti-CD4 (H129) or anti-CD8 (53.6.72) supernatant for one hour at RT in a humid box. After 2 washes in PBS for 5 min, a horseradish peroxidase (HRP) conjugated secondary antibody, goat anti-rat IgG, was added and incubated for another hour at RT. To locate pancreatic islets, insulin staining was performed with
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guinea pig (gp) anti-insulin and the secondary Ab of anti-gp-HRP. HRP was developed using a Vector® NovaRED™ substrate Kit for peroxidase (Burlingame, CA 94010). The reaction proceeded for 5-8 min and sections were counterstained with hematoxylin.

2.2.6 In vivo depletion of CD4+ T cells

Mice were injected (i.v.) with anti-mouse CD4 ascites (GK1.5) or anti-mouse CD8 ascites (YTS169.4) 4 times in 7 days.

2.2.7 Procedures for Dendritic cells (DCs) preparation

In vivo immunising the mice  B6 mice were primed by injection (i.v.) of 2 x 10^7 OVA-coated, irradiated bm1 spleen cells. Usually, 16 mice were required.

Isolation of murine DCs  This procedure was performed as described recently (Vremec et al., 2000). The spleens were harvested from the mice 13-14 hours after priming and cut into small fragments. Spleen fragments were suspended in 14 ml of KDS-RPMI-FCS containing 1 mg/ml freshly dissolved collagenase and 2 ml of 0.1% DNAse I. The collagenase/DNAse digestion was conducted at RT (~22°C) for 20 min with constant pipetting using a wide bore pasture pipette to facilitate digestion. Dissociation of T cell-DC complexes was achieved by the addition of EDTA (1.4 ml, 0.1M EDTA, pH7.2) for a further 5 min after the initial digestion. Residual stromal fragments were then removed by passing the suspension through a stainless steel sieve. Thereafter the samples were place on ice and all subsequent procedures were
performed at 4°C in a divalent-metal free medium (EDTA-BSS-FCS), in which FCS already contained 1/10 of 0.1 M EDTA.

The cells were recovered from the digested suspension by centrifugation at 500 g for 7 min through a FCS underlay. The cell pellet was immediately resuspended in cold, well-mixed 1.077 g/cm³ Nycodenz medium (5 ml of Nycodenz for 4 spleens) and layered onto a further 5 ml of Nycodenz. The Nycodenz were overlayed with 1-2 ml of EDTA-FCS and centrifuged for 10-15 min at 1700g (3100 rpm), at 4°C with no brake.

Light density cells were collected from all the upper zone to a little below the lower interface and the cells were washed in EDTA-BSS-FCS, then recovered by centrifugation at 500g (1700 rpm) for 7 min. The cells were incubated with 10 μl of mAb cocktail per 10⁶ cells for 30 min on ice. After incubation for 30 min, the cells were washed with EDTA-BSS-FCS and resuspended in a minimal volume of EDTA-BSS-FCS (400 μl/8 spleens). This thick suspension was transferred to the pre-washed Dynabeads. The beads and cells in a 3:1 ratio were mixed and incubated at 4°C for 20 min with continuous slow rotation in a 5 ml sealed tube. This mixture was then diluted with 2.5 ml EDTA-BSS-FCS and the unwanted cells with attached beads removed magnetically. After this stage, the splenic DCs were usually about 80% pure.

**Immunofluorescent labelling of DC preparations** To identify and sort total DCs, the pan-DC markers used were high levels of MHC class II and CD11c. Anti-CD11c
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(N418) was used as Cy5, FITC, or PE conjugates. Anti-MHC class II (N22 or M5/114) was used as FITC, PE, Cy5, Texas Red, or Alexa 594 conjugates; the conjugation levels were deliberately less than maximal to ensure that the strong staining for class II MHC at saturation did not cause inaccurate color compensation problems in other channels. The markers used to separate the spleen DC subpopulations were CD8 and CD4. Anti-CD8 (YTS169.4) and anti-CD4 (GK1.5 or YTA3.1) were used as FITC, PE, Cy5, Alexa 594, or biotin conjugates. The second-stage stain for biotin-conjugated mAb was PE-streptavidin (PharMingen, San Diego, CA). Propidium iodide (PI) was included at 1 μg/ml in the final wash after immunofluorescent staining to label dead cells.

Flow cytometric analysis and sorting of DCs Sorting was performed on a FACStarPlus instrument (Becton Dickinson, San Jose, CA), using up to four fluorescent channels for the immunofluorescent staining (FL1 for FITC, FL2 for PE, FL3 for Cy5, and FL4 for Texas Red or Alexa594), with the FL5 channel set to exclude PI-positive dead cells and autofluorescent cells. Care was taken during gating that any cells brightly fluorescent in FL3 and spilling over into FL5 were not gated out as dead cells. As well as the class II MHC and CD11c markers, the forward and side scatter gates were set to select for the light scatter characteristics of DC. When large DC numbers were needed, the MoFlo instrument (Cytomation) was used.
2.2.8 Quantitation of OVA on OVA-coated spleen cells by ELISA

The amount of OVA associated with OVA-coated spleen cells was quantitated by capture ELISA (Li et al., 2001). Cells isolated from bm1 mouse spleens were resuspended in HEM containing 10 mg/ml OVA protein and incubated at 2 × 10⁸ cells/ml at 37°C for 10 min. OVA-coated bm1 cells were then washed three times in HEM and lysed in buffer containing 1% Triton X-100 and proteinase inhibitors (Sambrook and Shapiro, 1989). The soluble fraction was recovered after centrifugation at 15,000 × g. For the ELISA, microtiter plates (Dynatech, Chantilly, VA) were coated with protein A-purified rabbit anti-OVA antiserum (Rudy and Lew, 1997) at 4 μg/ml in PBS overnight at 4°C, and washed 6 times with PBS/0.05% Tween 20. Serial dilutions of test lysates were incubated on the plates overnight at 4°C. Similarly, serial dilutions of OVA protein (Sigma) were used as standards. Plates were washed again, then mouse anti-OVA antiserum (Boyle et al., 1997) at 1:1000 in PBS-5% skim milk powder was added as the secondary antiserum for 3 h at RT. Plates were washed and incubated for 3 h at RT with HRP-conjugated rabbit anti-mouse IgG (Southern Biotechnology, Birmingham, AL), then washed before addition of a 3,3,5,5-tetramethylbenzidine substrate solution. The reaction was stopped with 0.5 M H₂SO₄ and the OD read at 450 nm. Data were analyzed using KC Jr. software (Biotek Instruments, Winooski, VT).

2.2.9 Radioisotopes and irradiation of cells and mice

Irradiation was performed using a ⁶⁰Co source on an Eldorado 8 instrument. Spleen cell suspensions were irradiated with a dose of 10 Gy in 10 ml HF2.5. DCs and EL4 cell suspensions were irradiated with a dose of 15 Gy in 10 ml HF2.5. Adult mice, at
least 6 weeks of age, were irradiated with a dose of 9 Gy once or 5.5 Gy twice, 4 hours apart prior to bone marrow reconstitution.

2.2.10 Removal of the OVA peptide from OVA protein

OVA peptides may be present in an OVA protein preparation and associate with MHC I molecules on the cell surface, which mediates direct presentation instead of being cross-presented. To remove the peptides from an OVA protein preparation, the soluble OVA solution was applied to a PD-10 column. The PD-10 column was equilibrated with PBS and 2.5 ml of sample (1 ml of 50mg chicken albumin/ml PBS plus 1.5 ml PBS) was run into the column. The high molecular weight component-protein was eluted with 3.5 ml PBS. OVA protein was collected and detected by testing OD (see the following formula), then sterilised by filtration through a 0.22 μm membrane filter with compressed nitrogen. Aliquots of soluble OVA protein were stored at –20°C.

\[
\text{OVA protein (mg/ml)} = \frac{\text{OD}_{280} - (0.35 \times \text{OD}_{495})}{0.7} \times \text{dilution}
\]

2.2.11 Miscellaneous techniques

2.2.11.1 Centrifugation

Lymph node or spleen cell suspensions were centrifuged in 14 ml or 50 ml tubes for 7 min at 450 g and 4°C. 5 ml polystyrene tubes (flow cytometry) were centrifuged for 5 min at 200 g and 4°C. 96-well plates were centrifuged for 5 min at 200 g and 4°C, with no brake.
2.2.11.2 Examination of glucose level of mouse urine and blood

Urine analysis was performed by dipping one test strip of Diastix Reagent Strips (Bayer Australia Ltd, NSW, Australia) into fresh mouse urine, removing immediately, and then 30 seconds later comparing the glucose test area to the Colour Chart.

Blood glucose was usually examined after two positive readings by urine analysis. This was performed by adding a drop of blood on the test area of a strip of BM-TEST-GLYCEMIE (Roche Diagnostics Australia Pty Ltd. NSW Australia). 1 min later, the blood was wiped off and the colour of the strip compared to the Colour Chart in 2-3 min.

2.2.11.3 Flow Cytometry

In general, approximately $10^6$ lymph node or spleen cells were added to 5 ml polystyrene tubes. In some experiments, the samples needed to be depleted of red blood cells by treatment with RCRB before being analysed by Flow Cytometry. For example, when examining CFSE-labelled T cell proliferation, spleen cells were treated with RCRB. 20 µl of FACS buffer containing the appropriate monoclonal antibodies was then added to the cells for 30 min on ice. The cells were then washed twice in FACS buffer and where necessary, 20 µl of the secondary antibody was added and the cells were incubated for a further 30 min on ice. After two washes, cells were resuspended in 100 µl FACS buffer and stored on ice. Just prior to analysis, propidium iodide was added to each tube to give a final concentration of 1 µg/ml. Three-colour flow cytometry was performed on a FACScan® using
CellQuest software (BD). Live gates were set on lymphocytes by forward and side scatter profiles. 5,000-10,000 live cells were usually collected for analysis. Analysis of fluorescent cells was carried out using WEASEL software (F. Battye, Walter and Eliza Hall Institute, Melbourne, Australia).

2.2.1.4 Injections

26 gauge needles were used for all intravenous, intraperitoneal and subcutaneous injections. The volumes injected were 0.2-0.5 ml for intravenous injections; 0.1 ml for intraperitoneal injections; and 0.1 ml for subcutaneous injections.

2.2.1.5 Live cell counts

50 μl was taken from a single cell suspension of known volume and was mixed, by pipetting, with 50 μl of Trypan Blue Solution. 50 μl of the resulting suspension was then mixed, by pipetting, with 50 μl of a 4% acetic acid solution to kill red blood cells. Live cells (Cells excluding Trypan Blue) were then counted under a light microscope using a 0.0025 mm² glass haemocytometer (Marienfeld, Germany).
Chapter 3 Antigenic Requirements in the Cross-presentation

3.1 Introduction

While most cells of the body cannot present exogenous antigens via their class I processing pathway, specialised antigen presenting cells (APCs) exist that have the capacity to capture such antigens and present them on MHC class I molecules (Carbone et al., 1998; Jondal et al., 1996; Yewdell et al., 1999). This process is referred to as cross-presentation and was first described for the generation of CTL to donor cell-associated minor histocompatibility antigens presented in the context of MHC class I molecules expressed by host APCs (Bevan, 1976). This pathway is accessible to various cell-associated antigens, and efficiently presents apoptotic cellular material (Albert et al., 1998; Bellone et al., 1997; Inaba et al., 1998). Cross-presentation has been reported to be involved in the induction of CTL immunity to various antigens, including minor histocompatibility antigen (Gordon et al., 1976), viral proteins (Gooding and Edwards, 1980; Knowles et al., 1979) and tumour-associated antigens (Huang et al., 1994; Pulaski et al., 1996). In this context, it has been referred to as cross-priming (Carbone et al., 1998). Cross-presentation has also been reported to be responsible for presentation of self-antigens, leading to tolerance (cross-tolerance) induction by deletion of self-responsive T cells (Adler et al., 1998; Heath et al., 1998; Kurts et al., 1997).

It was previously shown that OVA-specific CTL could be generated by injecting B6 mice intravenously with irradiated spleen cells, either loaded intracytoplasmically with OVA by osmotic shock, or simply coated with whole OVA protein (Carbone and Bevan,
In this model, OVA-coated splenocytes are not responsible for presentation of OVA, but are captured by host cross-priming APC and then associated OVA is presented to both CD8⁺ and CD4⁺ T cells.

To better understand the antigenic requirements for cross-presentation of self-antigens to CD8⁺ T cells, as well as to CD4⁺ T cells, several transgenic mouse lines were generated expressing different amounts of OVA under the control of the rat insulin promoter (RIP) (Kurts et al., 1998c; Kurts et al., 1999). Comparison of the activation of OVA-specific transgenic CD8⁺ T cells (OT-I cells) and CD4⁺ T cells (OT-II cells) revealed that it was somewhat more difficult to activate OT-II cells by OVA expressed by the pancreatic b cells. This raised the question of whether there was a bias for presentation of cell-associated antigens versus soluble antigens in the class I and class II pathways.

To examine the efficiency of cross-presentation for different antigens, a comparison of the in vivo efficiency of presenting cell-associated versus soluble OVA to CD8⁺ T cells versus CD4⁺ T cells was examined using the OT-I and OT-II TCR transgenic lines as responders. This research provided quantitative evidence that cell-associated antigens were very efficiently presented in both MHC class I and class II pathways. In addition, this data revealed the efficiency of activation of CD8⁺ and CD4⁺ T cells as a consequence of presenting different antigens was affected by multiple factors, including the dose of antigen, the route of administration and whether the antigen is cell-associated or soluble. The implications of these findings with respect to presentation of self-antigens are also discussed.
3.2 Results

3.2.1 Dose-dependent response of OT-1 to cell-associated OVA in vivo

Intravenous injection of spleen cells coated with OVA for 10 min at 37°C can induce an OVA-specific CTL response (Carbone and Bevan, 1990). This response is CD4+ T cell dependent, and requires cross-presentation of donor antigens on host APC (Bennett et al., 1997; Carbone and Bevan, 1990). To determine the amount of antigen required to activate CD8+ T cells by OVA-coated spleen cells, CFSE-labelled transgenic OT-I (CD8+) cells were transferred into B6 mice that were primed a day later with spleen cells coated with different amounts of OVA. Three days after priming, recipients were sacrificed and their spleen cells were analysed by flow cytometry (Figure 3.1, top row). When CFSE-labelled cells proliferate, their fluorescence is equally distributed between daughter cells, which results in a $2^n$-fold reduction in fluorescence intensity, where $n$ stands for cell divisions. Results of T cell proliferation revealed that OT-I cells could proliferate in response to cell-associated OVA when the coating concentration was as low as 0.01 mg/ml.

