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REGULATION OF GLUCOCORTICOID RECEPTOR EXPRESSION AND DOWNSTREAM GENE TARGETS IN MURINE LYMPHOCYTES

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GENERAL DECLARATION

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General Declaration
In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in peer reviewed journals and two unpublished publications. The core theme of the thesis is Glucocorticoid signalling in lymphocytes. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Biochemistry and Molecular Biology department under the supervision of Tim Cole.

In the case of chapters 3 and 4 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Publication status	Nature and extent of candidates contribution
3	*Nfil3* is a glucocorticoid-regulated gene required for glucocorticoid-induced apoptosis in T-cells	Published in *Endocrinology* 20/2/2013	70% Project direction, development and writing up of paper, qRT-PCR analysis, ChIP analysis, immunohistochemistry, siRNA transfection, Flow cytometry

4	Differential activity of the GR gene 1A promoter in the pituitary and B cell lineages of the mouse	Under review	70% Project direction, development and writing up of paper, qRT-PCR, *in situ* hybridization

I have / have not (circle that which applies) renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: ……

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ABSTRACT

Glucocorticoids (GCs) are homeostatic steroid hormones with essential roles in the regulation of development, integrated metabolism, immune and neurological responses. GCs act via the widely expressed Glucocorticoid Receptor (GR), which is expressed from multiple untranslated exon 1s to yield 11 alternatively spliced transcripts in humans (1A-1H) and five in mice (1A-1E). These transcript isoforms are under the control of their own promoters which confers tissue specificity and a higher level of regulation to this transcription factor. In thymocytes activity from the GR1A promoter is implicated with increasing sensitivity to Glucocorticoid Induced Cell Death (GICD). $CD4^+CD8^+$ Double Positive (DP) cells and NKT cells in particular are hypersensitive to GICD.

The main objectives of this study were to investigate further the molecular mechanisms involved in GICD, to examine more closely the role of GR in the development of T-lymphocytes, and to characterise the expression and regulation of the GR1A promoter in mouse tissues previously not examined.

To explore the molecular pathway driving GICD in thymocytes we performed whole genome microarray analysis in mouse GR null thymocytes. Interesting direct GR targets included *P21*, *Bim* and *Nfil3*. Regulation of these targets by GCs was validated using qRT-PCR in WT thymocytes. *Nfil3* in particular has been studied further. Previous studies demonstrated that GC-mediated up-regulation of Nfil3 is dependent on intracellular calcium levels, and correlates with GICD of GC-sensitive leukemic CEM cells. *In silico* promoter analysis revealed a putative Glucocorticoid Response Element in the *Nfil3* 5'UTR which was confirmed to interact with the GR by ChIP. Immunohistochemical staining of *Nfil3* in whole thymus has localised NFIL3 protein primarily to the medullary region. Double labelling has co-localised NFIL3 to apoptotic cells and macrophages. Using siRNA technology we have shown that NFIL3 does in fact confer greater sensitivity to GICD in Ctll-2 cells.

Previous to our studies transcripts initiating from the GR1A promoter had only been localised to the cortex of the brain and to T-lymphocytes. Using qRT-PCR and *in situ* hybridisation we have detected transcripts initiating from the GR1A promoter in the anterior lobe of the pituitary. However, the role of the GR1A promoter activity in the brain is unknown. In the brain, particularly in the cortex, and also in the hypothalamus and pituitary (components of the Hypothalamic-Pituitary-Adrenal axis) GCs and their receptors have a key role in the response to stress. A 2.5 fold increase in the level of GR1A promoter usage in the pituitary was observed in response to treatment with the synthetic GC Dexamethasone. It is possible that a tissue/cell specific increase in activity of the GR1A promoter during periods of elevated levels of circulating GCs may help to make those cells more sensitive to these rising levels of GCs and serve as a fine tuning mechanism to aid in a rapid return to the normal state after stress.

The significance of increased sensitivity of some T-cell lineages to GICD is controversial, with a proposed function being that it is involved in T-cell development. The direct role of the GR in development of T-cell populations and some specific lymphocyte lineages in T-cell specific GR-null mice (TGRKO) was examined using FACS. Major differences in CD8 CD4 cellularity was observed in spleen and liver and to a lesser extent in thymus. In Spleen NKT cellularity was reduced and T_{Reg} cell CD25 populations were altered.

This study has shed light on some of the regulatory mechanisms and molecular interactions associated with GR function in the pathways of stress, T-cell development and GICD.

We have identified rapidly induced GR target genes in GR null thymocytes and characterized the regulation of the transcriptional repressor *Nfil3* by GR in normal mouse thymocytes. We have shown that NFIL3 is necessary for GICD in Ctll2-T cells.

Additionally we have shown that the regulation of GR expression in the pituitary may occur via auto-regulation and maintenance of the GR1A promoter,

an action which likely increases sensitivity to elevated GC levels and may account for the rapid return to a normal state following stress.

Finally we have demonstrated T-cell development is subtly affected by loss of GR in T-cells.

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Chapter 4: Differential activity of the GR gene 1A promoter in the pituitary and B cell lineages of the mouse

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PUBLICATION RECORD

Refereed Journal Publications

Carey KT, Tan KH, Ng J, Liddicoat DR, Godfrey DI, Cole TJ 2013 Nfil3 Is a Glucocorticoid-Regulated Gene Required for Glucocorticoid-Induced Apoptosis in Male Murine T Cells. Endocrinology 154(4):1540-52

Trounce IA, Crouch PJ, Carey KT, McKenzie M 2013 Modulation of ceramide-induced cell death and superoxide production by mitochondrial DNA-encoded respiratory chain defects in *Rattus* xenocybrid mouse cells. Biochimica et Biophysica Acta (BBA)-Bioenergetics 1827(7):817-25

Wong S, Tan K, Carey KT, Fukushima A, Tiganis T, Cole TJ 2010 Glucocorticoids stimulate hepatic and renal catecholamine inactivation by direct rapid induction of the dopamine sulfotransferase Sult1d1. Endocrinology 151(1):185-194

Proceedings of meetings

Oral Presentations

“Identification of direct Glucocorticoid-Regulated genes in T-Lymphocytes by whole genome expression microarray” ComBio, Christchurch, New Zealand (2009)

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Poster Presentations

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Kirstyn T Carey, Judy Ng, Douglas R Liddicoat, Dale I Godfrey and Timothy J Cole, Glucocorticoid Induced Cell Death in T-Lymphocytes. Lorne Cancer, Lorne, Victoria (2009)