3.2.2 Quantitation of OVA on OVA-coated spleen cells

To determine the amount of OVA associated with the injected spleen cells, protein was isolated from OVA-coated bm1 splenocytes by Triton X-100 solubilization and quantitated using ELISA by Dr. Robyn Sutherland. In duplicate samples, coating with OVA at 10 mg/ml yielded 160 and 162 ng OVA per $2 \times 10^7$ cells (average 161 ng), and coating with OVA at 1 mg/ml yielded 18 and 24 ng OVA per $2 \times 10^7$ cells (average 21 ng). This meant that the minimum amount of cell-associated OVA (0.01 mg/ml) required to activate OT-I cells was approximately 0.2 ng per mouse.
Figure 3-1 Response of OT-I and OT-II cells to spleen cells coated with various concentrations of soluble OVA. Mice were injected with CFSE-labelled transgenic OT-I or OT-II T cells, and one day later primed with $2 \times 10^7$ irradiated spleen cells coated with different amounts of OVA. Three days later, these mice were sacrificed and their spleen cells analysed by flow cytometry. Profiles were gated on $\text{CD8}^+$ or $\text{CD4}^+$, CFSE$^+$, propidium iodide-negative cells. *Approximate amount of OVA in μg delivered to each mouse in association with the OVA-coated spleen cells, which is described in chapter 2 (2.2.8). The experiment was performed three times, with two mice in each group.
Chapter 3  
Antigenic requirement in cross-presentation

3.2.3 Soluble OVA is not presented as efficiently as cell-associated OVA for recognition by OT-I cells

The above data indicates that very small amounts of cell-associated OVA were able to stimulate OT-I cells. This observation, however, contrasts with the generally held belief that exogenous proteins have poor access to the class I pathway. Perhaps this was related to the fact that we used cell-associated OVA, which may behave very differently from traditionally examined soluble protein antigen (Carbone and Bevan, 1990). To address this issue, mice were injected intravenously with soluble OVA at different doses and then the proliferation of CFSE-labeled OT-I cells was examined (Figure 3.2, top row). While soluble OVA was able to stimulate OT-I cells, it required a minimum dose of 10 μg per mouse, which was 50,000-fold more antigen than needed to stimulate when in a cell-associated form (approximately 0.2 ng) (Figure 3.1, top row).

One explanation for the poor response to soluble OVA relative to cell-associated OVA may be that the former did not supply any type of inflammatory stimulus. Thus, the difference in the sensitivity of proliferate responses may reflect differences in the co-stimulatory state of the APC. To test this possibility, various doses of soluble OVA were injected intravenously with 1 μg of LPS, and then the proliferation of OT-I cells was examined. The presence of LPS did not apparently enhance the dose response of OT-I cells. This experiment was performed once only (Figure 3.3).

3.2.4 Response of OT-II cells to cell-associated and soluble OVA in vivo

So far, we had examined the MHC class I-restricted response to cell-associated OVA
Figure 3-2  Response of OT-I and OT-II cells to soluble OVA injected intravenously. Mice were injected with CFSE-labelled transgenic OT-I or OT-II T cells and then one day later primed with various amounts of soluble OVA i.v. Three days later, these mice were sacrificed and their spleen cells analysed by flow cytometry. Profiles were gated on CD8+ or CD4+, CFSE+, propidium iodide-negative cells. The experiment was performed twice.
Figure 3-3 Response of OT-I cells to soluble OVA in the presence of LPS. Mice were injected intravenously with CFSE-labelled transgenic OT-I T cells, and one day later primed intravenously with various amounts of soluble OVA in the presence or absence of LPS (1 μg/mouse). Three days later, these mice were sacrificed and OT-I proliferation in the spleen was analysed by flow cytometry. Profiles were gated on CD8+, CFSE+, propidium iodide-negative cells. The proportion of proliferated OT-I cells in two mice injected with 10.0 μg sOVA in the presence of LPS was 85% and 77.2%.
versus soluble OVA. To gain understanding of the MHC class II-restricted response to these forms of OVA, CFSE-labelled transgenic OT-II (CD4\(^+\)) T cells were transferred into B6 mice that were primed the next day with titrated amounts of OVA-coated spleen cells (Figure 3.1, bottom row) or soluble OVA (Figure 3.2, bottom row). Three days after priming, recipients were sacrificed and their spleen cells analysed by flow cytometry. This revealed OT-II cells required a minimum concentration of 1.0 mg/ml OVA during spleen cell coating (Figure 3.1, bottom row), which translates to approximately 21 ng OVA associated with the injected cells. This was 500-fold less than the 10 \(\mu\)g of soluble OVA required to activate OT-II cells (Figure 3.2, bottom row).

These data indicated that both OT-I and OT-II cells were more sensitive to cell-associated OVA than to soluble OVA, with a 50,000-fold increase in sensitivity for OT-I cells and a 500-fold increase in sensitivity for OT-II cells. Furthermore, they indicated that while soluble OVA equally stimulated OT-I and OT-II cells, cell-associated OVA was approximately 100-fold more efficient at activating OT-I cells.

We have previously reported that cross-priming with cell-associated OVA requires CD4\(^+\) T cells help (Bennett et al., 1997) raising the question of why OT-I cells were able to proliferate to doses of cell-associated OVA that were too low to stimulate OT-II help. This may be explained by the observation that high frequencies of CD8\(^+\) T cells, such as those used in our adoptive transfer experiments, can induce CTL immunity by cross-priming in the absence of CD4\(^+\) T cell help (Mintern and Heath, personal communications).
3.2.5 Are the sensitivities of OT-I and OT-II cells similar?

One explanation for the greater sensitivity of OT-I cells to cross-presented cell-associated OVA could be that these cells have a greater sensitivity to antigen than OT-II cells. In an attempt to compare their sensitivities, we examined the proliferative response of OT-I and OT-II cells to peptide in vivo and in vitro. In vivo, OT-II cells required only a little more peptide than OT-I cells for activation (Figure 3.4). Interestingly, however, when examined in proliferative responses in vitro, OT-I cells were approximately 500-fold more responsive than the OT-II cells to peptide stimulation (Figure 3.5). The differences between these two conditions highlight the limitations in comparing these two T cell subsets.

3.2.6 Are OT-I cells more sensitive than OT-II cells to other forms of cell-associated OVA?

To determine the generality of the biased sensitivity of OT-I cells over OT-II cells in response to in vivo stimulation with cell-associated OVA (Figure 3.1), we examined the response of OT-I and OT-II cells to spleen cells transgenically expressing OVA instead of coated with OVA. To do this, mice expressing a membrane-bound form of OVA (mOVA) under the control of the MHC class I promoter were generated (I-mOVA mice). Irradiated spleen cells from these mice were then used as a source of cell-associated OVA to stimulate mice containing CFSE-labelled OT-I or OT-II cells (Figure 3.6). While OT-I cells proliferated in response to I-mOVA spleen cells, OT-II cells were unresponsive to this level of transgenically expressed mOVA. Thus, even when OVA was expressed as a cellular antigen, it still preferentially stimulated OT-I
Figure 3-4  Response of OT-I and OT-II cells to different amounts of their cognate peptide in vivo. Mice were injected with CFSE-labelled transgenic OT-I or OT-II T cells and then one day later primed i.v. with the appropriate peptide: OVA\textsubscript{257-264} for OT-I cells and OVA\textsubscript{323-339} for OT-II cells. Three days later, these mice were sacrificed and their spleen cells analysed by flow cytometry gated on CD8\textsuperscript{+} or CD4\textsuperscript{+}, CFSE\textsuperscript{+}, propidium iodide-negative cells. The minimum dose required for activation of OT-I cells was found to be 0.1 nM (2/2) and the minimum doses for OT-II cells were 0.5 nM (2/4) and 1.0 nM (2/4).
Figure 3-5  Proliferation of OT-I and OT-II T cells responding to corresponding OVA peptides *in vitro*. OT-I cells and OT-II cells were prepared from the mice lymph nodes respectively. 2.5 × 10^5 /well OT-I cells and 5 × 10^5 /well OT-II cells were seeded in 96 well plates in triplicate and stimulated by OVA peptides (OVA_{257-264} for OT-I, OVA_{323-339} for OT-II cells) at indicated concentrations from 1 to 3 days with 1 μCi [3H]-thymidine per well for the last 18 h. The proliferative response to OVA peptides was determined by [3H]-TdR incorporation (CPM).
Figure 3-6 Response of OT-I and OT-II cells to priming with spleen cells expressing mOVA. Spleen cells from mice expressing mOVA under the control of the class I promoter (I-mOVA mice) were irradiated and used to prime B6 mice injected one day prior with CFSE-labelled OT-I or OT-II cells. Three days later, these mice were sacrificed and their spleen cells were analysed by flow cytometry. Profiles were gated on CD8$^+$ or CD4$^+$, CFSE$^+$, propidium iodide-negative cells. The experiment was performed twice.
cells. To check that the I-mOVA mice produced the class II-restricted determinant, CFSE-labelled OT-II cells were injected into these mice and then proliferation was examined 2 to 3 days later. This showed strong proliferation of OT-II cells, indicating the presence of the MHC class II determinant in these mice (Figure 3.7).

3.2.7 Presentation of self-antigens to the T cell subsets

The data outlined above suggested that cell-associated OVA was presented more efficiently than soluble OVA to both OT-I and OT-II cells. Furthermore, while soluble OVA was presented equivalently to OT-I and OT-II cells (Figure 3.2), cell-associated OVA was more efficiently presented to OT-I cells (Figure 3.1). Knowledge of this pattern of relative responses to different forms of antigen allowed us to examine the form of cross-presented antigen when soluble OVA was expressed as a model self antigen in the pancreas. If OVA was simply secreted and presented in the draining lymph node then it should equally stimulate both OT-I and OT-II cells. If, however, it was captured in a cell-associated form then we might expect to see biased presentation to OT-I cells. To address this issue, we examined the response of OT-I and OT-II cells in transgenic lines expressing OVA under the control of the rat insulin promoter. We have three transgenic lines that express different amounts of secreted OVA: RIP-OVA hi mice, which express 1 μg/mg of islet tissue (Kurts et al., 1999), RIP-OVA int, which express 0.04 μg/mg of islet tissue, and RIP-OVA lo mice, which express less than 0.03 μg/mg (Kurts et al., 1999). These mice were injected with CFSE-labelled OT-I or OT-II T cells and then 3 days later their pancreatic lymph nodes were harvested and single cells were examined by flow cytometry (Figure 3.8). Like exogenous foreign antigens,
**B6** | **I-mOVA**
--- | ---
**day 2** | **day 2**
**day 3** | **day 3**

**Figure 3-7 Proliferation of OT-II cells in I-mOVA.** B6 and I-mOVA mice were injected intravenously with CFSE-labelled transgenic OT-II cells, these mice were sacrificed 2 day and 3 days later and the cells from the spleen were analysed by flow cytometry. Profiles were gated on CD8⁺, CFSE⁺ and propidium iodide-negative cells. This experiment was performed by Gayle Davey.
Figure 3-8 Response of OT-I and OT-II cells to different doses of transgenically expressed OVA. Three lines of pancreatic OVA expressing transgenic mice were injected with CFSE-labelled OT-I or OT-II cells. These lines expressed secreted OVA at (A) 1 μg/ mg of islet tissue (RIP-OVA^{hi} mice), (B) 0.04 μg/mg of islet tissue (RIP-OVA^{int} mice) and (C) less than 0.03 μg/mg of islet tissue (RIP-OVA^{lo} mice). 3 days later their pancreatic lymph nodes were harvested and single cells examined by flow cytometry. Profiles were gated on CD8^{+} or CD4^{+}, CFSE^{+}, propidium iodide-negative cells. The experiment was performed three times.
transgenically expressed self-antigens could also enter the MHC class I presentation pathway, as previously reported (Kurts et al., 1996). OT-I cells proliferated well in RIP-OVAhi mice, showed a much weaker response in RIP-OVAint mice, and failed to proliferate in RIP-OVAb, whereas OT-II cells only proliferated in the RIP-OVAhi line. Thus, it appeared that transgenically expressed OVA produced by islet β-cells behaving like cell-coated OVA, not soluble OVA, as it favoured the activation of OT-I T cells over OT-II T cells.

3.2.8 Response of OT-I T cells and OT-II T cells to subcutaneous soluble antigen

In an attempt to understand how different routes of antigen administration affect presentation to CD8+ and CD4+ T cells, subcutaneous injection of soluble OVA was examined for its ability to stimulate T cells. B6 mice were transferred intravenously with CFSE-labelled OT-I or OT-II T cells and then the next day titrated amounts of soluble OVA were administered subcutaneously into the flank of B6 mice. After three days, the draining inguinal LN (ILN) cells were analysed by flow cytometry (Figure 3.9). Surprisingly, OT-II T cells responded to soluble OVA at a concentration as low as 0.5 μg/mouse, which was 20 fold less than required to stimulate proliferation of OT-I T cells (which required 10 μg/ mouse). Interestingly, s.c. injection was 20 fold more efficient than i.v. injection for activation of OT-II cells.

3.2.9 Response of OT-I T cells and OT-II T cells to subcutaneous cell-coated antigen

Differences in the response of OT-I and OT-II cells to soluble OVA injected i.v. versus s.c., led us to ask whether there would also be differences in the response to cell-
Figure 3-9  Response of OT-I and OT-II cells to soluble OVA injected subcutaneously. Mice were injected intravenously with CFSE-labelled transgenic OT-I or OT-II cells, and one day later primed with various amounts of soluble OVA subcutaneously. Three days later, these mice were sacrificed and their cells from both draining lymph node and non-draining lymph node were analysed by flow cytometry. Profiles were gated on CD8$^+$ or CD4$^+$, CFSE$^+$, propidium iodide-negative cells. This experiment was done twice.
associated OVA by these two routes. On day two after intravenous transfer of CFSE-labelled OT-I and OT-II T cells, B6 mice were primed subcutaneously with OVA-coated spleen cells. Three days later, the cells isolated from inguinal draining LN were analysed by flow cytometry to identify proliferating OT-I or OT-II cells. In contrast to soluble OVA, the response to cell-associated OVA by either OT-I or OT-II T cells was very poor, with weak proliferation seen only at the highest coating concentration (10μg/ml) (Figure 3.10).

The fact that we observed a weak response indicated that cell-associated OVA was presented very poorly when injected sc. In fact, it was possible that it was not presented at all, but that the weak response we observed was due to presentation of soluble OVA that had leaked from the cell-associated antigen and entered the draining LN. In case we had simply missed the appropriate time for presentation, we next examined the kinetics of presentation in the draining LN. B6 mice were transferred i.v. with CFSE-labelled OT-I cells and then primed with OVA coated spleen cells 4 days later. To limit the release of soluble OVA, we now coated cells with 1 mg/ml. Draining lymph node cells were harvested and their proliferation analysed on day 6, 7 and 8 respectively (Figure 3.11). In this case, again only a very weak OT-I response could be seen, and this was on day 7. These data strongly suggest that OVA-coated spleen cells are not cross-presented when injected s.c.:

3.2.10 Apoptotic cells are not essential for cross-presentation

Although apoptotic cells have been implicated as targets for cross-priming APCs (Albert et al., 1998; Arrode et al., 2000; Bellone et al., 1997), there is no evidence to
<table>
<thead>
<tr>
<th>Subcutaneous cell-coated OVA (mg/mouse)</th>
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<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>OT-I</td>
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<td>OT-II</td>
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CFSE →

Figure 3-10 Response of OT-I and OT-II cells to cell-coated OVA injected subcutaneously. Mice were injected intravenously with CFSE-labelled transgenic OT-I or OT-II cells, and one day later primed with various amounts of cell-coated OVA subcutaneously. Three days later, these mice were sacrificed and their cells from draining lymph node were analysed by flow cytometry. Profiles were gated on CD8⁺ or CD4⁺, CFSE⁺, propidium iodide-negative cells.
Figure 3-11 Response of OT-I cells to cell-coated OVA injected subcutaneously. Mice were injected intravenously with CFSE-labelled transgenic OT-I cells, and then, 4 days later primed with $1 \times 10^7 / 0.1 \text{ ml}$ cell-coated OVA (1.0mg/ml) subcutaneously. The mice were sacrificed on day 6 to day 8 respectively, and their cells from draining lymph node were analysed by flow cytometry. Profiles are gated on CD8$^+$ or CD4$^+$, CFSE$^+$, propidium iodide-negative cells. The experiment on day 7 was performed twice.
exclude the possibility that live cells are also targets. To examine whether live cells could be captured and cross-presented into the MHC class I restricted pathway, both irradiated and non-irradiated splenocytes were coated with OVA protein, and then used to prime B6 mice containing CFSE-labelled OT-I cells. To minimise dead cells, non-irradiated cells were separated by centrifugation on Nycodenz (density = 1.091 g/cm³), and viable cells obtained from above the interface. Viability was about 99% when injected. Three days after priming, spleens were collected and single cells analysed by flow cytometry for proliferation of OT-I cells. It was found that OT-I proliferation was equally effective whether OVA-coated cells were irradiated or non-irradiated, live cells (Figure 3.12). This suggested that apoptotic cells were not essential for cross-presentation of cellular antigens.

3.3 Discussion

In this chapter, we examined the effect of antigen dose, antigen type (cell-associated or soluble) and route of administration, on the activation of OT-I and OT-II T cells. Two models were employed. In the first model, we examined the response of antigen specific transgenic OT-I and OT-II T cells to exogenous OVA introduced intravenously in either a cell-associated or soluble form. In the second model, activation of OVA specific TCR transgenic T cells was examined in the draining lymph nodes of mice expressing OVA transgenically in the pancreas.

The efficiency of cross-presentation of cell-associated OVA versus soluble OVA was examined using OT-I and OT-II transgenic T cells to detect class-I and class II-restricted presentation, respectively. Our data provides evidence that cell-associated OVA is
Figure 3-12 Response of OT-I cells to irradiated/non-irradiated cell-coated OVA. B6 mice were adoptively transferred with CFSE-labelled OT-I cells, and primed with OVA-coated irradiated or non-irradiated splenocytes on the second day. OVA-bearing cells were collected from bm1 spleens, and selected by centrifugation on Nycodenz (1.091g/cm$^3$) density gradient to minimise dead cells from the pool of non-irradiated cells. The viability of the non-irradiated cells were about 99% before injected. Three days later after priming, the spleens were removed from the mice, and OT-I proliferation was analysed by flow cytometry. Profiles were gated on CD8$^+$, CFSE$^+$, propidium iodide-negative cells. The experiment was performed twice.
presented very efficiently in both the class-I and class II pathways. Compared with soluble OVA, intravenously injected cell-associated OVA was presented 500-fold more efficiently to CD4\(^+\) OT-II T cells and 50,000-fold more efficiently to CD8\(^+\) OT-I T cells.

It should be stressed that we have no way to determine whether the response of OT-I or OT-II cells reflects the strength of the response of normal naïve OVA-specific CD8 or CD4 T cells. Thus, we cannot draw general conclusions about the comparative response of normal CD4 versus CD8 T cells from our data. What can be compared is the relative efficiency of class I-restricted presentation for the different forms of antigen (cell-associated, soluble and pancreatic), and the same applies to class II-restricted presentation. Thus, while a 100-fold difference in the sensitivity of OT-I and OT-II cells to OVA-coated spleen cells might simply reflect a difference in the sensitivity of these two individual T cell clones, the 50,000-fold greater sensitivity of OT-I cells to OVA-coated spleen versus soluble OVA clearly indicates a superiority of class I-restricted presentation of OVA-coated spleen.

The dramatic increase in the presentation efficiency of cell-associated versus soluble material had been noted for class II-restricted cross-presentation \textit{in vitro}, where DCs were shown to efficiently present cell-associated class II molecules (Inaba \textit{et al.}, 1998), but our data represents the first quantitative analysis \textit{in vivo} for either pathway. These data support the idea that cell-associated antigens are specifically targeted for presentation by the immune system. This suggests that the immune system might be dedicated to capturing and presenting cellular material, presumably because CTL immunity is only important for responses to such antigens.
Earlier work demonstrated that soluble antigen could be processed \textit{in vivo} for presentation to class-I restricted T cells provided that the antigen was introduced in a cell-associated form (Carbone and Bevan, 1989; Carbone and Bevan, 1990; Moore \textit{et al.}, 1988). Data presented in this chapter shows that activation of antigen specific T cells by cross-presented exogenous antigen is more efficient for cell-associated compared to soluble antigen when injected intravenously. However, this was not the case when these antigens were injected subcutaneously. When soluble OVA was inject i.v., OT-I and OT-II showed similar dose responses. In contrast, s.c. injection revealed that OT-II cells were 20-fold more sensitive than OT-I cells and responded to 20-fold less antigen than required i.v.. Whereas, cell-associated OVA administered subcutaneously essentially failed to activate OT-I or OT-II T cells. This suggests that soluble OVA is handled differently when injected i.v. versus sc. One possibility for the enhanced response of OT-II cells could be that antigen was better targeted to the draining LN when injected s.c. than it is targeted to the spleen when injected i.v.. This would, however, not explain why OT-I cells were equivalently sensitive to antigen by either route. An alternative explanation could be the availability of different APCs for each route. For example, Langerhans cells are able to capture the antigen in the skin, where s.c. antigens might be captured, whereas these cells are not present in the spleen where i.v. antigens would localise. To address this issue, it would be interesting to determine which DC subsets capture FITC-labelled OVA when introduced by either route.

Activation of OT-I and OT-II cells was also examined in response to tissue-specific OVA expressed in the pancreatic islet β cells under the control of the rat insulin promoter. In these experiments, three lines of mice expressing whole, secreted OVA at
different concentrations were employed. This showed that cross-presentation of tissue-expressed OVA was biased towards activation of OT-I cells over OT-II cells. It is important to stress that even though the transgenically-expressed OVA was of a secreted form, the phenotype of the T cell responses was similar to that seen for the cell-associated OVA of OVA-coated spleen cells injected i.v., which was biased toward activation of OT-I cells over OT-II cells. Given that exogenous soluble OVA was presented equally well to both OT-I and OT-II cells when injected i.v. in the B6 mice (Figure 3.2) and preferentially activated OT-II cells when injected s.c. (Figure 3.9), our data implied that transgenically-expressed OVA was presented as if it were cell-associated with biased activation of OT-I cells over OT-II cells, even though OVA was expressed as a secreted form in the pancreata of RIP-OVA mice. This favours the idea that APCs capture transgenically-expressed OVA directly from the islets and traffic to the draining LN, where they present it to T cells. It is possible, however, that cellular material carrying OVA, such as heat shock proteins, traffic to the draining LN and are captured by resident APCs. Clearly, the source of antigen in the pancreatic lymph nodes is not the secreted soluble form of OVA, which would be expected to better activate OT-II cells.

Using various RIP-OVA lines expressing different amounts of OVA in the pancreas, we found that the strength of OT-I and OT-II proliferation in the pancreatic draining lymph nodes was related to the amount of antigen expressed. This extends an earlier observations which showed that low dose OVA in the pancreas was not cross-presented whereas high dose OVA induced delation via cross-presentation on bone marrow derived APC (Kurts et al., 1999). Here, we additionally examined an intermediate dose of OVA
(0.04 μg/mg of protein) and showed that it was weakly cross-presented to OT-I cells and did not stimulate OT-II cells. These data suggest that this dose is close to the threshold for stimulating by cross-presentation.

In examining the requirement of apoptotic cells for cross-presentation of exogenous antigen in vivo, we found that OT-I cells were activated equally by either irradiated or non-irradiated OVA-coated cells and efficient CTL responses were elicited following cross-presentation of these forms of antigen. This suggests that apoptotic cells are not obligatory for cross-priming, and that healthy live cells might also be targeted to be presented to T cells. Although numerous mechanisms have been proposed for the entry of exogenous antigens into the class I pathway (Yewdell et al., 1999), it is unclear how cell-associated antigens gain access during cross-presentation. Previous reports suggest that apoptotic cells are targeted by the cross-presenting APC, but not all cases of cross-presentation are associated with obvious apoptosis (Kurts et al., 1997b). Alternative routes are likely, such as reported by Harshyne and colleagues who showed that antigen from live cells could be captured by DCs for priming MHC class I restricted CTL (Harshyne et al., 2001). It is important to point out, however, that our studies do not exclude the possibility that some of the live OVA coated spleen cells might have died once they were injected i.v. Thus, a requirement for apoptoses can not be excluded. Perhaps one way to circumvent this problem would be to use spleen cells expressing bcl-2, which dramatically enhances cell survival after adoptive transfer.

In summary, the data presented in this chapter demonstrates that multiple factors can influence the extent of cross-presentation. These include antigen dose, antigen form and
the route of delivery. Our data confirms the idea that high dose antigens are more effectively cross-presented and lead to stronger T cell responses for both exogenous foreign antigen or cellular self-antigens. Furthermore, it was found that for intravenous immunisation, MHC-I restricted T cell responses were more efficiently induced by cell-associated antigens than soluble antigens in vivo and that living cells represented an effective antigen source. Such favourable cross-presentation of cell-associated OVA suggests that the immune system is particularly responsive to cellular antigens, presumably to monitor intracellular pathogens, such as viruses.
Chapter 4 Helper Requirements for Induction of CTL-mediated Auto-immunity in RIP-OVA\textsuperscript{hi} Mice

4.1 Introduction

Cytotoxic T lymphocytes (CTL) are responsible for lysing target cells presenting the appropriate peptide/MHC class I complex. Naïve CTLs are not lytic, however, and only become effector CTL, capable of target cell lysis, when they are primed (Koniaras et al., 1998). Priming is thought to depend on the provision of antigenic and co-stimulatory signals by professional APC (Matzinger, 1994), which express molecules such as B7 family members, and secrete cytokines such as IL-12. In some cases, induction of CTL immunity is dependent on CD4\textsuperscript{+} T cell help (Bennett et al., 1997; Fayolle et al., 1991; Guerder and Matzinger, 1992; Husmann and Bevan, 1988; Jennings et al., 1991; Kast et al., 1986; Keene and Forman, 1982; Kirberg et al., 1993; Porgador and Gilboa, 1995; Rees et al., 1990; Sauzet et al., 1996; Yang et al., 1995), but this is not always the case (Ahmed et al., 1988; Buller et al., 1987; Liu and Mullbacher, 1989; Moskophidis et al., 1987; Nash et al., 1987; Rahemtulla et al., 1991). Previously it was shown that generation of ovalbumin (OVA)-specific CTL by cross-priming was CD4\textsuperscript{+} T cell dependent, and that the CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells must see antigen on the same APC (Bennett et al., 1997). This was later found to relate to the need for the CD4 T cell to deliver a CD40L signal to CD40 on the APC, ‘licensing’ it to prime naïve CTL precursors (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). At present, it is unclear how this ‘licensing’ allows the APC to prime naïve CTL, but it has been shown that CD40 signaling upregulates B7 molecules and induces the secretion of IL-12 (Cella et al., 1996; Grewal et al., 1998).
1996; Yang and Wilson, 1996). More recently, other CD40-independent means of APC activation have been described (Bachmann et al., 1999; Green and Flavell, 1999; Lu et al., 2000), but just how important each of these is to CTL induction is unclear.

In addition to understanding how naïve CD8 T cells are primed to become effector CTL, we have been interested in the mechanisms underlying peripheral tolerance, particularly for CD8+ T cells. In previous studies, it has been shown that tissue antigens may be cross-presented on bone marrow-derived APC in the draining LN and that this leads to the activation followed by deletion of naïve CTL (Kurts et al., 1996; Kurts et al., 1997b). This has been referred to as cross-tolerance (Heath et al., 1998). To better understand the factors that influence the induction of tolerance versus immunity, the effect of providing CD4+ T cell help during the induction of CTL cross-tolerance was examined (Kurts et al., 1997a). This was based on the notion that the availability of CD4+ T cell help can determine the status of a CTL response, with naïve CTL being driven towards tolerance in the absence of help (Guerder and Matzinger, 1992). More specifically, this model consisted of transgenic mice expressing the neo-autoantigen OVA in the pancreatic islets and the kidney proximal tubular cells (Kurts et al., 1997b). In this case, OVA was membrane bound, but could be cross-presented in the draining lymph node (LN) of the pancreas and kidney by a bone marrow-derived APC. Adoptive transfer of OVA-specific CD8+ T cells from the OT-I transgenic line into transgenic mice expressing OVA led to the activation, proliferation and eventual deletion of the OT-I cells (Kurts et al., 1997b). These observations led to the conclusion that cross-presentation of self-
antigens can lead to cross-tolerance induction. If, however, OVA-specific CD4⁺ T cells from the OT-II transgenic line were co-transferred, the deletion of the OT-I T cells was impaired and this resulted in diabetes induction in a large proportion of the mice (Kurts et al., 1997a). Therefore, tolerance of autoreactive CTL could be impaired by the provision of CD4⁺ helper cells and the response directed towards autoimmunity.

In this chapter, the mechanism by which CD4⁺ T cell help converts CTL tolerance to autoimmunity was further dissected. Efforts were made to understand the nature of this CD4⁺ T cell help and the role of CD40 signaling in switching tolerance towards autoimmunity. These studies were performed in a new transgenic model, RIP-OVA⁶, primarily because RIP-mOVA mice could not be made homozygous and were therefore difficult to obtain in large numbers. The results of this study confirm that under certain conditions, CD4⁺ T cell help can impair CTL tolerance and facilitate an autoimmune response. Importantly, they provide evidence that signals in addition to the supply of CD40 ligand are associated with CD4⁺ T cell help for CTL-mediated autoimmunity.