Kirstyn T Carey, Kheng Tan, Judy Ng, Douglas R Liddicoat, Dale I Godfrey and Timothy J Cole, Identification of direct Glucocorticoid-Regulated Genes in T-Lymphocytes by whole genome expression microarray. Network in Genes and Development Conference and ECR Workshop, Palm Cove, Australia (2009)

Kirstyn T Carey, Kheng Tan, Judy Ng, Douglas R Liddicoat, Dale I Godfrey and Timothy J Cole, Identification of direct Glucocorticoid-Regulated Genes in T-Lymphocytes by whole genome expression microarray. Lorne Genome, Lorne, Australia (2010)

Kirstyn T Carey, Kheng Tan, Judy Ng, Kon Kyparissoudis, Douglas R Liddicoat, Dale I Godfrey and Timothy J Cole, Identification of Nfil3 as a direct glucocorticoid regulated gene target in T-Lymphocytes. Ozbio, Melbourne, Australia (2010)

ABBREVIATIONS

ACTH	Adrenocorticotropin Hormone
AD	Activation Domain
AICD	Activation Induced Cell Death
AF	Activation Function
ALL	Acute Lymphoblastic Leukemia
AP-1	Activator Protein 1
BAX	BCL2-Associated X protein
BAK	BCL2-Antagonist/Killer 1
BCl2l11/Bim	B-cell Lymphoma like 11
BM	Bone Marrow
CD	Cluster of Differentiation
CDC6	Cell Division Cycle 6
CLP	Common Lymphoid Progenitor
C-MYC	Myelocytomatosis oncogene
CNS	Central Nervous System
CRH	Corticotropin Releasing Hormone
CRH-R	Corticotropin Releasing Hormone Receptor
CTLA-4	Cytotoxic T-lymphocyte-Associated protein 4 precursor
DBD	DNA Binding Domain
DDIT4	DNA Damage Inducible Transcript 4
DN	Double Negative
DP	Double Positive
EGFP	Enhance Green Fluorescent Protein
ELP	Early Lymphoid Progenitor
ETP	Early T-lineage Progenitors
FasL	Fas Ligand
FoxP3	Forkhead box P3
FKBP5	FK506 Binding Protein 5

FM	Follicular Mature
FP	Foot Print
FSH	Follicle Stimulating Hormone
FTOC	Fetal Thymus Organ Culture
GATA3	GATA binding protein 3
GC	Glucocorticoid
GHRH	Growth Hormone Releasing Hormone
GHRH-R	Growth Hormone Releasing Hormone Receptor
GITR	Glucocorticoid-Induced TNFR-related
GnRH	Gonadotropin Releasing Hormone
GnRH-R	Gonadotropin Releasing Hormone Receptor
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
hGR	human Glucocorticoid Receptor
HPA	Hypothalamic Pituitary Adrenal (axis)
HSC	Haematopoietic Stem Cell
HSP	Heat Shock Protein
IBD	Inflammatory Bowel Disease
IL	Interleukin
IPEX	Immune Dysregulation, Polyendocrinopathy, Enteropathy and X-linked Syndrome
IRF	Interferon Regulatory Factor
LCK	Lymphocyte-specific protein tyrosine Kinase
LBD	Ligand Binding Domain
LH	Leuteinizing Hormone
MC2R	Melanocortin 2 Receptor
MHC	Major Histocompatibility Complex
mGR	mouse Glucocorticoid Receptor
MPP	Multipotent Progenitors

MR	Mineralocorticoid Receptor
MZ	Marginal Zone
NF-κB	Nuclear Factor κB
NLS	Nuclear Localisation Sequence
NKT	Natural Killer T-cell
NK	Natural Killer
PLZF	Promyelocytic Leukemia Zinc Finger
POMC	Pro-opiomelanocortin
PRL	Prolactin
PVN	Paraventricular Nucleus
SLAM	Signaling Lymphocytic Activation Molecule
SP	Single Positive
Sp-1	Specificity Protein 1
TCR	T-cell Receptor
TGF-β	Transforming Growth Factor β
TH	Thyroid Hormone
TNFAIP3	Tumour Necrosis Factor, Alpha-Induced Protein 3
TRH	Thyrotropin Releasing Hormone
TRH-R	Thyrotropin Releasing Hormone Receptor
TSH	Thyroid Stimulating Hormone
TXNIP	Thioredoxin Interacting Protein
UTR	Untranslated Region
V(D)J	Variable-Diverse-Joining

CHAPTER 1:

INTRODUCTION

Chapter 1: Introduction

1.1: Overview

Glucocorticoids (GCs) are a class of steroid hormones that play an essential role in the regulation of biological processes such as growth, development, metabolism, immune reactions, stress responses, behaviour and apoptosis. Clinically GCs are a widely prescribed class of drug. Their immunosuppressive and anti-inflammatory actions are exploited to control autoimmune diseases and airway inflammation in respiratory diseases such as asthma (Pujols, et al. 2004). In the brain GCs primarily act on receptors localised within the hippocampus, cortex, hypothalamus and pituitary (the latter regions are components of the Hypothalamic-Pituitary-Adrenal (HPA) axis) where they have a key role in the response to stress and aiding a rapid return to the normal state after stress. In addition, the ability of GCs to induce apoptosis in cells; Glucocorticoid Induced Cell Death (GICD) and malignancies of lymphoid origin is central in the treatment of lymphocytic leukaemia's and lymphomas, for example childhood Acute Lymphoblastic Leukemia (ALL) although mechanisms underlying this process are incompletely understood (Sapolsky, et al. 2000).

The actions of GCs are mediated via the Glucocorticoid Receptor (GR) which belongs to a superfamily of nuclear receptors that also includes receptors for mineralocorticoid, thyroid and sex hormones, vitamin D and retinoic acid (Pujols et al. 2004). The receptors of this superfamily share a common domain structure consisting of a modulatory domain, a DNA Binding Domain (DBD) and a Ligand Binding Domain (LBD) (Giguere, et al. 1986). The GR gene is composed of 9 exons with exons 2 - 9 encoding portions of the receptor. Exon 1 is untranslated and is alternately spliced to yield at least 13 transcripts in humans and at least 10 in mice (Presul, et al. 2007; Turner and Muller 2005; Breslin, et al. 2001; Chen, et al. 1999b). It is believed that at least 5 of the alternate transcripts are under the direction of their own promoter conferring tissue specific distribution to each of the individual transcripts through interaction with unique combinations of transcription

factors, contributing to the fine regulation of GR expression which is essential for this ubiquitous multifunctional transcription factor. Transcripts initiating from the 1A promoter in particular have so far been localised only to T-lymphocytes, spleen and the cortex of the brain. In T-lymphocytes elevated GR1A promoter activity correlates with increased sensitivity to GICD (Purton, et al. 2004). GICD was believed to be a mechanism involved in negative selection in T-cell development however this has largely been disputed, thus the physiological function of GICD remains largely unknown.