4.2 Results

4.2.1 Autoreactive CTL require help to mediate tissue destruction

To examine the requirements for induction of autoimmunity by CD8⁺ T cells, we adoptively transferred naïve OVA-specific CD8⁺ T cells from the OT-I transgenic line into RIP-OVA⁶ transgenic mice, which express a relatively high level of
Chapter 4  Helper requirements for CTL-mediated autoimmunity

secreted OVA in the pancreatic islet β cells (Kurts et al., 1998b). Interestingly, following transfer into RIP-OVA<sup>hi</sup> mice, naïve OT-I T cells became activated and proliferated in the pancreatic lymph node, but this did not result in diabetes, even when as many as 10 million cells were transferred (Table 4-1, Experiment A). To determine whether OVA-specific CD4<sup>+</sup> T cells derived from the OT-II transgenic line could help OT-I cells cause autoimmunity, RIP-OVA<sup>hi</sup> mice were adoptively cotransferred with naïve OT-I and naïve OT-II cells, and diabetes monitored (Table 4-1, Experiment B). Surprisingly, this did not lead to diabetes induction, despite cotransferring 2.5 x 10<sup>6</sup> naïve OT-I, which is 10-fold more OT-I cells than previously shown to cause diabetes in the RIP-mOVA transgenic model (Kurts et al., 1997b) and (Table 4-1, Experiment C).

Table 4-1  OT-I cells do not cause diabetes in RIP-OVA<sup>hi</sup> mice, even when co-injected with naïve OT-II cells

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Number of* OT-I cells</th>
<th>Number of* OT-II cells</th>
<th>Diabetes&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIP-OVA&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>1.0 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>nil</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>nil</td>
<td>0/10</td>
</tr>
<tr>
<td>Experiment B.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIP-OVA&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>2.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>nil</td>
<td>2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0/5</td>
</tr>
<tr>
<td>Experiment C.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIP-mOVA</td>
<td>0.25 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>nil</td>
<td>1/8</td>
</tr>
<tr>
<td></td>
<td>0.25 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>6/10</td>
</tr>
</tbody>
</table>

*Indicated numbers of OT-I and OT-II cells prepared from Rag-<sup>1<sup>−</sup>−</sup>OT-I and Rag-<sup>1<sup>−</sup>−</sup>OT-II mice were injected i.v. into the recipients between 6-12 wk of age.

<sup>+</sup>Diabetes was monitored by examining the level of mouse urine glucose 4 times/wk for at least 3 wk. These data have been accumulated over 3 experiments.
To explain the differences between these two models, we examined the proliferation of CFSE-labeled OT-I and OT-II cells in the pancreatic LN (PLN) of both RIP-OVA\textsuperscript{hi} and RIP-mOVA mice. This showed that in RIP-mOVA mice both cells proliferate well, although a stronger response was seen for OT-I cells. In contrast, only OT-I cells proliferated well in RIP-OVA\textsuperscript{hi} mice (Figure 4.1). Thus, the poor \textit{in vivo} activation of OT-II cells in RIP-OVA\textsuperscript{hi} mice might explain their failure to help OT-I cells cause autoimmunity.

To test this possibility, OT-II cells were first activated \textit{in vitro}, and then various doses of activated OT-II cells were adoptively transferred together with 2.5 \times 10^6 naïve OT-I cells into RIP-OVA\textsuperscript{n} mice (Table 4-2). This led to insulitis and diabetes induction, which revealed that activated OT-II cells were efficiently able to help OT-I cells cause diabetes. Poor \textit{in vivo} activation of naïve OT-II cells, therefore, appeared to explain their failure to help OT-I cells cause diabetes in RIP-OVA\textsuperscript{hi} mice. For the remainder of this chapter, naïve OT-I cells were used in combination with activated OT-II cells in various experiments to examine the underlying mechanisms of help for the diabetes induction in RIP-OVA\textsuperscript{hi} mice.
Figure 4-1 Cross-presentation of OVA to OT-I and OT-II T cells in RIP-OVA$^{hi}$ and RIP-mOVA mice. $2 \times 10^6$ OT-I cells (top) and OT-II cells (bottom) were labelled with CFSE and adoptively transferred i.v. into RIP-OVA$^{hi}$ mice (left column) or RIP-mOVA mice (right column). Three days later, cells isolated from the pancreatic lymph node of each mouse were analysed by flow cytometry, gating on CFSE$^+$ PI$^+$ CD8$^+$ cells for OT-I cells, or CFSE$^+$ PI$^+$ CD4$^+$ cells for OT-II cells.
> Table 4-2 OT-I cells can cause diabetes in RIP-OVA<sup>hi</sup> mice, when co-injected with activated OT-II cells

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Number of* OT-I cells</th>
<th>Number of* activated OT-II cells</th>
<th>Diabetes&lt;sup&gt;†&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIP-OVA&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>2.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>nil</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>7/7</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2/6</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>nil</td>
<td>2 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>6/7</td>
</tr>
<tr>
<td></td>
<td>nil</td>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>8/12</td>
</tr>
<tr>
<td></td>
<td>nil</td>
<td>2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0/5</td>
</tr>
</tbody>
</table>

*OT-I or/and titrated in vitro activated OT-II cells were injected i.v. into RIP-OVA<sup>hi</sup> mice.

†Urine glucose level was monitored 4 times/wk for at least 3 wk. These data have been accumulated over 4 experiments.

The above data showed that co-injection of activated CD4<sup>+</sup> T cells with naïve CD8<sup>+</sup> T cells could lead to autoimmunity in this model. It should be noted that activated OT-II cells alone could cause diabetes when 2 x 10<sup>7</sup> of these cells were transferred. This might have been because large numbers of OT-II cells could cause damage themselves or because they recruited low affinity OVA-specific CD8 T cells remaining in these mice. To address this question, induction of diabetes by large numbers of OT-II cells was examined in CD8<sup>+</sup> T cell-deficient RIP-OVA<sup>hi</sup> mice, which were generated by intravenous injection of anti-CD8 mAb. 0.5 ml of a 1:5
dilution of YTS169 ascites in PBS was injected i.p. 3 times within 6 days, with $2 \times 10^7$ activated OT-II transferred on the day before the last injection. Activated OT-II cells were still able to cause diabetes even in those CD8-depleted mice (3/3), suggesting that with extremely large numbers of activated CD4$^+$ T cells, these cells may on their own induce diabetes. It is important to stress, however, that autoimmune diabetes induced by activated OT-II cells alone was never seen with $2 \times 10^6$ cells (Table 4-2) which was the highest dose used in combination with naive OT-I cells.

To establish the minimum number of OT-I cells required to cause diabetes, a series of cell combinations was set up in which OT-I T cells were titrated while activated OT-II cells were kept constant (Table 4-3). This indicated that as few as $2.5 \times 10^3$ naive OT-I cells co-injected with $2 \times 10^5$ activated OT-II cells were diabetogenic. The relatively small numbers of co-transferred OT-I and OT-II cells that were required to cause islet destruction indicated a co-operative interaction between the two subsets. In other words, the CD4$^+$ T cells appeared to help the CD8$^+$ T cells induce diabetes.
Table 4-3  Titration of OT-I cells with activated OT-II cells into RIP-OVA<sup>hi</sup> mice

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Number of* OT-I cells</th>
<th>Number of* activated OT-II cells</th>
<th>Diabetes&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIP-OVA&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>2.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>nil</td>
<td>0/10</td>
</tr>
<tr>
<td>nil</td>
<td>2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>2.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>7/7</td>
<td></td>
</tr>
<tr>
<td>2.5 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>25/25</td>
<td></td>
</tr>
<tr>
<td>2.5 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>33/39</td>
<td></td>
</tr>
<tr>
<td>2.5 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5/7</td>
<td></td>
</tr>
<tr>
<td>2.5 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1/6</td>
<td></td>
</tr>
</tbody>
</table>

*Titrated OT-I cells and in vitro activated OT-II cells were co-transferred into RIP-OVA<sup>hi</sup> mice.

<sup>+</sup>Diabetes monitor was done as described before. The data was collected over 10 experiments.

4.2.2 OT-I cells are responsible for directly recognizing and destroying islet β cells

To determine whether CD8<sup>+</sup> OT-I T cells were responsible for directly destroying islet cells, chimeric mice were generated in which the bone marrow compartment was from B6 mice, but the host was either of B6 (RIP-OVA<sup>hi</sup>), or bm1 (RIP-OVA<sup>hi</sup>/bm1) origin. This takes advantage of the fact that OVA can not be presented to OT-I cells by the K<sup>bm1</sup> molecules (Nikolic-Zugic and Carbone, 1990). B6 and bm1 mice are congenic, differing only at the K locus, with B6 mice expressing K<sup>b</sup> and bm1 mice expressing K<sup>bm1</sup>. Using this model, K<sup>b</sup>-restricted OT-I T cells would not be able to recognise OVA presented by islet cells of K<sup>bm1</sup> host origin, but would recognise
OVA presented on B6 bone marrow-derived APC (Kb). Since both bml and B6 mice express the same MHC class II molecule, I-A^b, all class II-expressing cells would have equal potential to present to OT-II cells. Using these combinations, autoimmune diabetes only occurred when the islet targets were of B6 origin (Table 4-4). Thus, direct recognition of islet cells by OT-I cells was essential for autoimmunity.

### Table 4-4 Recognition of the islets cells by OT-I cells is essential for induction of diabetes

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Number of OT-I cells</th>
<th>Number of activated OT-II cells</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6-&gt;B6* (RIP-OVA\textsuperscript{hi})</td>
<td>2.5 x 10^5</td>
<td>nil</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>nil</td>
<td>2 x 10^5</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10^5</td>
<td>2 x 10^5</td>
<td>11/12</td>
</tr>
<tr>
<td>B6-&gt;bml* (RIP-OVA\textsuperscript{hi})</td>
<td>2.5 x 10^5</td>
<td>nil</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>nil</td>
<td>4 x 10^5</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10^5</td>
<td>2 x 10^5</td>
<td>0/14</td>
</tr>
</tbody>
</table>

*Bone marrow from B6 mice was injected i.v. into irradiated RIP-OVA\textsuperscript{hi} and RIP-OVA\textsuperscript{hi}/bml mice. 8 wk later, those chimera were transferred with indicated numbers of OT-I or/and OT-II cells.

\textsuperscript{1}Diabetes was examined by urine glucose test 4 times/wk for 3 wk. Mice that did not develop diabetes were monitored for 4-5 wk. The data was accumulated over 4 experiments.

#### 4.2.3 Activated OT-II cells enhance OT-I cell expansion in RIP-OVA\textsuperscript{hi} mice

One way by which help from activated OT-II cells might favour the induction of autoimmune diabetes is by enhancing OT-I cell expansion (ie. increase their
number). To test this possibility, I examined OT-I expansion in the presence or absence of activated OT-II. In this experiment, $10^6$ OT-I cells were transferred into RIP-OVA hi mice with or without $2 \times 10^5$ activated OT-II cells, and then the recipients were sacrificed 4 days later to determine the number of OT-I cells by flow cytometry (Figure 4.2). Interestingly, the number of OT-I cells was increased up to 3.6-fold when activated OT-II cells were administered with OT-I cells. This preliminary experiment indicated that activated OT-II T cells contributed to the clonal expansion of OT-I cells.

4.2.4 Activated OT-II cells do not help through increasing the accumulation of lymphocytes to the pancreas

One possibility was that activated OT-II cells helped by generating cytokines that attracted OT-I T cells into the islet tissue. To determine whether activated OT-II T cells increased OT-I infiltration of the islet, two preliminary experiments were performed to examine the islet infiltration by histological staining. The pancreases were removed from the recipients of $2.5 \times 10^5$ naïve OT-I cells with or without $2 \times 10^5$ activated OT-II cells on days 3, 4 and 5 respectively. Haematoxylin and eosin stain suggested that OT-I cells do not migrate to the pancreatic islets on day 3-5 when injected alone (Figure 4.3). In contrast, heavy islet infiltration by lymphocytes was observed on days 4 and 5 in the recipients transferred with the combination of both types of cells. In the case of OT-II cells transferred alone, no evidence of infiltration of the islets was found on days 3 or 4, although 2 out of 13 islets were observed to contain lymphocytes on day 5. This detection of some insulitis when
Figure 4-2 Expansion of OT-I cells in RIP-OVA\textsuperscript{hi} mice given activated OT-II cells. 10\textsuperscript{6} naïve OT-I cells were i.v. transferred into RIP-OVA\textsuperscript{hi} mice with (n = 6) or without (n = 5) 2 \times 10^5 activated OT-II cells. Four days later, the spleens and lymph nodes were removed from each recipient and pooled cells counted and then analysed by flow cytometry for the proportion of V\textalpha{}\textbeta{}\textsuperscript{2}\textbeta{}\textsuperscript{5}\textsuperscript{+} CD8\textsuperscript{+} cells. The total number of OT-I cells was calculated using the formula: ([% of CD8 T cells that are V\textalpha{}\textbeta{}\textsuperscript{2}\textbeta{}\textsuperscript{5}\textsuperscript{+} in the test mice] - [% of CD8\textsuperscript{+} T cells that are V\textalpha{}\textbeta{}\textsuperscript{2}\textbeta{}\textsuperscript{5}\textsuperscript{+} cells in normal RIP-OVA\textsuperscript{hi} mice]) \times \% of live cells that are CD8\textsuperscript{+} \times total number of live cells. This result is from one of two consistent experiments.
Figure 4-3 The accumulation of lymphocytes in the islets. Sections were prepared on day 3, 4 and 5 from the pancreata of RIP-OVA$^{hi}$ recipients of $2.5 \times 10^5$ naïve OT-I cells with or without $2 \times 10^5$ activated OT-II cells. Haematoxylin and eosin stain suggested that OT-I cells did not migrate to the pancreatic islets (see arrows) when transferred alone, while heavy infiltration of inflammatory cells was found on day 4 and 5 in the recipients transferred with both types of cells. Most islets appear normal in OT-II cell recipients except 2/13 islets were observed to contain a cellular infiltration on day 5.
OT-II cells were transferred alone suggested that they might be able to access the islets and attract in OT-I cells. However, this mild infiltration was only seen on day 5, whereas when both OT-I and OT-II cells were co-transferred there was strong islet infiltration seen a day earlier (day 4), suggesting that the combination of both cell types somehow improves infiltration and that this is not likely to be due to the OT-II cells attracting the OT-I cell into the islets.