This review covers aspects of GR function, expression and regulation in lymphocytes and the brain, as well as GR regulated genes associated with T-cell development and GICD.

1.2 Glucocorticoids: their mechanism of action via the Glucocorticoid Receptor

In response to a stress signal or a change in blood levels of GCs, the hypothalamus, the coordinating centre of the neuroendocrine system and a component of the Hypothalamic-Pituitary-Adrenal (HPA) axis, secretes Corticotropin Releasing Hormone (CRH)(Charmandari, et al. 2004). CRH subsequently acts on the anterior pituitary, stimulating the secretion of Adrenocorticotropin Hormone (ACTH), which in turn promotes the synthesis of corticosteroids, such as cortisol, from cholesterol in the adrenal cortex of the adrenal gland (Figure 1.1). It is then carried to target cells through the bloodstream. Target cells are those expressing the Glucocorticoid Receptor (GR), which is almost ubiquitously expressed in all human and murine cells (Charmandari et al. 2004). Due to their lipophilic nature, GCs are able to diffuse freely across the cell membrane to their receptors (Zhou and Cidlowski 2005).

1.2.1: Activation of the Glucocorticoid Receptor

Inactive GR, unbound by GC ligand is sequestered within the cytoplasm by

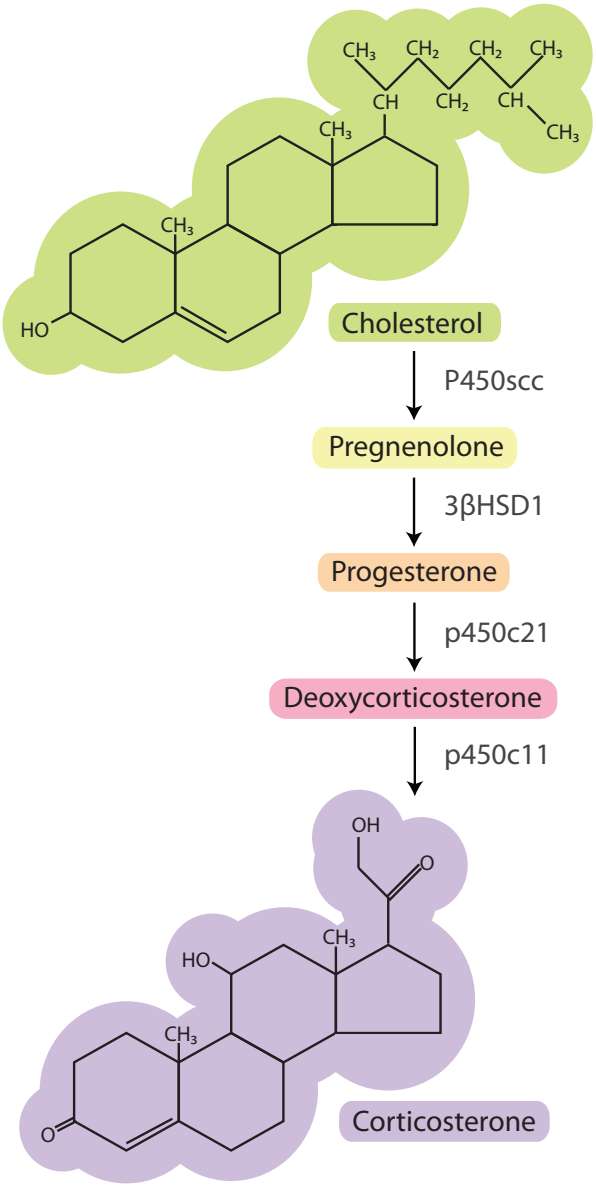


Figure 1.1 Synthesis of Corticosterone.

Corticosterone, a lipophilic corticosteroid hormone is synthesised from cholesterol in the cortex of the adrenal gland via a number of intermediates with the requirement for many enzymatic reactions.

a protein complex consisting of an immunophilin protein such as FKBP51 (Zhou and Cidlowski 2005) as well as two molecules of Heat Shock Protein (HSP) 90 which bind to the Ligand Binding Domain (LBD) of the receptor (Figure 1.2A) (Pujols et al. 2004). The interaction of the receptor with this protein complex holds the protein in a stable conformation with a competent ligand binding site (Bresnick, et al. 1989), in addition to inhibiting subcellular trafficking of GR into the nucleus by stabilising a conformation in which the Nuclear Localisation Sequences (NLS) are shielded (Yang and DeFranco 1996). When the ligand is bound by the LBD, a conformational change is induced which allows 1) homodimerisation of the GR within the cytoplasm via interactions between the C-terminal ligand binding domain (Dahlman-Wright, et al. 1992) and the receptor hinge region (Savory, et al. 2001) which links the steroid and DNA Binding Domains (DBD) (Giguere et al. 1986) (Figure 1.2B), and 2) exposure of the NLS's which are recognised by nuclear translocation proteins allowing nuclear import of the active GR dimer (Barnes 1998; Pujols et al. 2004; Zhou and Cidlowski 2005).

The dimeric form of GR has a high affinity for Glucocorticoid Response Elements (GREs) (Cairns, et al. 1991) which are located in the 5' upstream promoter regions of target genes. The consensus GRE sequence for these target genes is the partially palindromic 15bp sequence GGTACAnnnTGTTCT, where 'n' is any nucleotide (Barnes 1998; Pujols et al. 2004). The DNA binding domain of a single GR consists of two zinc finger motifs (Zhou and Cidlowski 2005). The amino terminal α helix of the zinc finger for each subunit of the dimer symmetrically interacts with successive major grooves of the target DNA (GREs) via hydrogen bonds (Luisi, et al. 1991) (Figure 1.2C). While the DNA is in its compacted form, i.e. associated with proteins in chromatin, the GR may bind to GREs of linker DNA between nucleosomes or if a GRE happens to be exposed in DNA that is wound around histones. Binding of the GR to the GRE may then modify the local chromatin structure, allowing GR access (Hayashi, et al. 2004).