To further identify the accumulated cells in the pancreatic islets, immunohistological stain for CD4 and CD8 was performed with the frozen sections prepared from the same recipients as described above. Preliminary analysis provided a consistent observation that there was no OT-I cell infiltration to the pancreatic islets on days 4 or 5 when transferred alone, whereas dense infiltration of T cells was observed on day 4 and 5 when a combination of both cell types were injected (Figure 4.4). When OT-II were transferred alone, there was little CD4 T cell infiltration in the islets. The dramatic insulitis resulting from co-injection of OT-I and OT-II cells provided evidence that in the presence of activated OT-II cells, large numbers of CD8$^+$ T cells were recruited to the pancreas, leading to the destruction of pancreatic islets. However, the overall lack of infiltration when either population was transferred alone, versus the intense infiltration when co-transferred, suggested that improved infiltration in the presence of help was more likely due to increases in the number of OT-I cells generated, rather than a specific improvement in their recruitment to the islets. On the other hand, it is possible that OT-II cells migrate into the islets earlier than OT-I T cells, but whether this contributes to the accumulation of lymphocytes in the islets and induction of diabetes is unclear.
Figure 4-4 The accumulation of lymphocytes in the islets. Frozen sections were prepared on day 3, 4 and 5 from the pancreata of the same RIP-OVA<sup>hi</sup> recipients as described in figure 4-3 and stained with anti-CD4 or anti-CD8 Abs. Immunohistological examination suggested that neither activated OT-I nor naive OT-I cells migrated to the pancreatic islets in appreciable numbers on day 4 or 5 when transferred alone, though occasional islet infiltration of CD4<sup>+</sup> T cells was seen on day 4 in 1 out of 2 mice. Mice receiving both OT-I and OT-II cells showed infiltration by both cell types on day 4 and 5.
4.2.5 Cognate CD4+ T cell help is not essential for the generation of effector
CTL in autoimmune diabetes

Previous studies examining CTL cross-priming showed that help was essential for
CTL cross-priming, and that CD4+ T cells mediated their help by signaling through
CD40 on the APC in a process where both CD4+ and CD8+ T cells interacted with
the same APC (Bennett et al., 1998; Bennett et al., 1997). To determine whether
CD4 help occurred in a similar way in this autoimmunity model, we first tested
whether OT-I and OT-II cells needed to recognise the same APC. This was tested by
generating chimeric RIP-OVA hi mice that were either reconstituted with B6 bone
marrow (B6→B6), or a combination of equal amounts of class II-deficient bone
marrow and bm1 bone marrow (IIa+ bm1→B6). In the mixed bone marrow
chimera, two types of APCs were present, each of which could only interact with
either OT-I or OT-II T cells. APCs from MHC II+ bone marrow have K b, but no I-A b
for presentation to OT-II; while APCs from bm1 bone marrow have I-A b for
presentation to OT-II, but can not present OVA to OT-I cells. In our first set of 2
experiments where these two groups of chimeras were co-injected with 2 x 10⁵
activated OT-II cells and 2.5 x 10⁶ naïve OT-I cells, all mice became diabetic (Table
4-5). Thus, unlike OVA-specific CTL cross-priming (Bennett et al., 1997), the two T
cell sub-populations did not appear to recognise antigen on the same bone marrow-
derived APC to cause autoimmunity. As a control for irradiation efficiency, the
bm1→B6 RIP-OVA hi chimeras did not get diabetic given this same dose of T cells.
Table 4-5 Co-recognition of the same APC by CD4 and CD8 T cells is not essential for mediating autoimmunity

<table>
<thead>
<tr>
<th>Recipients*</th>
<th>Number of OT-I cells</th>
<th>Number of OT-II cells</th>
<th>Diabetes†</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6 -&gt; B6 (RIP-OVA&lt;sup&gt;hi&lt;/sup&gt;)</td>
<td>2.5 x 10⁶</td>
<td>Nil</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>nil</td>
<td>2 x 10⁵</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10⁶</td>
<td>2 x 10⁵</td>
<td>6/6</td>
</tr>
<tr>
<td>II&lt;sup&gt;−&lt;/sup&gt; + bm1 -&gt; B6 (RIP-OVA&lt;sup&gt;hi&lt;/sup&gt;)</td>
<td>2.5 x 10⁶</td>
<td>nil</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>nil</td>
<td>2 x 10⁵</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10⁶</td>
<td>2 x 10⁵</td>
<td>6/6</td>
</tr>
<tr>
<td>bm1 -&gt; B6 (RIP-OVA&lt;sup&gt;hi&lt;/sup&gt;)</td>
<td>2.5 x 10⁶</td>
<td>2 x 10⁵</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10⁵</td>
<td>2 x 10⁵</td>
<td>1/12</td>
</tr>
</tbody>
</table>

*Bone marrow from B6 or bm1 mice or class II<sup>−</sup> and bm1 mice were grafted i.v. into irradiated RIP-OVA<sup>hi</sup> mice. †The chimera were transferred with indicated numbers of OT-I or/and OT-II cells 8 wk after bone marrow reconstitution. ‡Diabetes was examined regularly, mice that did not develop diabetes were monitored for 4-5 wk. The data was accumulated over 4 experiments.

4.2.6 Activated OT-II cells help more efficiently in the presence of MHC class II molecules

The above analysis of the requirement for co-recognition of the same APC suggested co-recognition was not required, therefore we asked whether activated OT-II cells needed to recognize OVA in the context of I-A<sup>b</sup> to provide help. To test the requirement of the MHC-II restricted re-activation of OT-II cells, a combination of naïve 2.5 x 10⁵ OT-I and 2 x 10⁵ in vitro activated OT-II cells was transferred into MHC II<sup>−</sup> -> RIP-OVA<sup>hi</sup> bone marrow chimeric mice or B6->RIP-OVA<sup>hi</sup> controls, and the blood glucose level examined over time. Surprisingly, 7/8 recipients became
Chapter 4

Helper requirements for CTL-mediated autoimmunity

diabetic (Table 4-6, part A), suggesting that MHC-II re-stimulation was unnecessary for activated OT-II cells to provide help. However, it was still possible that a few radio-resistant APCs persisted in the MHC II^+→RIP-OVA^hi chimeras, supplying the class II molecules required for re-stimulating OT-II cells. To exclude this possibility, MHC II-deficient (II^−) RIP-OVA^hi were generated by crossing RIP-OVA^hi mice with MHC II^+ mice. It was found that co-transfer of 2.5 x 10^5 naïve OT-I with 2.0 x 10^5 activated OT-II T cells caused diabetes in 5/11 (46%) MHC II^+ and 9/9 MHC II sufficient RIP-OVA^hi mice (MHC-II^w/w or MHC-II^+/−) (Table 4-6, part B), again supporting the idea that class II recognition was not essential.

Table 4-6 Class II-restricted recognition is important, but not essential for induction of diabetes in RIP-OVA^hi mice

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Number of* OT-I cells</th>
<th>Number of* activated OT-II cells</th>
<th>Diabetes*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Part A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6→B6 (RIP-OVA^hi)</td>
<td>2.5 x 10^5</td>
<td>nil</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>nil</td>
<td>2 x 10^5</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10^5</td>
<td>2 x 10^5</td>
<td>11/12</td>
</tr>
<tr>
<td>MHC-II^+→B6 (RIP-OVA^hi)</td>
<td>2.5 x 10^5</td>
<td>nil</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>nil</td>
<td>2 x 10^5</td>
<td>0/6</td>
</tr>
<tr>
<td><strong>Part B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHC-II^w/w (RIP-OVA^hi)</td>
<td>2.5 x 10^5</td>
<td>2 x 10^5</td>
<td>7/8</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10^5</td>
<td>2 x 10^5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10^4</td>
<td>2 x 10^5</td>
<td>2/2</td>
</tr>
<tr>
<td>MHC-II^+/− (RIP-OVA^hi)</td>
<td>2.5 x 10^5</td>
<td>2 x 10^5</td>
<td>5/11</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10^4</td>
<td>2 x 10^5</td>
<td>0/7</td>
</tr>
</tbody>
</table>

*Indicated numbers of T cells were injected into chimeric mice and RIP-OVA^hi mice (either MHC-II^+/− or II^w/w). †Urine glucose level of mice was examined 4 times/wk for at least 3 wk.
However, transfer of low number of OT-I cells showed that while class II presentation is not essential, it could nonetheless contribute to disease development. Thus when only \(2.5 \times 10^4\) OT-II cells were transferred, diabetes was seen only in the RIP-OVA\(^{hi}\) mice bearing intact class II (compare Table 4-6, part B line 2 with line 4). Therefore, OT-II cells do not necessarily need to recognise professional APCs for induction of diabetes, although this recognition enhances disease progression.

### 4.2.7 Requirement for CD40 signalling by activated CD4 T cells

Since there was limited need for co-recognition of the APC by the CD4 and CD8 T cells, we wondered whether activated OT-II cells were required to supply a CD40 ligand signal. To address this question, OT-II mice were crossed to CD40L-deficient mice to generate CD40L-deficient OT-II mice (CD40L\(^{-}\)OT-II). RIP-OVA\(^{hi}\) mice were co-injected with naïve OT-I cells plus activated OT-II cells that were either wild-type or deficient in CD40 ligand. This showed that \(2 \times 10^5\) activated CD40L\(^{+}\)OT-II T cells plus \(2 \times 10^4\) naïve OT-I caused diabetes in majority of RIP-OVA\(^{hi}\) mice (Table 4-7, Experiment B). In contrast, the same number of activated CD40L\(^{+}\)OT-II cells given with the OT-I cells was poorly diabetogenic, with 3/24 mice becoming diseased. This suggested that CD40 ligand expression by OT-II cells was important for their provision of help. The impaired help from the CD40L\(^{-}\) OT-II cells could, however, be overcome to some degree by increasing the number of OT-I cells. When \(2 \times 10^5\) activated CD40L\(^{+}\)OT-II cells were co-transferred with \(2.5 \times 10^5\) naïve OT-I cells, 10/19 mice became diabetic, and when OT-I cells were increased 10 more folds, all mice became diabetic (Table 4-7, Experiment A). It appears that induction of autoimmunity requires CD40 ligand expression by the CD4\(^{+}\) T helpers.
when the number of OT-I cells transferred is small, but for higher doses, diabetes is less dependent on CD40 ligand.

### Table 4-7. Requirement of CD40 Ligand for provision of help in RIP-OVA hi mice

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Number of OT-I cells</th>
<th>Number of Activated OT-II cells (o/o or wt for CD40L)</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment A.</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIP-OVA hi</td>
<td>nil</td>
<td>2 x 10⁶ (wt)</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10⁶</td>
<td>2 x 10⁶ (wt)</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10⁶</td>
<td>2 x 10⁵ (wt)</td>
<td>7/7</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10⁵</td>
<td>2 x 10⁵ (wt)</td>
<td>21/21</td>
</tr>
<tr>
<td></td>
<td>nil</td>
<td>2 x 10⁵ (o/o)</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10⁶</td>
<td>2 x 10⁶ (o/o)</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10⁶</td>
<td>2 x 10⁵ (o/o)</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10⁵</td>
<td>2 x 10⁵ (o/o)</td>
<td>10/19</td>
</tr>
<tr>
<td><strong>Experiment B.</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIP-OVA hi</td>
<td>2.5 x 10⁴</td>
<td>2 x 10⁵ (wt)</td>
<td>33/39</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10⁴</td>
<td>2 x 10⁵ (o/o)</td>
<td>3/24</td>
</tr>
</tbody>
</table>

*CD40L+OT-II mice used in this experiment were derived from backcrossing CD40L+OT-II mice to B6 background OT-II mice from F to J generations.

'Diabetes was examined by urine glucose test 4 times/wk for at least 3 wk. The data was accumulated over 7 experiments.

Finally, to directly address a role for CD40 in the provision of help for autoimmunity, RIP-OVA hi mice were co-injected with naïve OT-I cells and a mAb specific for CD40 (FGK45) that could replace CD4 T cell help in our cross-priming model (Bennett et al., 1998). As shown, stimulation of CD40 by FGK45 alone did
not provide sufficient help for induction of autoimmunity, although direct CD40 engagement could synergize with CD40L^{−/−} OT-II cells to induce diabetes (Table 4-8, line 3 and 4). Since FGK45 could not help on its own, but could provide help when injected together with activated CD40L-deficient OT-II cells, this suggests that helper factors in addition to CD40L are required to induce autoimmunity.

Table 4-8. Requirement of CD40 ligand for provision of help in RIP-OVA^{hi} mice

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Number of* OT-I cells</th>
<th>Number of activated OT-II cells (o/o** or wt for CD40L)</th>
<th>Antibody injected i.v.</th>
<th>Diabetes^{1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIP-OVA^{hi}</td>
<td>$2.5 \times 10^4$</td>
<td>$2 \times 10^5$ (wt)</td>
<td>-</td>
<td>3/3</td>
</tr>
<tr>
<td>RIP-OVA^{hi}</td>
<td>$2.5 \times 10^4$</td>
<td>$2 \times 10^5$ (o/o)</td>
<td>-</td>
<td>0/8</td>
</tr>
<tr>
<td>RIP-OVA^{hi}</td>
<td>$2.5 \times 10^4$</td>
<td>-</td>
<td>FGK45</td>
<td>0/2^{2}</td>
</tr>
<tr>
<td>RIP-OVA^{hi}</td>
<td>$2.5 \times 10^4$</td>
<td>$2 \times 10^5$ (o/o)</td>
<td>FGK45</td>
<td>8/8</td>
</tr>
<tr>
<td>RIP-OVA^{hi}</td>
<td>$2.5 \times 10^4$</td>
<td>$2 \times 10^5$ (o/o)</td>
<td>GL117^{3}</td>
<td>0/5</td>
</tr>
</tbody>
</table>

*OT-I cells were injected with anti-CD40 antibody FGK45 or OT-II cells (either CD40L^{−/−} or CD40L^{−/−}) or both into the recipients between 6-12 wk of age.

^{1}GL117 is Rat IgG2a against bacteria β-galactosidase, acting as isotype control for FGK45.

^{2}CD40L^{−/−} OT-II mice used in these experiments were derived from backcrossing CD40L^{−/−} OT-II mice to B6 background OT-II mice from F to J generations.

^{3}Diabetes was examined by urine glucose test 4 times/wk for at least 3 wk. These data have been accumulated over 5 experiments.

^{4}An additional 5 mice were given $2.5 \times 10^6$ naive OT-I cells and FGK45. None of these developed diabetes.
4.3 Discussion

CD4⁺ helper T cell responses are intimately involved in many autoimmune diseases, as evident from the MHC class II association with disease susceptibility in many animal models of autoimmunity (Wraith et al., 1989; Yagi et al., 1992) and the linkage to human autoimmune diseases (Altmann et al., 1991; Todd et al., 1987). Nonetheless, it has also become apparent that CD8⁺ T cells play a crucial role in disease induction. For example, removal of CD8⁺ T cells from animals belonging to the NOD inbred mouse strain protects this strain from diabetes (Taki et al., 1991; Wang et al., 1996). Recent studies using transgenic mice have also underlined the importance of CD8⁺ T cells in the development of autoimmune responses (Kurts et al., 1998a; Kurts et al., 1998b). Many factors have been implicated in the balance between tolerance and autoimmunity, including target antigens (Kurts et al., 1998a; Kurts et al., 1997b), the micro-environment (de Saint-Vis et al., 1998; Pulendran et al., 2001) and CD4⁺ T cell help (Akkaraju et al., 1997; Kirberg et al., 1993; Kurts et al., 1997a). Unfortunately, how these factors, particularly CD4⁺ T cells, affect the ultimate consequence of tolerance versus immunity or autoimmunity remains poorly understood. In this chapter, the nature of the help provided by activated OT-II cells was characterised.

Adoptive transfer of naïve OT-I cells into RIP-OVA⁽¹⁾ mice leads to OT-I proliferation in the pancreatic draining lymph node, but they are unable to mediated destruction of islet β cells. Instead, they undergo activation induced apoptosis, as
reported in studies demonstrating that cross-presentation results in peripheral self-tolerance (Kurts et al., 1999; Morgan et al., 1999b).

We observed that naive OT-II cells could not provide sufficient help to allow OT-I cells to cause autoimmunity in RIP-OVA hi mice. This was most likely because the expression level of transgenic OVA protein was not high enough to efficiently activate OT-II cells. Therefore, to initiate autoimmunity, CD4+ T cell help had to be mediated by in vitro activated OT-II T cells, rather than cells naturally activated in the draining LNs of RIP-OVA hi mice. This was shown in adoptive transfer experiments, where co-transfer of as few as $2 \times 10^6$ activated CD4+ OT-II cells could help to cause diabetes with 2,500 autoreactive CTL (Table 4-2), while $10^7$ naive OT-I alone or $2.5 \times 10^6$ naïve OT-I plus $2 \times 10^5$ naïve OT-II were not diabetogenic. This study demonstrated that autoimmune induction in RIP-OVA hi mice was CD4+ helper dependent, and that activated CD4+ T cell help could drive the OT-I T cells response away from tolerance towards autoimmunity.

To prove that CD8+ T cell recognition of islet cells was essential for islet destruction, chimeric mice (B6 bone marrow→RIP-OVA hi/bm1) were generated and adoptively transferred with autoreactive T cells. This showed that without recognition of islet MHC class I molecules, diabetes could not ensue, supporting the view that CTLs were the final effectors in this model. It remains possible that CD4+ OT-II cells provided some effector function, but clearly, they were excellent helpers but poor effectors. This was illustrated by the observation that as few as $2 \times 10^7$ OT-II cells
could help OT-I cells cause diabetes, whereas transfer of as many as $2 \times 10^6$ activated OT-II cells on their own failed to induce diabetes.

To investigate the role of the OT-II helper T cells in this autoimmune response, we determined whether OT-II help led to an expansion of OT-I cells. Provision of help dramatically enhanced OT-I cell numbers in the peripheral lymphoid compartment of RIP-OVA$^{hi}$ mice, suggesting that activated OT-II T cells enhanced CD8$^+$ T cell proliferation or survival, perhaps through provision of IL-2. However, preliminary analysis of proliferation in the first 2-3 days indicated that activated OT-II T cells did not improve initial OT-I proliferation (data isn't shown). An alternative explanation is that IL-2 produced by OT-II cells might increase CD8$^+$ T cell survival or improved their proliferation later in the response (post day 2-3). IL-2 has been regarded to be an important helper factor in mediating autoimmune diseases (Heath et al., 1992; Morgan et al., 1999a) and has been suggested to impair CD8$^+$ T cell deletion (Kurts et al., 1997a). To further address this issue, it would be interesting to use IL-2$^-$OT-II cells as the source of the helper T cells in the RIP-OVA$^{hi}$ model. This is currently under investigation.

In studies examining how CD4 T cells help CTL cause autoimmunity in RIP-OVA$^{hi}$ mice, we found that under condition where both CD4$^+$ and CD8$^+$ T cells could not see the same APC (mixed bone marrow chimeras), autoimmune diabetes was induced. This suggested that CD8$^+$ T cells do not need to interact with the same APC as seen by CD4 T cells for generation of autoimmunity. This was surprising,
since other experiments examining priming of CTL immunity showed that both CD4+ and CD8+ T cells needed to interact with the same APC (Bennett et al., 1997; Cassell and Forman, 1988). Further observation using RIP-OVA^II+ mice provided evidence strongly supporting the idea that both OT-I and OT-II do not need to see the same APC, since transfer of naïve OT-I T cells and activated OT-II T cells led to autoimmune diabetes in RIP-OVA^II+ mice in which class II restricted recognition was excluded. This implied that while class II restricted recognition by OT-II T cells was not essential, it appeared to optimise the autoimmune response in RIP-OVA^II+ mice.

As co-recognition of the same APC did not appear absolutely necessary for autoimmunity, we asked whether activated OT-II cells needed to supply a CD40L signal. This showed that while CD40L-deficient OT-II cells could help cause autoimmunity, they were very inefficient and could be aided by the addition of an anti-CD40 mAb. The reduced efficiency of help from CD40L^OT-II cells highlighted the importance of the CD40L signal. Hence, a role for CD40 signaling in autoimmune induction was identified. Because anti-CD40 mAb alone could not provide help, but activated CD40L^OT-II cells in combination with this mAb was very efficient, this suggested that CD40L^ OT-II cells provided additional helper signal(s).

These data raised the question of why cross-priming CTL in B6 mice and generation of autoimmunity in the RIP-OVA^II+ model showed quite different requirement for recognising the same APC by CD8+ and CD4+ T cells, while both clearly needed a CD40 signal. One explanation is that in vitro activated OT-II cells might act by
providing CD40-dependent and CD40-independent help. With the expression of CD40L, they can license APC for priming CTL, perhaps without the need to see class II on the APC (since they are pre-activated). Secondly, they could help by secreting soluble mediator(s). This concept is consistent with many studies showing that molecules such as IL-2 (Cassell and Forman, 1988; Kirberg et al., 1993) and IFN-γ (Maraskovsky et al., 1989; Simon et al., 1986) can act as important amplification or differentiation factors for CTL. Furthermore, activated OT-II cells may stimulate APCs through cell-cell interactions, involving molecules such as TRANCE or OX40, which may stimulate APC to secret soluble factors such as IL-12 (Gately et al., 1994; Gately et al., 1992; Mehrotra et al., 1993) or TNFα (Green et al., 2000). Recently, Pardoll's group provided in vitro evidence for three forms of CD4 help, two of which were CD40-independent, one of these mediated by soluble factor(s) (Lu et al., 2000). At present, the factor(s) responsible for the non-cognate help acting in our model have not been identified, but they could be as simple as the supply of IL-2, which has been previously shown to dramatically enhance the destructive potential of autoreactive CTL (Heath et al., 1992; Miller and Heath, 1993) and influence CTL priming or survival (Keene and Forman, 1982; Kirberg et al., 1993).

An alternative explanation for the observed lack of a requirement for co-recognition of the APC is that such co-recognition was essential, but a few radio-resistant APC supplied this requirement in the chimeras. We think that this is unlikely, since parallel experiments examining CTL cross-priming confirmed a need for co-recognition of the same APC (data not shown), indicating our ability to effectively
eliminate host APC in the mixed bone marrow chimera. The effectiveness of irradiation is further supported by the failure of the RIP-OVA\textsuperscript{hi} chimera reconstituted with bm1 bone marrow to become diabetic after administration of OT-I cells and activated OT-II cells.

In summary, this chapter describes the nature of CD4\textsuperscript{+} T cell help for induction of autoimmunity by CTL in RIP-OVA\textsuperscript{hi} transgenic mice. This model represents a novel \textit{in vivo} approach for examining the helper requirements for a CTL response to self-antigens. Here we show that CD4 helper T cells play a critical role in promoting the expansion of CTL effectors, most likely by decreasing apoptosis of activated CD8 T cells rather than increasing their rate of proliferation. In addition, delivery of optimal CD4 help required \textit{in vivo} stimulation of helper T cells, as the lack of an antigen signal in MHC class II deficient mice decreased the incidence of autoimmune diabetes. A similar requirement for CD40-CD40L interactions was observed. Most importantly, this report provides the first \textit{in vivo} example that help for CTL can be supplied via a soluble mediator without the need for co-recognition of the APC by the CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells.
Chapter 5 Bone Marrow-derived Antigen-presenting Cells in Cross-priming CTL versus Cross-tolerance

5.1 Introduction

To generate a CTL response, MHC-bound peptides must be presented to naïve CD8+ T cells by professional APCs that express high levels of MHC-I molecules, adhesion receptors, and costimulatory molecules (Chambers and Allison, 1997; Coyle et al., 2000; Iwang et al., 2000; Sabzevari et al., 2001; Sperling et al., 1996; Sperling and Bluestone, 1996; Witherden et al., 2000; Yu et al., 2000). Normally, naïve T lymphocytes do not directly enter peripheral tissues to scan for invading pathogens. Instead, they recirculate throughout the secondary lymphoid compartment, from lymph nodes to blood then to spleen, searching the surface of professional APC for their specific antigen. To stimulate MHC class I restricted naïve CTL, APCs located in the peripheral tissues must first capture pathogen-derived antigens, and then cross-present them as antigenic peptides in the context of MHC class-I molecules. In cases where the pathogen can infect professional APC, cross-presentation may not be necessary for antigen to access the class I pathway. Antigen-bearing APC that have captured antigen (or those infected by the pathogen) must then migrate to the draining LNs where they can stimulate specific T cells to expand in numbers and differentiate into effector CTL (Bevan, 1976a; Carbone et al., 1998; Gooding and Edwards, 1980; Huang et al., 1994; Lenz et al., 2000; Morgan et al., 1999a; Morgan et al., 1999b; Sigal et al., 1999; Sigal and Rock, 2000; Wolfers et al., 2001). In this way, professional APCs act as immune initiators for the induction of CTL immunity. For pathogens that do not infect professional APC, it is necessary for
their antigens to be captured and cross-presented to naïve CTL. The generation of responses in this manner has been referred to as cross-priming.

In addition to generating CTL immunity by cross-priming, the cross-presentation pathway may be used by APC to induce self tolerance (Adler et al., 1998; Heath and Carbone, 1999; Heath and Carbone, 2001; Miller et al., 1998; Morgan et al., 1999a; Morgan et al., 1999b). It is unclear, however, whether the APCs involved in cross-tolerance are the same as those involved in cross-priming. In this chapter, I attempted to identify the cross-priming and cross-tolerizing APCs. In these studies, two different models were used. For cross-priming, B6 mice were primed with OVA-coated spleen cells; whereas, for cross-tolerance, I used the RIP-OVA^1 transgenic model, where OVA is expressed as a self-antigen by pancreatic islet cells. As shown in Chapter 4, OVA specific CD8^+ T cells when adoptively transferred into RIP-OVA^hi respond to OVA cross-presented in the pancreatic draining LN. However, this response does not lead to immunity, but instead, OVA-specific T cells are deleted (Kurts et al., 1999). In this chapter, the nature of the cross-priming and cross-tolerizing APC is examined in detail.

5.2 Results

5.2.1 Identification of the cross-priming APC

5.2.1.1 The site of cross-priming with OVA-coated spleen cells

In order to identify the cross-priming APC, it was first necessary to determine the site at which cross-priming occurred when OVA-coated spleen cells were used to prime. Previous studies showed that intravenous injection of OVA-coated spleen cells induced an OVA-specific CTL response (Carbone and Bevan, 1990). To determine whether this
form of priming occurred in the spleen or lymph nodes, B6 mice were transferred with CFSE-labelled OT-I T cells, and one day later were injected with OVA-coated irradiated bm1 splenocytes. OT-I proliferation was then assessed in the spleen and inguinal lymph nodes at 1, 2 and 3 days after immunization. One day after priming, there was no evidence of proliferation of CFSE-labelled OT-I cells in either the spleen or lymph nodes. Proliferation was, however, evident in the spleen on day 2, but was not seen in the inguinal lymph node until at least day 3 (Figure 5.1) (Mintern et al., 1999). This suggests that cross-priming of OT-I cells is initiated in the spleen, and that some proliferated cells recirculate to the lymph nodes by late on day 3.

5.2.1.2 Cross-priming with OVA-coated spleen cells requires host APCs

Bone marrow-derived APCs have been shown to be responsible for cross-presentation of tumour cell antigens (Huang et al., 1996; Huang et al., 1994), viral pathogens (Lenz et al., 2000; Sigal and Rock, 2000) and OVA-loaded spleen cells (Bennett et al., 1997). Previous studies had used spleen cells loaded with OVA by osmotic shock, whereas we now use cells that have simply been coated with OVA. To verify that this form of priming also occurred via cross-priming on host APC, chimeric (B6 × bm1) F1 mice previously reconstituted with either B6 or bm1 bone marrow were primed with OVA-coated spleen cells. Only those mice reconstituted with B6 bone marrow were able to be cross-primed (Figure 5.2), indicating that like OVA-loaded cells, OVA-coated cells require presentation by host APC.

To determine the APC requirement for presentation of OVA-coated spleen cells to CD4+ OT-II cells, B6 mice were transferred with CFSE-labelled OT-II cells and then primed
Figure 5-1 Proliferating OT-I cells are first seen in the spleen after intravenous injection of OVA-coated spleen cells. B6 mice were injected i.v. with $2 \times 10^6$ CFSE-labelled OT-I T cells and the next day, primed with $2 \times 10^7$ irradiated splenocytes previously coated with 10mg/ml OVA for 10 min at 37°C. On days 1, 2 and 3 after priming, host spleen and lymph node cells were analysed by flow cytometry. Histograms were gated on CD8+ CFSE+ PI- cells.
Figure 5-2  Class I-restricted presentation of OVA-coated spleen cells is via a bone marrow-derived APC. Hosts including B6 BM chimeric mice (B6 APC), MHC II-/- mice (II-/- APC) and bm1 BM chimeric mice (bm1 APC) were transferred i.v. with 2 x 10^6 OT-I cells labelled with CFSE. On the next day, mice were immunized with OVA-coated, irradiated bm1 spleen cells (bm1 OCS) or irradiated bm1 cells alone (unprimed). Three days later, the cells were collected from the spleens and analysed by flow cytometry gated on CD8+ CFSE+ PI- cells. The experiments were performed twice with two mice in each group.
with irradiated OVA-coated splenocytes that were either MHC class II sufficient or deficient. OT-II proliferation occurred efficiently with either form of antigen (Figure 5.3), indicating (i) that donor cells did not have to express MHC II for the response to proceed, and (ii) that host APCs were therefore able to re-present this antigen to the OT-II cells. To determine whether donor cells, or only host APC, were able to present in this model, MHC class II sufficient or deficient recipients were transferred with OT-II cells and then primed with OVA-coated bml spleen cells (expressing normal I-A\textsuperscript{b} molecules). OT-II cells only proliferated when host APC expressed MHC II molecules (Figure 5.3). Thus, even if the donor spleen cells expressed the correct MHC-II haplotype for presentation of OVA antigen, no response was seen. Together, these results indicate that the host APCs and not the donor APCs were responsible for presentation of class II-restricted OVA determinants when irradiated OVA-coated splenocytes were used to prime.