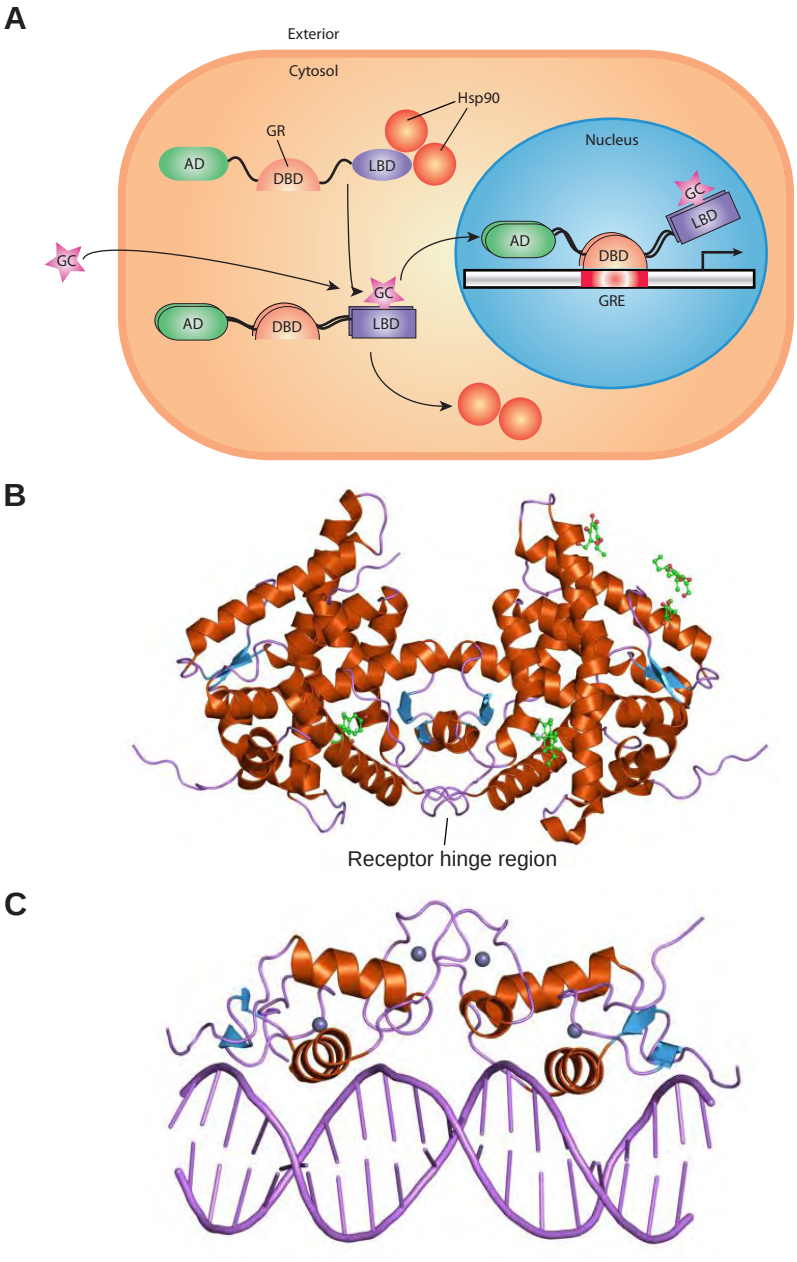


Figure 1.2 Glucocorticoid Receptor structure and action via direct DNA binding

A: Prior to hormone binding, GR is mostly cytosolic and is bound by HSP90 in addition to immunophilin protein. Upon ligand binding HSP90 is released and GR enters the nucleus where it binds to GRE's as a homodimer, allowing stimulation or respression of transcription (Lodish, 1999). B: Crystal structure of the ligand bound GR homodimer. Interactions occur between the C-terminal ligand binding domain and the receptor hinge region (as indicated). α-helices are presented in orange, β-sheets in blue and GC ligand is shown in green. C: Model of the GR homodimer DBD interacting with the GRE sequence of DNA. α-helices shown in orange represent the zinc finger motifs. Zinc ions are represented in grey. AD: Activation Domain, DBD: DNA Binding Domain, LBD: Ligand Binding Domain, HSP90: Heat Shock Protein 90. Protein structures for figures 1.2B and 1.2C were obtained from the EMBL European Protein Data bank. URLs: http://www.ebi.ac.uk/pdbe-srv/view/entry/1m2z/summary and http://www.ebi.ac.uk/pdbe-srv/view/entry/1r4o/summary respectively.

1.2.2 Mechanisms regulating GR activation

With the extensive functions of the GR it is important that it is tightly regulated. This occurs via regulation and autoregulation at multiple levels; Nr3c1(GR) gene transcription, GR protein production and turnover, and also GR activation by ligand binding. Thus the access of GCs to the receptor is under tight control.

p-Glycoprotein transporters located within the cell membrane regulate intracellular GC levels by actively pumping GCs out of cells thus reducing GR activation (reviewed in (Pariante 2008)). There is evidence for this process occurring in murine lymphocytes (Bourgeois, Gruol et al. 1993) and is particularly relevant in regulating passage of GCs across the blood brain barrier in the mouse brain (Uhr, Holsboer et al. 2002). Intracellularly, GR activation can be limited via the activity of two opposing enzymes, 11β–hydroxysteroid dehydrogenase 1 (11β-HSD1) and -2 (11β-HSD2). 11β-HSD2 converts active corticosterone to inactive 11-dehydrocorticosterone. 11β-HSD1 has bidirectional function however it acts primarily as a reductase converting inactive 11-dehydrocorticosterone to corticosterone. These enzymes show tissue specific expression. 11β-HSD1 is highly expressed in GC target tissues such as liver, lung, gonads, pituitary, brain and adipose tissue where it facilitates GC exposure to the GR. In particular regions of the brain including hippocampus, cerebellum, and neocortex 11 β-HSD1 is particularly important in increasing corticosterone levels, modulating mood, memory and learning (De Kloet, Vreugdenhil et al. 1998; Holmes, Yau et al. 2003; Draper and Stewart 2005). The mineralocorticoid receptor (MR) acts to regulate salt concentration within the body promoting sodium reabsorption and potassium excretion. GCs bind with equal affinity to MR as the primary ligand, aldosterone. Inappropriate activation of MR results in polyuria, hypertension, sodium retention, potassium loss and distal nephron pathology. Thus 11β-HSD2 acts to inactivate corticosterone in tissues such a kidney, colon, salivary glands and placenta where MRs is required. 11β-HSD2 function in these tissues prevents excess activation of MR and allows aldosterone access (Holmes, Yau et al. 2003; Draper and Stewart 2005).

1.2.3: Regulation of Gene Transcription

Ligand-activated GR can contribute to the regulation of gene transcription in at least three different ways. The cellular context and physiological conditions is of great importance in determining whether the steroid receptor complex either enhances or represses gene transcription. The first classic mode of regulation include activation or repression of gene expression mediated by direct binding of GR to positive or negative GREs of glucocorticoid-responsive genes (Figure 1.2A). The transcriptional activity of GR, i.e. as an activator or a repressor, depends on its interaction with other factors that regulate the chromatin structure and facilitate recruitment of the basal transcriptional machinery e.g. RNA polymerase II and general transcription factors, and allow access of this machinery to the DNA (De Kloet, et al. 1998). In addition, active GR is able to repress gene transcription via protein-protein interactions with other transcription factors which have already activated the gene, without GR binding to DNA, this is known as “tethering”. For example in combating inflammation, the major anti-inflammatory effects of GCs are through repression of inflammatory genes and genes involved in the immune system. The inhibitory effect of GRs occurs via interactions between the activated GR and transcription factors that mediate expression of inflammatory genes e.g. NF-κB and AP-1, which may be already bound to their respective DNA binding sites (De Kloet et al. 1998; Hayashi et al. 2004; Pujols et al. 2004). The “composite“ mode of regulation occurs when GR is bound to a GRE and interacts simultaneously with transcription factors bound to adjacent response elements. In addition, a fourth, ill-defined, mode of regulation involves the formation of heterodimers between different nuclear receptors, for example the Mineralocorticoid Receptor (MR) and GR do in fact share complete sequence identity within their dimer interfaces thus heterodimers between these receptors have been identified. It seems likely that MR-GR heterodimers play an essential role in mediating responses to corticosteroids in cells where they are co-expressed, however it remains unclear as to whether the effect is to inhibit transcriptional activity or

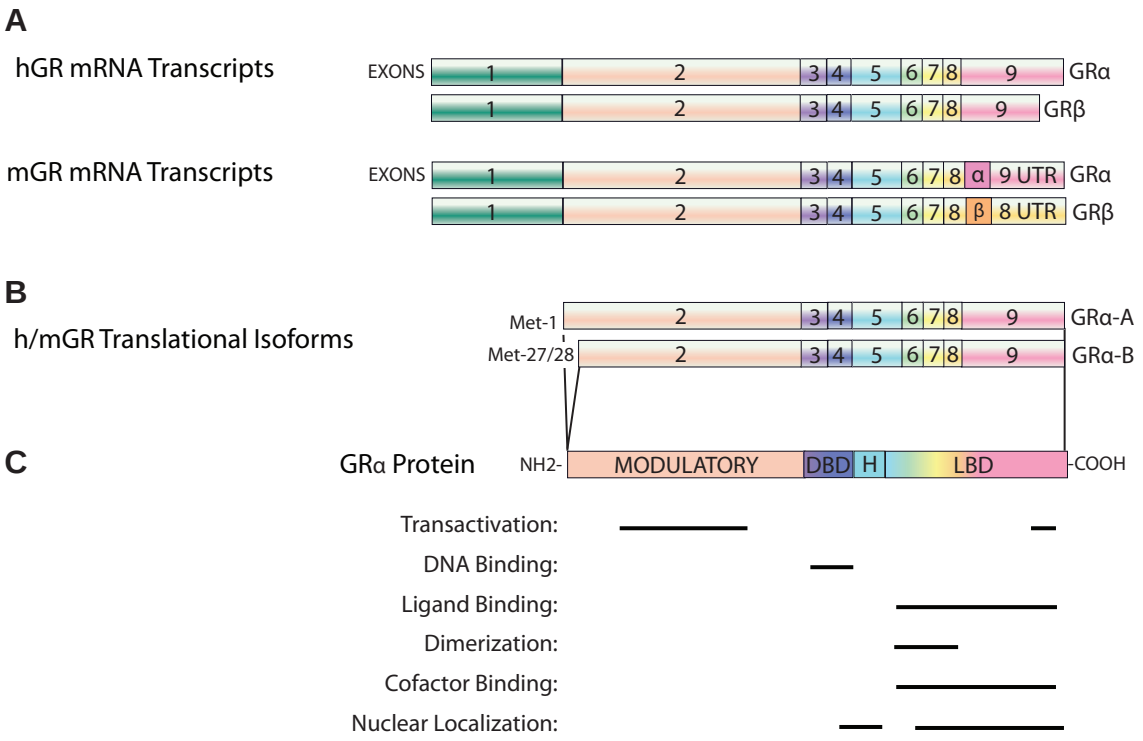


Figure 1.3 Human and mouse GR gene structure and transcript and protein isoforms

A: Depicts human (h) and mouse (m) GR mRNA transcripts, showing exons 1-9. Two main transcriptional isoforms which come about by differential splicing exist in human and mouse, the main isoform GRα and the truncated isoform GRβ. B: There are two major translational isoforms of GR which are almost identical in mouse and human. The major translational isoforms are GRα-A and GRα-B and these occur via translation initation from different start codons, Met-1 and Met-27 (human)/Met-28 (mouse). C: This portion of the figure depicts the functional domains of the mGRα protein, which are colour coded according to the exon which encodes that portion of the protein. The N-terminus encodes the “Modulatory” or transcriptional “activation function” (AF1) domain. Exons 3 and 4 encode the DNA Binding Domain (DBD). The C-terminus, encoded on Exons 5, 6, 7, 8, and 9 contains the Ligand Binding Domain (LBD), and specifies dimerisation, cofactor binding and nuclear localisation (Zhou and Cidlowski, 2005).

(Adapted from Zhou and Cidlowski 2005)

to activate it. It may be a case of cell-specific activation or repression (Liu, et al. 1995; Trapp, et al. 1994).

1.3: Structure of the Glucocorticoid Receptor Gene

1.3.1 Transcriptional isoforms

In 1991, Encio and Detera-Wadleigh determined the structure of the human glucocorticoid receptor gene (hGR). The hGR is encoded from *NR3C1,* a single gene of at least 80kb which contains a total of ten exons. The hGR gene encodes two primary receptor isoforms, hGRα and hGRβ, which come about by differential splicing of exon 9 with either the 9α or 9β portion of the exon being included (Encio and Detera-Wadleigh 1991) (Figure 1.3A). The alpha form is a fully functional receptor consisting of 777 amino acids, the beta form, however, is truncated to 742 amino acids (Yudt and Cidlowski 2002) and does not show hormone binding ability due to the loss of the hormone binding domain (Encio and Detera-Wadleigh 1991). The two isoforms are identical up to residue 727, with hGRα having an additional 50 amino acids and hGRβ having only an additional 15 non-homologous amino acids. Truncation of hGRβ at this point abolishes hormone binding ability, however within the unique 15 amino acids lies its ability negatively regulate hGRα (Oakley, et al. 1999). A portion of the dimerisation domain is maintained in the truncated isoform thus hGRβ is able to heterodimerise with hGRα, however as the β subunit does not bind hormone, dimerisation results in a transcriptionally inactive complex (Oakley et al. 1999).