5.2.1.3 Isolation of a cross-priming DC

In vitro studies had indicated that DCs are more potent in cross-presenting antigens than B cells and macrophages (Regnault et al., 1999; Rodriguez et al., 1999; Yrlid and Wick, 2000). However, it was still unknown which type of APC was responsible for cross-presentation of antigens in vivo for CD8\textsuperscript{+} T cell priming. To better define the cross-priming APC, mature DCs that had captured antigen were isolated from B6 mice and examined. B6 mice were first injected i.v. with OVA-coated cells and the spleens were removed 14 hours later, then digested with collagenase. Resulting DC preparation contained >80% CD11c\textsuperscript{+} MHC II\textsuperscript{+} cells. The cells were irradiated before being seeded
Figure 5-3 Cross-presentation of OVA to CD4+ OT-II cells depends on host APCs. All hosts were transferred i.v. with $2 \times 10^6$ OT-II cells labelled with CFSE and, the next day, primed with OVA-coated, irradiated spleen cells (OCS) from either B6 mice ($II^w$ OCS) or MHC class II deficient mice ($II^{-/-}$ OCS). As a control, one group of mice was primed with irradiated B6 spleen cells that were not coated with OVA (unprimed). Three days later, spleen cells were collected and analysed by flow cytometry. Profiles were gated on CD4+ CFSE+ PI- cells. This experiment was performed twice with two mice in each group.
into a 96 well plate where they were cultured with freshly isolated, naïve OT-I T cells. OVA-specific OT-I proliferation as a consequence of antigen presentation by DCs that had captured the antigen in vivo was measured by $^3$H-TdR incorporation. The results showed that the DCs isolated from mice primed with OVA-coated spleen cells but not from mice given spleen cells alone were able to induce OT-I proliferation (Figure 5.4). This implied that the APC responsible for cross-presenting OVA to OT-I cells was a DC.

5.2.1.4 CD8$^+$ DCs are responsible for in vivo cross-presentation

There are three distinct DC subpopulations present in the mouse spleen. These cells are CD4$^+$8" DEC 205" CD11b$^+$, CD4$^+$8" DEC 205" CD11b$^+$, or CD4$^+$8" DEC 205$^+$ CD11b$^+$ (Vremec et al., 2000). To investigate which subset(s) of DCs are responsible for cross-presentation, the total splenic CD11c$^+$ DCs from B6 mice which were primed with OVA-coated spleen cells were sorted into CD4$^+$CD8$^-$, CD4$^-CD8^+$ and CD4$^-CD8^-$ subsets, and cultured with naïve OT-I cells. Activation of OT-I T cells distinguished by $^3$H-TdR incorporation indicated that only CD8$^+$CD4$^-$ DCs were capable of cross-presenting OVA antigen (Figure 5-5), with no evidence of activation by CD4$^+$ DCs or double negative DCs. Therefore, CD8$^+$ DCs could be the subset responsible for cross-priming in vivo.

5.2.1.5 In vivo study of the cross-priming APC

So far, I had shown that CD8$^+$ DC isolated from mice immunised with OVA-coated cells could activate OT-I cells in vitro. While this implies that they are responsible for in vivo
Figure 5-4 Initiation of OT-I cell proliferation by in vivo primed DC. Total DCs prepared from mice primed with OCS were used as stimulators for naive OT-I cells and gBT-I cells (as a control) in a [³H]-thymidine incorporation assay. A. Indicated numbers of irradiated DCs were incubated with 2.5 x 10⁴ T cells. B. As positive controls, DCs were coated with 1 μM OVA₂₅⁷-₂₆₄ peptide or gB₄₉₈-₅₀₅ peptide. This experiment was repeated three times.
Figure 5-5 Initiation of OT-I cell proliferation by distinct DC subpopulation primed *in vivo*. Distinct DC subpopulations were prepared from OCS primed mice and used as stimulators for naïve T cells in a $[^3]$H-thymidine incorporation assay. A. Indicated numbers of irradiated DCs were incubated with $2.5 \times 10^4$ OT-I cells or gBT-I cells (as a control), only CD8$^+$ DCs could cross-prime OT-I cells. B. gBT-I cells were shown to be functional by stimulating with DCs coated with 1 $\mu$M of gB$_{498-505}$ peptide, but not with DCs coated with OVA$_{257-264}$ peptide. This experiment was performed three times.
cross-priming, it does not strictly prove this point. To examine which DC subset was important for activation of T cells in vivo, mice deficient in either CD4+ DCs or CD8+ DCs were examined for their ability to initiate T cell proliferation in vivo.

Two approaches were applied to deplete CD4+ DC from the mice. In the first, mice were injected (i.v.) with anti-mouse CD4 ascites (GK1.5) 4 times in 7 days, then transferred with CFSE-labelled OT-I cells on day 5, and primed with OVA-coated bm1 spleen cells on day 6. 3 days later their spleens were removed and analysed by flow cytometry. In the second model, transgenic mice (GK5 mice) expressing anti-mouse CD4 mAb (GK1.5) under the control of both CMV promoter and the RIP promoter were used (Han et al., 2000). This transgenic model has recently been shown lack of CD4+ DCs but retaining CD8+ DCs and double negative DCs (Kamath et al., 2000). CFSE-labelled OT-I cells were injected into the mice before priming with OVA-coated spleen cells the next day. Their spleen cells were analysed by flow cytometry 3 days later. Analysis of T cell proliferation showed that OT-I cells proliferated equally well in both CD4 cell deficient and wild type controls (Figure 5.6, top), indicating that the APCs responsible for cross-presenting cell-bound OVA to OVA specific OT-I cells were not CD4+ DC subset.

Unfortunately, cross-presenting OVA antigen to CD8+ T cells cannot be directly assessed in vivo when a CD8-depleting mAb is used, because the responding CD8+ T cells will also be depleted. As an alternative, we asked whether CD8+ DCs were also responsible for presenting cell-associated OVA antigens to CD4+ T cells in vivo. To assess activation of CD4+ T cells by CD8+ DCs, CFSE-labelled OT-II cell proliferation was examined in mice treated with anti-CD8 mAb (Figure 5.6, bottom). In two
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![Graph showing CFSE levels for OT-I and OT-II cells under different conditions.](image)

**Figure 5-6** OT-I and OT-II cell proliferation in CD4 or CD8 cell depleted mice. CD4⁺ cell deficient mice (sufficient with CD8⁺ cells including anti-CD4 transgenic GK5 mice and depleting CD4 cells via i.v. anti-CD4) were transferred i.v. with CFSE-labelled 2 × 10⁶ OT-I cells (top), and CD8⁺ cell deficient mice (i.v. anti-CD8 ascites, CD4 cell sufficient) transferred i.v. with OT-II cells (bottom) labelled with CFSE. The next day, mice were primed with OVA-coated, irradiated bm1 spleen cells (OCS) or bm1 cells alone (no OVA). Three days later, their spleens were removed and cells were analysed by flow cytometry. Profiles were gated on CD8⁺ or CD4⁺ cells that were CFSE⁺ PI⁻. This experiments were repeated twice with two mice in each group.
experiments, proliferation of OT-II cells was dramatically decreased in 3/4 recipients in the absence of CD8+ cells which included CD8+ DCs as well as T cells. This observation provided us with the first in vivo evidence that CD8+ DCs participated in cross-presentation, albeit to CD4+ T cells. Combined, the results showed that CD8+ DCs that had captured antigen from OVA-bound cell primed mice could activate CD8+ T cells both in vitro and in CD4+ cell deficient mice. In addition, these DCs are apparently involved in cross-presentation to CD4+ T cells. In contrast CD4+ DCs failed to activate CD8+ T cells in vitro and did not affect CD8+ OT-I proliferation in mice devoid of them. Our results are therefore consistent with the notion that the APC responsible for CD8+ T cell cross-priming belongs to the CD8+ DC subset, although, ultimate proof will require the effective in vivo depletion of this DC subset prior to CD8+ T cell priming.

5.2.2 Identification of the cross-tolerizing APC

DCs are not only critical for inducing immunity but may also play an important role in both central and peripheral tolerance. In previous studies from our laboratory, it has been shown that when OVA is expressed in the pancreatic islets it can be captured by bone marrow derived APC and cross-presented in the draining pancreatic lymph node (Kurts et al., 1996; Kurts et al., 1998a; Kurts et al., 1998b). This leads to activation of OVA-specific CD8 T cells, although these autoreactive T cells are eventually deleted. This process is referred to as cross-tolerance, since tissue antigens are being cross-presented on host APC in a process that leads to tolerance induction. Recently, Kurts and associates provided evidence that the bone marrow derived APC responsible for cross-tolerance is CD11c+ and therefore most likely a DC (Kurts et al., 2001). However,
it is unclear whether the same DC subset is responsible for cross-tolerance as causes cross-priming.

5.2.2.1 Attempted isolation of the DC responsible for cross-tolerance

As indicated in the previous sections, the CD8+ DCs were the most likely cell subset involved in cross-priming. Employing the same approach, I attempted to isolate the DCs responsible for cross-tolerance. To do this, mixed DC populations were isolated from the pancreatic LN (draining OVA-expressing tissue) or inguinal LNs (as the control) of RIP-mOVA mice and cultured with naïve OT-I cells. The activation of OT-I cells was then assessed by ³H-TdR incorporation 3 days later. This showed that while very weak proliferation of OT-I cells was specifically induced by DCs from the pancreatic lymph nodes of RIP-mOVA mice (Figure 5.7), the approach would need to be improved before the cross-tolerizing DC could be unequivocally identified.

5.2.2.2 Depleting CD4+ DC does not prevent cross-tolerance

To test whether the DC subset responsible for cross-tolerance was CD4+, RIP-OVA hi mice, which express OVA in the pancreas, were depleted of CD4+ DC by crossing to GK5 mice expressing the CD4-depleting mAb in the pancreas. When CD4+ cell-deficient RIP-OVA hi mice were injected with CFSE labelled OT-I cells, they showed equivalent proliferation in the pancreatic LN to that of wild-type RIP-OVA hi mice (Figure 5.8). This indicates that CD4+ DCs are not responsible for cross-presenting this self-antigen to CD8+ OT-I cells.
Figure 5-7 Attempted isolation of the lymph node DCs responsible for cross-tolerance. Total DC populations were isolated from the pancreatic LN and renal LN (draining OVA-expressing tissue, DLN) or inguinal LN (as the control) of RIP-mOVA mice and cultured with naïve OT-I cells ($2.5 \times 10^4$). The activation of OT-I cells was then assessed by $[^{3}H]$-TdR incorporation 3 days later. A. Stimulation of OT-I cells by lymph node DCs; B. Stimulation of OT-I cells by OVA$_{257-264}$ pulsed DCs.
Figure 5-8  Cross-presentation of self-antigen to CD8⁺ OT-1 cells.

CD4⁺ cell deficient or sufficient RIP-OVA⁺ mice were transferred i.v. with a total of 2 × 10⁶ OT-1 cells labelled with CFSE. Three days later, the pancreatic or inguinal LN were removed from the mice and the cells were analysed by flow cytometry. Profiles were gated on CD8⁺ CFSE⁺ Pi⁺ cells. This experiment was repeated twice with 2-3 mice in each group.
5.3 Discussion

Cross-presentation of cell-associated antigen to T cells can mediate either cross-priming or cross-tolerance (Adler et al., 1998; Bevan, 1976b; Carbone et al., 1998; Cella et al., 1997; Heath and Carbone, 2001; Heath et al., 1998; Huang et al., 1994; Sigal et al., 1999). Under certain condition the response can be switched from one to the other (Garza et al., 2000; Kurts et al., 1997a; Miller et al., 1998). The former activates T cells and generates CTL immunity against invading pathogens or tumour cells. The latter, also activates T cells, but leads to the induction of tolerance. Although cross-priming was discovered in the mid-1970s, and the APC was found to be of bone marrow origin, little success was made in identifying the phenotype of this APC until recently. Recently, Bevan and colleagues discovered that CD8$^+$ DC can cross-present antigen acquired in vivo (den Haan et al., 2000). However, they did not present data strictly showing that those DCs were effective at in vivo cross-priming.

During our studies aimed at identifying the cross-priming APC, it was shown that bone marrow-derived cells were essential for cross-priming OT-I cells and for presenting cell-bound OVA to OT-II T cells. This supported the idea that the cross-priming APC was a DC. In vivo depletion of DC sub-populations revealed that CD4$^+$ DCs were not required for cross-presentation. In contrast, presentation of cell-bound OVA to OT-II cells was inhibited in the recipients depleted of CD8$^+$ DCs, suggesting that the cross-priming APC may be a CD8$^+$ DC. This idea was supported by a cell-isolation study, in which mice were primed with cell-bound OVA and then DC isolated from the spleen were tested for their ability to cross-present OVA to OT-I cells. Only CD8$^+$ DCs were able to cross-present in this system. In addition, our in vivo depletion experiment showed that CD8$^+$
DC subset was effective at in vivo presentation albeit to CD4\(^+\) T cells supporting the proposal that they are the cross-priming APCs. This observation confirms and extends the studies from Bevan’s group, which showed that CD8\(^+\) DCs are capable of cross-presenting cell-bound OVA to OT-I cells (den Haan et al., 2000).

Recent research has introduced the notion that DC might be crucial in determining the outcome of tolerance versus autoimmunity (Fazekas de St Groth, 1998; Garza et al., 2000; Shortman and Caux, 1997). There is evidence that the activation status of DC is important in induction of tolerance or autoimmunity (Garza et al., 2000). With respect to cross-presentation, the cross-tolerizing APC has not been identified and it is still unclear whether the same DC subset is responsible for induction of both cross-tolerance and cross-priming. We found that a bone marrow derived APC is required to initiate cross-tolerizing responses in RIP-OVA\(^{\beta}\) mice. In the absence of CD4\(^+\) DC, this response was still evident, suggesting that this cross-tolerating APC does not express CD4. A report by Kurts et al. (Kurts et al., 2001) suggests that the cross-tolerizing APC is a CD11c\(^+\) cell, and therefore most likely a DC. Our observations are consistent with the idea that it belongs to the same CD8\(^+\) subset responsible for cross-priming.

In summary, this study focused on identification of the APC responsible for cross-priming and cross-tolerance. Our studies showed that CD8\(^+\) DCs are most likely responsible for cross-priming. Identification of cross-tolerizing APC has been more difficult, and other than knowing that it is likely to be a DC that is not CD4\(^+\), its identity remains elusive. However, it is worth speculating that it is likely that CD8\(^+\) DCs are
involved in both cross-priming and cross-tolerance and that additional signals determine whether this subset induces priming or tolerance.
Chapter 6  General Discussion

Cross-presentation provides an important mechanism for immunosurveillance of localized infection and to maintain self-tolerance of peripheral antigens. This process is mediated by bone marrow derived APCs, most likely DCs (Heath and Carbone, 2001; Steinman and Nussenzweig, 2002). DCs function by capturing antigen and initiating immune responses. These processes are carried out at different stages of DC maturation and affected by different factors (Cella et al., 1997; Lanzavecchia and Sallusto, 2001; Randolph et al., 1999; Steinman, 2001). Antigen concentration (Kurts et al., 1998; Kurts et al., 1999), accessory molecules (Ridge et al., 1998; Sabzevari et al., 2001; Schoenberger et al., 1998) and CD4+ T cell help (Husmann and Bevan, 1988; Keene and Forman, 1982) have all been shown to contribute to CTL cross-priming. However, how these factors control this event remains to be assessed. Our hypothesis is that some or all of these can change the DC status and promote a switch from priming to the induction of self-tolerance. In order to dissect cross-presentation in both priming and tolerance, we have developed a transgenic model system in which MHC class I and class II restricted presentation of OVA protein could be visualized by T cell proliferation in draining lymph nodes (Kurts et al., 1999; Li et al., 2001). In addition, OVA specific CTL activation could be monitored by the destruction of pancreatic islet cells leading to autoimmune diabetes in transgenic mice expressing OVA under the control of the insulin promoter. Using this system, I examined the influence of CD4 T cell help in the generation of CD8+ T cell-mediated autoimmunity. In particular, I looked at the CD4 T cell-APC interaction, the nature of antigen involved in cross-presentation as well as attempting to identify the cross-presenting APC.
6.1 CTL-mediated autoimmunity depends on CD4\(^+\) T cell help and relies on the concentration and the form of the antigen

Previous studies have shown that B6 mice can generate OVA-specific CTL when stimulated with cell-associated OVA protein (Carbone and Bevan, 1990). This response is initiated by bone marrow derived cross-priming APCs and depends on specific CD4\(^+\) T cell help (Bennett et al., 1997). The results presented in this thesis demonstrating cross-presentation by a host, not a donor, bone marrow-derived APC for activation of OVA-specific CD8 and CD4 T cells are consistent with the earlier observations. I have also shown that the higher the dose of antigens, the more efficient the cross-presentation and the stronger the T cell responses to both foreign and self-antigen mediated. In addition, cross-presentation of cell-associated OVA was found to favor MHC-I restricted T cell responses consistent to earlier suggestion (Bevan, 1987; Carbone and Bevan, 1990).