In mice the β form arises from an alternate splice acceptor site to that seen in humans, which resides in intron 8 and this alternate exon is terminated by a stop codon within intron 8 also. This exon confers an extra 15 amino acids to GRβ which are 87% homologous to the hGRα and similar in function and cellular distribution (Hinds, et al. 2010).

1.3.2: Translational isoforms

In addition to transcriptional isoforms, multiple translational isoforms of GRα ex-

ist, produced by alternative translation initiation of the same gene. The translational isoforms are termed GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2 and GRα-D3 and are conserved across humans and rodents. Of particular interest are GRα-A and GRα-B (shown in figure 1.3B). GRα-A is the 777 amino acid, 94kDa, GR product translated from the first initiator AUG codon. GR-B is a 751 amino acid, 91 kDa species, and is translated from a downstream AUG codon, met 27 in humans or met 28 in rodents. Mutation of either of the ATG codons in humans and rodents resulted in absence of GRα-B synthesis. Both GRα-A and GRα-B are consistently observed in western blots and are thought to be translated at approximately an equal level, however the GRα-B species appears to be more susceptible to degradation. Interestingly, the shorter hGRα-B is up to two times more effective as hGRα-A in activating transcription from a GRE, however the reason for this remains to be determined (Yudt and Cidlowski 2001), as does the tissue distribution of the various isoforms, although the GRα-B isoform has been seen in high levels in T-cells and this increased level correlated with increased activity from the GR1A promoter (Pedersen, et al. 2004).

Of the nine exons of the GR gene Exon 1 is untranslated, but does contribute to the many transcript isoforms. Exon 2 encodes the first 395 amino terminal residues (Encio and Detera-Wadleigh 1991) some of which correspond to the constitutive Activation Function (AF) 1 transcriptional activation domain (Zhou and Cidlowski 2005) (Figure 1.3C). Exons 3 and 4 each encode a zinc finger of the DNA Binding Domain (Encio and Detera-Wadleigh 1991), composed of two repeated units each containing a Cys-Lys-Arg rich sequence which are essential for zinc coordination (Giguere et al. 1986). Exon 5 also encodes residues involved in transactivation (Encio and Detera-Wadleigh 1991) and acts as a hinge region linking the steroid and DNA binding domains (Giguere et al. 1986). Five exons; the 3' end of exon 5, exons 6, 7 and 8, and the 5' end of exon 9α encode the steroid binding domain at the carboxyl terminal end of the receptor (Encio and Detera-Wadleigh 1991) in addition to the ligand dependent AF2 (Zhou and Cidlowski 2005).

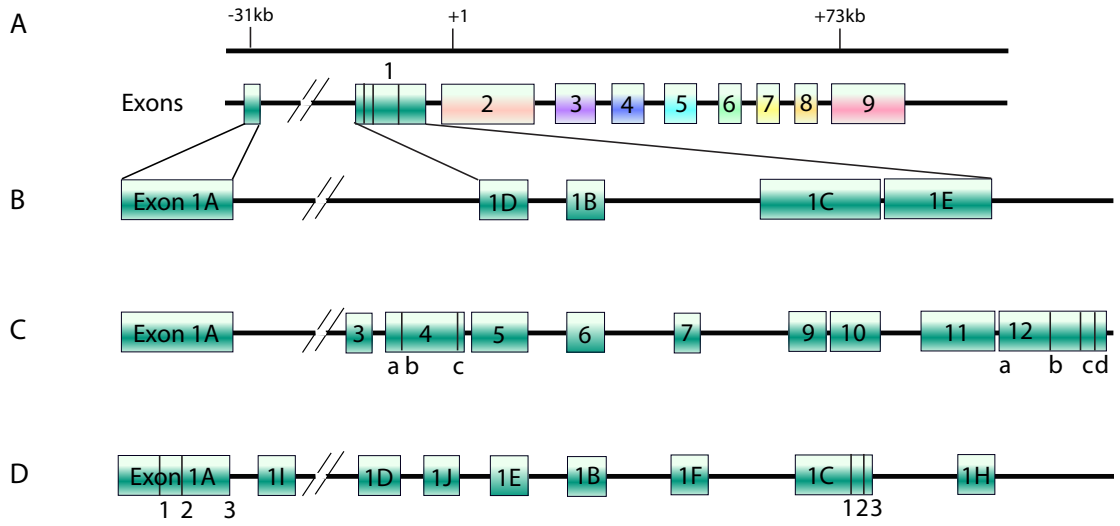


Figure 1.4 Mouse and human GR exon 1 transcript isoforms

A: The genomic structure of the mouse GR (mGR) gene showing exons 1-9. The exons are aligned to their relative genomic location, with the transcription start site set to "+1". The distal exon 1A sits 31kb downstream from the TSS, while exon 9 begins 73kb upstream of the TSS. B: Alternate splicing of the 5 exons 1 to a common acceptor site within exon 2 as understood until 2011. C: updated exon 1 structure based on data from Bockmuhl et al., 2011. Bockmuhl et al., renumbered the exons to be consistent with rat nomenclature. Exons 4 and 12 were found to have internal splice sites thus generating exons Exons 4a, 4b, and 4c as well as 12 a, 12b, 12c and 12d respectively (Bockmuhl et al., 2011). D: Alternative human exons 1 aligned to their mouse homologues. In humans the nomenclature is that the exons are alphabetised, and internal splicing is indicated by numbers. Thus for exon 1A there are three isoforms; 1A1, 1A2 and 1A3 and for exon 1C there are also three; 1C1, 1C2 and 1C3.

Adapted from Bockmuhl et al. 2011.