Bone marrow derived APCs were also found responsible for induction of cross-tolerance. It was shown that OVA specific CD8\(^+\) OT-I cells, which adoptively transferred into RIP-OVA\(^{hi}\) mice, were activated and proliferated but did not lead to the onset of pancreatitis or autoimmune diabetes since these T cells are known to be deleted (Kurts et al., 1999). However, diabetes did occur when naïve OT-I cells were co-transferred with in vitro activated OT-II cells into the RIP-OVA\(^{hi}\) mice. Although OT-I cells are responsible for the direct killing of OVA-presenting β cells, it clearly indicates that OVA specific OT-II cells help OT-I T cells induce CTL-mediated autoimmune diabetes.
Interestingly, antigen form and expression levels were able to determine whether the CD4\(^+\) T cell help was required to induce autoimmunity. This was revealed by comparing the incidence of autoimmune diabetes between RIP-OVA\(^{hi}\) and RIP-mOVA mice after adoptive transfer of T cells into the mice. Transfer of naïve OT-I cells into RIP-mOVA mice which express a high level of a membrane-bound form of ovalbumin (mOVA) in the pancreatic islet β cells and the kidney proximal tubular cells (Kurts et al., 1997b) led to stronger OT-I T cell proliferation and incurred a more severe deletion when compared with RIP-OVA\(^{hi}\) mice (Kurts et al., 1999). Furthermore, the majority of RIP-mOVA recipients became diabetic in the absence of exogenous OT-II cells after receiving 5 \(\times\) 10\(^6\) naïve OT-I cells alone (Kurts et al., 1997a), whereas double this number of naïve OT-I cells never caused diabetes in RIP-OVA\(^{hi}\) mice, except where exogenous activated OT-II T cells were also provided. It appears that a higher level of self-antigen not only induces a stronger CD8\(^+\) T cell response, but also decreases the dependency on CD4\(^+\) T cell help for the generation of autoimmunity.

### 6.2 The help from activated OT-II T cells can be both CD40-dependent and independent

Since the ligand for CD40 is predominantly expressed on activated CD4\(^+\) T cells, and CD40 is constitutively expressed on APCs, it is thought that during cross-priming, CD4\(^+\) T cell help involves CD40 signalling. Ligation of CD40 on APCs has been shown to up-regulate the expression of accessory molecules (Shinde et al., 1996; Stout and Suttles, 1996; Yang and Wilson, 1996), enhance APC co-stimulatory activity, support CD8\(^+\) T cell activation (Bennett et al., 1998; Schoenberger et al., 1998) as well as
promote IFN-γ production (Stout and Suttles, 1996; van Kooten and Banchereau, 1997).

In the present analysis, it was shown that CTL-induced autoimmunity was efficiently achieved with the help from activated OT-II cells with intact CD40L. As shown in chapter 4, a deficiency in CD40 inhibited the CD4 T cell-mediated enhancement of diabetes occurrence about 100-fold, which indicated a critical role for CD40 signalling in mediation of CD4+ T cell help. However, transfer of large numbers of naïve OT-I cells overcame this impaired help. This suggests that increasing the strength of interaction between CD8 T cells and APC minimize the dependency of these T cells on CD4 T cell help. One possibility is that high numbers of CD8+ T cell may simply up-regulate expression of accessory molecules such as CD40L (Cronin et al., 1995; Hermann et al., 1995). Alternatively, large numbers of OT-I cells may produce cytokines that support CTL progenitors becoming effectors before they are deleted.

Interestingly, a direct CD40 stimulus in the form of soluble anti-CD40 antibody could not substitute for the help from OT-II cells. This failure of anti-CD40 antibody to replace the CD4 T cell help highlights that this signal does not represent the sole form of help from activated OT-II cells. Therefore, both a CD40 ligand and additional unknown factor(s) supplied by the OT-II cells are required to help CTL cause autoimmune diabetes in the transgenic system.

6.3 Non-cognate CD4+ T cells help

It was revealed that to cross-prime CTL, both CD4+ and CD8+ T cells were required to recognize the antigen presented by the same APC (Bennett et al., 1998; Cassell and Forman, 1988), suggesting that CD4+ T cells might help by modifying APC activity. In
contrast, the results presented in this thesis showed that cognate help was not required to induce autoimmune diabetes after CTL cross-priming, although CD4 T cell help enhanced disease induction. While both types of responses are helper T cell-dependent, the mechanism of this help appears different. It is possible that pre-activated OT-II cells may function differently from resting cells that need \textit{in vivo} activation. For example, the activated T cells may interact directly with APCs through already upregulated accessory molecules such as CD40L and TRANCE without need for MHC-restricted recognition. Alternatively, they may also produce cytokines that promote the expansion and survival of the CD8\(^+\) T cells once the latter are activated. Indeed, CD4\(^+\) T help cells improved CD8\(^+\) T cell expansion, which has been shown to be critical in the autoimmunity induction. In addition, cytokine may simply enhance the effector capability of any activated CTL. This may be important in the RIP-OVA\(^{hi}\) adoptive transfer system, where these mice have received relatively large number of OVA-specific OT-I T cells. In this case, the helper T cells might simply enhance killing after relatively in-efficient OT-I T cell activation by providing a favorable cytokine milieu. Consistent with this, MHC II\(^+\) RIP-OVA\(^{hi}\) mice also became diabetic after receiving the combination of activated helper T cells along with the naïve OT-I population. Regardless, the result highlights the conclusion that there is a component of CD4\(^+\) T cell help that does not require cognate recognition of APC.

6.4 CD8\(^+\) DC: cross-priming or cross-tolerizing APC?

It has recently been revealed that CD8\(^+\) DCs can act as cross-presenting APC since they initiate CD8\(^+\) T cell proliferation as measured \textit{in vitro} (den Haan \textit{et al.}, 2000). Consistent with this, I observed that OVA-bearing CD8\(^+\) DCs were not only able to
initiate OT-I cell proliferation in vitro, they were also able to activate OVA specific OT-I and OT-II cells in the mice immunized with cell-associated OVA. Thus, CD8+ DCs capture foreign antigen and then carry it into draining lymph nodes or spleen for specific T cell priming. However, CD8+ DCs from RIP-OVA h mice appear to also present self-antigen, suggesting that this DC subset may participate in both cross-priming and cross-tolerance. So what selects between priming and tolerance?

Several possibilities have been suggested as to how cross-presentation leads to immunity or tolerance. Originally, it was thought that immature DCs, lacking co-stimulatory molecules and bearing low levels of surface MHC molecules, could tolerate T cells, while mature DCs were involved in T cell activation (Hawiger et al., 2001; Jiang et al., 1995). It was later found that distinct DC subsets exert different functions. For example, CD8+ DCs produce large amounts of IL-12 (Hochrein et al., 2001; Pulendran et al., 1999), while CD8- DCs are capable of stimulating Th2 responses (Maldonado-Lopez et al., 1999; Maldonado-Lopez and Moser, 2001; Pulendran et al., 1999). More recently, it has been shown that the both CD8+ and CD8- DC are able to activated T cell responses in vitro and (Maldonado-Lopez et al., 1999; Pooley et al., 2001). In addition, antigen-specific CD4+ T cells provide signals that activate APCs rendering them immunogenic (Bennett et al., 1998; Heath and Carbone, 2001; Jenkins et al., 2001; Shortman and Heath, 2001). It is therefore possible that multiple mechanisms are involved in the switch of immunity versus tolerance (Steinman and Nussenzweig, 2002). As noted, the data presented in the thesis seems favor the notion that CD8+ DCs appear to participate in both cross-priming and cross-tolerance. Therefore, I favor the possibility that a single type of professional APC could be immunogenic under some conditions and tolerogenic
under others. In this respect, factors such as helper T cell interaction may be crucial in determining the outcome of T cell activation and the selection between immunity and tolerance.

6.5 Summary

The thesis describes the nature of CD4 T cell help in cross-priming CTL, the antigenic requirements during cross-presentation and attempts to identifying the cross-presenting APC. Firstly, the data indicate that antigen-specific CD4+ T cell help is critical in promoting CTL priming. The help might be supplied in both the priming phase and the effector phase. The former is characterized by the requirement for interaction of CD4+ T cells with APCs, as small numbers of CD8+ T cells are unable to respond normally without signalling from CD40 and MHC class II. T cell help greatly promoted CD8+ T cell expansion to produce large numbers of effector CTLs. These events probably occur in the priming phase. On the other hand, CD4+ T help is also important in the effector phase. Both T cell subsets did not need to recognize the same APC and CD40 signalling was not essential for β-cell destruction leading to autoimmunity. The data presented in this thesis also showed that the CD8+ DC subpopulation may function in either cross-priming or cross-tolerating depending on different conditions such as the availability of helper T cells or the amount of antigens. Finally, it was shown that cellular antigen is much more efficiently cross-presented than soluble antigen when administered i.v., and antigen dose may determine whether CTL cross-priming is CD4-dependent. Overall, the thesis describes the optimum helper and antigenic requirements for cross-presentation and provides some insight into the mechanisms that select cross-priming versus cross-tolerizing.
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ADDENDUM

Page 3, 7th line from the bottom: should read “Cleavage of li allows MHC class II molecules to bind peptides present in endosomes.” In the same section, the last sentence of the page should read “This accessory molecule catalyzes the exchange of CLIP bound to MHC class II molecules with peptides.”

Page 3, part 1.1.2, the last sentence should read “peptides binding to MHC class I molecules in the ER are required for their transportation to the cell surface and their presentation to CD8+ T cells.”

Page 4, part 1.1.3, line 4: should read “It was first revealed 26 years ago by Bevan.”

Page 4, part 1.1.3 last line: add references for TAP requirement in cross-presentation: (Sigal et al., 1999; den Haan et al., 2000).

Page 6, part 1.1.3.2, line 13: should read “Binding antigen-Ig complex to APCs mediates internalization of the complexes”. In the same paragraph, add “More details are described in part 1.1.3.3.5.” to the end of the paragraph.

Page 20, part 1.2.3.2, line 5: delete “proliferate and”, the sentence should read “ligation of the TCR alone does not allow naïve T cells to differentiate into effectors, …..” In the same paragraph, line 11: delete “and their ligand”.

Page 21, line 5: delete “and T cells”, should read “……such as DCs, macrophages and B cells.”

Page 23, part 1.2.3.3, line 6: The following sentence should be added before “In addition to…….”, “The fact that CD28-/- mice can mount an efficient response against some viruses like LCMV provides evidence for the existence of other costimulatory molecules (Suresh M et al. 2001. J. Immunol. 167(10):5565:73).”

Page 62, Fig.3-1, change the last sentence of the figure legend to: “The minimum dose for activation of OT-I cells was 0.01 mg (4/5) and 0.01 – 0.1 mg (1/5). While, for OT-II cells, in 4/7 recipients it was 1.0 mg and in 3/7, 10 mg."

Page 63, the last sentence in the first paragraph of part 3.2.3 should read: “……, it required a minimum dose of 10-100 μg/ml, which was 5,000 - 50,000 fold more antigen than needed to …..”.

Page 64, 70, 73 and 78, the last sentences in the figure legends should read “The experiment was performed…… times with similar results.”
Page 75, Fig.3-9, the following sentence should be added to the figure legend “The minimum dose required for OT-I proliferation was found to be 10 μg OVA on two separate occasions and the minimum dose for OT-II cells was 1.0 μg OVA on 3 occasions and 0.5 μg on one occasion.”

Page 102, part 4.2.6, The first two sentences should read “Based on the above analysis, it was suggested that co-recognition of the same APC by both CD4+ and CD8+ T cells was not required. To further address whether the help from OT-II cells was antigenic specific, we examined whether activated OT-II cells needed to recognize OVA in the context of I-A^b to provide help.”
Chapter 1
In section 1.1.3.4 it is suggested that antigen presentation in draining lymph nodes is carried out by the transfer of antigen on a cross-priming APC, which migrates to draining lymph nodes where it is presented to antigen-specific T cells. In other words, the actual cross-priming APCs are mobile, rather than the antigen. Randolph and his colleagues (Randolph et al., 1999) clearly showed that a substantial minority of inflammatory monocytes carried phagocytosed micropheres to the T cell area of draining lymph nodes and differentiated into DCs. Microsphere-transporting cells were distinct from resident skin DCs and this transport was reduced by more than 85% in monocyte-deficient osteopetrotic mice. Although the possibility that an antigen can be transported in a soluble form to lymph nodes has not been excluded, it is unlikely to be an effective antigen-transporting pathway. There are also no reports to suggest that antigen moves by some other, non-cellular mechanism such as exosomes. Finally, it has been reported that HSPs may facilitate the entry of peptides into the MHC class I restricted pathway and induce tumour specific CTL responses, as described in section 1.1.3.3.2. The detailed mechanism remains to be clarified and whether HSP is also involved in the transfer of peripheral antigen remains to be determined.

Chapter 4
Results from Chapter 4 showed that the presence of MHC class II molecules was very important, but not essential, for induction of diabetes. The data also showed that both OT-I and OT-II T cells did not need to recognize the same APC. This suggested some other components unrelated to MHC class II and TCR interactions were involved. However, the data showed that “help” in the absence of MHC class II molecules was very poor and required the transfer of large numbers of antigen specific OT-II cells. As a consequence, this help is unlikely to be achieved during a natural inflammatory process and its role in an adaptive immune response remains unclear.

Finally, in order to generate efficient CD4\(^+\) T cell help for induction of antigen-specific cytotoxic responses, OT-II cells were activated \(\textit{in vitro}\) prior to transfer into RIP-OVA\(^{hi}\) mice since naïve OT-II cells were unable to help efficiently to induce
autoimmune diabetes. The selection of activation conditions, such as antigen dose and stimulation time, was based on T cell proliferation and flow cytometry analysis and was designed to give optimal stimulation.