1.3.3: Multiple promoters of the GR gene and alternate splicing of exon 1

Early analysis of the genomic structure of the GR gene revealed that the promoter region of the 5' UTR lacks either a TATA box or a CAAT box, however an abundance of GC boxes, the consensus binding site for the transcription factor, SP1 was observed in the human form (Encio and Detera-Wadleigh 1991), as well as Yin-Yang 1 and GR itself (Breslin et al. 2001; Breslin and Vedeckis 1998). Analysis of the promoter region has indicated that multiple promoters exist for the human GR gene (hGR) (Breslin et al. 2001; Turner and Muller 2005), consistent with findings up to 2011 that five promoters direct expression of the mouse GR (mGR) gene with each promoter differing in its cell specificity (Chen et al. 1999b; Strahle et al. 1992).

In mice these five promoters have been named 1A, 1B, 1C, 1D and 1E and each drive expression of a transcript containing a corresponding exon 1 (Figure 1.4). In April 2011 Bockmuhl et al. demonstrated splice sites and transcription start sites for 10 exons, although individual promoter regions for these exons were not established (Bockmuhl et al. 2011). The authors renamed the exons from 1-12 in keeping with nomenclature of rat exon 1s. The alignment of these with the traditional exons 1A – E is seen in Figure 1.4. As this new nomenclature has not been adopted elsewhere, the old naming system is maintained in this thesis. Regardless of name, these exons 1 are spliced alternatively into the common exon 2, whose splice acceptor site lies 12nt upstream of the initiation codon. Thus exons 1 are noncoding (Chen et al. 1999b; Strahle et al. 1992). In mice exon 1A is located much further upstream from the other exons, approximately 32kb upstream of exon 1D in the distal region of the 5' untranslated region (5'UTR)(Strahle et al. 1992).

In humans the 5'untranslated promoter region of the GR gene has also been characterised and it has been found that at least thirteen hGR transcripts are expressed from nine separate exons 1 (Figure 1.4) (Turner and Muller 2005). In addition to exons 1B and 1C, which are homologous to the mouse exons 1B

and 1C (Breslin et al. 2001), exons 1D, 1E, 1F, 1H, 1I and 1J have been identified, with another, 1G hypothesised (Turner and Muller 2005). Exons 1B through H have known homologues of their exon 1 counterparts in the rat GR gene (Turner and Muller 2005). Alignment of the exon splice sites has predicted potential rat homologues for exons 1I and 1J, a potential mouse homologue for exon 1J, as well as predicting the presence of many more promoters in the distal and proximal regions of the human and rodent GR gene, however these remain to be experimentally confirmed (Turner and Muller 2005). As in mice, the human exon 1A is the furthest upstream of the initiation codon in exon 2, located in the distal promoter region, approximately 31kbp upstream of the translation start site (Turner and Muller 2005) and contains three separate alternative splice sites that give rise to three GR transcripts containing exons 1A1, 1A2 or 1A3 (Breslin et al. 2001). Exon 1C has three internal splice donor sites and thus there are at least three splice variants, named 1C1, 1C2 and 1C3. This gives thirteen hGR transcripts; 1A1, 1A2, 1A3, 1B, 1C1, 1C2, 1C3, 1D, 1E, 1F, 1H, 1I and 1J. All the proximally located exons (from 1B – 1F, 1-H and 1J) are localised to a CpG island, with 1I being located upstream of this island along with exons 1A, in the far upstream distal region. All individual exons have a unique exon-specific splice donor site and are all spliced to a common exon 2 splice acceptor site (Turner and Muller 2005). An in frame stop codon is located three codons
upstream of the initiator codon in exon 2, thus, as in mouse GR, transcripts from exon 1 are not translated into the hGR protein (Breslin et al. 2001).

Expression of all the exon 1s has been observed in human and mouse tissue and follows a highly specific expression pattern. For example, expression from exons 1E and 1F is biased toward immune cells, whereas 1D is found only in hippocampal cells. Exon 1I has been observed in T-lymphocytes of humans and exon 1A has been observed in T-lymphocytes and the brain of humans and mice (Breslin et al. 2001; Purton et al. 2004; Strahle et al. 1992). It is believed that each exon 1 has its own individual promoter region, as observed in mice, which

may explain tissue specific expression of the individual GR isoforms (Turner and Muller 2005). DNase I Footprint analysis has shown that there are in fact sequences resembling a TATA box and a CAAT box within FP4 and FP2 respectively which are localised within the 1A promoter of the hGR (Breslin et al. 2001). In addition examination of the exon 1 region has revealed the presence of a TATA box upstream of exon 1E. With the exception of these two exons, none of the putative proximal promoter regions were found to have TATA or TATA like boxes, consistent with earlier findings for 1B and C (Breslin and Vedeckis 1998). They do, however, contain Sp1 transcription factor binding sites which are generally associated with housekeeping genes, although, the presence of so many exon 1s with individual promoter regions suggests that regulation of the hGR is much more complicated (Turner and Muller 2005). It is thought that the difference in tissue and cell specific patterns for each of the transcripts is in part due to the ability of unique combinations of transcription factors to interact with individual promoter regions, thus establishing these complex expression patterns (Chen et al. 1999a).

Many studies have been performed in mice and humans, with transcripts initiating from the 1A promoter observed in T-lymphocyte cells, spleen, the cortex of the brain from mice, a membrane GR+ pituitary tumour cell line and recently the hippocampus, hypothalamus and pituitary (Bockmuhl et al. 2011; Chen et al. 1999b; Purton et al. 2004; Strahle et al. 1992). In cells and tissues of human origin, expression from the 1A promoter and its splice variants have been observed and quantified. In cancer cells of hematopoietic origin e.g. CEM-C7 and IM-9 cells, the 1A3 variant in particular is expressed (Breslin et al. 2001; Presul et al. 2007; Russcher et al. 2007). Variants 1A1 and 1A2 were low or not detected in cancer cells of hematopoietic origin, however they were observed in various other cancer cell lines, such as MCF-C7 (breast carcinoma) and H1299 (Lung carcinoma), cells in which the 1A3 variant was not observed. Expression of the exon 1A3 containing transcripts was also detected in human adult brain tissue and the adrenal gland (Breslin et al. 2001; Presul et al. 2007).

1.3.4: Involvement of the GR1A promoter in autoregulation of GR gene activity

1.3.4.1: Sensitivity to GICD

As mentioned previously, expression of the GR from the 1A promoter is highly tissue specific. The response of the GR1A promoter to hormone treatment also varies between different cell types. In most cells the response to GC treatment is the down regulation of GR mRNA and protein levels and a decrease in the steroid binding capability, however in T-lymphocytes, GR mRNA and protein are up-regulated and apoptosis can occur (Burnstein et al. 1991). It has been shown that a certain threshold of intracellular GR must be achieved for GC-induced cell death to occur, with the increase in GR thought to amplify the hormonal signal in cells destined to undergo apoptosis (Ramdas et al. 1999). In Purton's (Purton et al. 2004) study the relative level of expression of the 1A exon to the 1B-1E exons within a lymphocyte population was found to directly correlate with susceptibility to GC-induced cell death. They proposed that increased activity of the GR gene 1A promoter may serve to make thymocytes more sensitive to increased levels of GC. A study by Pedersen et al. (Pedersen et al. 2004) has shown that for all types of GR transcripts (containing any of the exons 1) GC regulation occurs primarily at the level of transcription and that the relative concentration of transcripts reflects the relative transcription rate from the corresponding promoters. Their study revealed that there is no difference in the stability of transcripts containing the various exons 1 and there is no significant difference between translation rates of all GR transcripts (Pedersen et al. 2004). Additionally, in dexamethasone (dex) treated human CEM-C7 T-lymphoblast cells, which contain relatively high levels of GR1A3 containing transcripts, the GC treatment does not decrease GR protein stability as does occur in some cells such as human IM-9 B-lymphoblast cells. In human Eb.2 B-lymphoblast cells transfected with a plasmid containing exon 1A3 upstream of the complete GR encoding sequence, the ratio of GRα-B protein to GRα-A protein is higher than observed in Eb.2 cells transfected with plasmids

containing GR exons 1B or 1C (Pedersen et al. 2004). GR-B protein is known to have a higher transactivation potential than GR-A protein.

The physiological effect of having a high GRα-B:GRα-A ratio appears to fine tune the transactivation potential of GCs (Pedersen et al. 2004). Pedersen et al. have proposed that three factors contribute to the increased sensitivity of GCs and thus result in a strong apoptotic response (Pedersen et al. 2004). These factors are: Increased levels of GR mRNA, increased expression of GR-B via an increase in exon 1A3 containing transcripts and no decrease in the stability of GR protein in the presence of GCs.

1.3.4.2: A Molecular Switch Model

In experiments performed in 2001, GR transcripts containing exon 1A3 were down-regulated in human IM-9 B-lymphoblast cells in response to treatment with the synthetic GC, Dexamethasone (Dex). In contrast, treatment of human CEM-C7 T-lymphoblastic cells with Dex resulted in an increase in the level of transcripts containing 1A3 in CEM-C7 cells (Breslin et al. 2001). The CEM-C7 cell line is derived from a T-lymphocyte ALL patient and is an established line for studying GC induced apoptosis in T-lymphocytes. It is known that in T-lymphocytes hGR mRNA and protein are up-regulated in response to GC hormone treatment, and this is followed by apoptosis. The results of Breslin's experiments suggest that the hGR1A promoter and exon region contain sequences necessary for the cell type specific regulation of GR in T-lymphocytes (Breslin et al. 2001).

Footprint analysis of the hGR1A promoter region in T-lymphocytes has revealed binding sites for transcription factors that are able to modulate activity of the GR1A promoter. These include binding sites for NFKB, IRF and four sites for GR binding at foot prints 6, 7, 8 and 11(Breslin et al. 2001), in addition to binding sites for C-MYB and C-ETS. The binding sites for C-MYB and C-ETS actually overlap and are present in FP12. FP12 is adjacent to FP11, which as mentioned, is able to bind GR. It was determined that either C-MYB or C-ETS can be bound

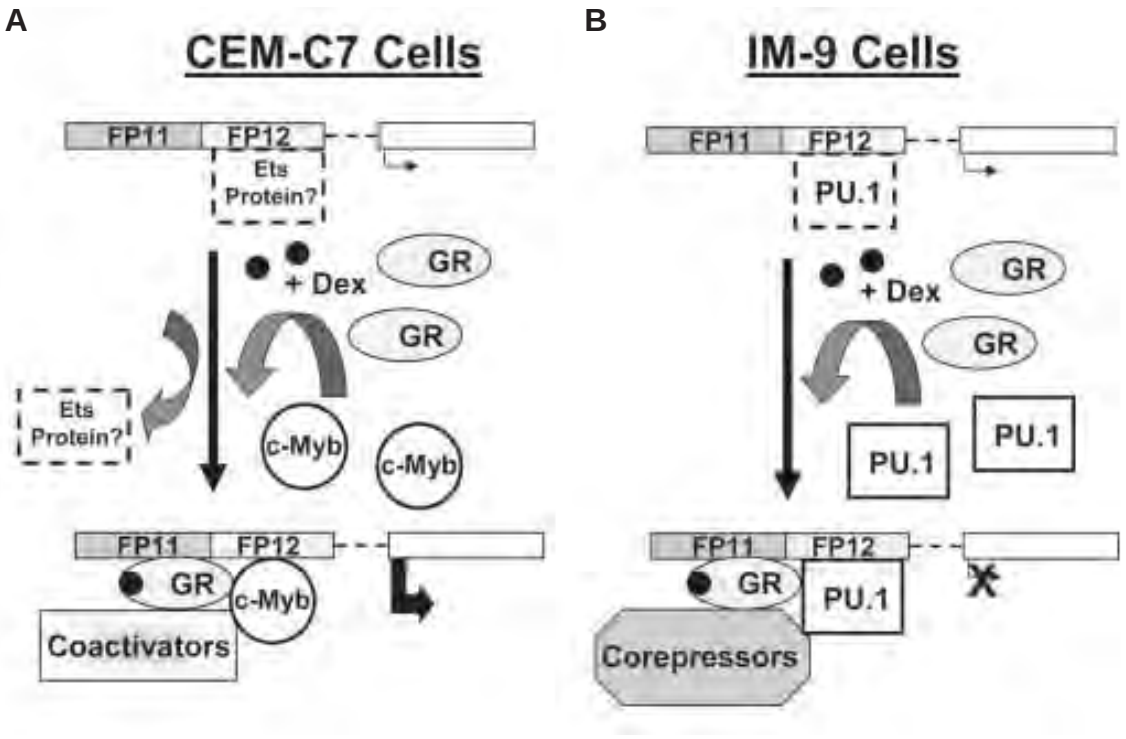


Fig 1.5 The "Molecular Switch" model

A: In CEM-C7 T-lymphoblast cells, in the presence of hormone (such as Dex, represented by black circles), c-Myb is recruited to FP12, presumably with recruitment of unknown coactivators, resulting in hGR1A upregulation and apoptosis. B: In IM-9 B-lymphoblast cells, which are not sensitive to hormone mediated apoptosis, in the presence of hormone, PU.I is recruited to FP12 and hGR1A is down regulated in these cells.

Reproduced from Geng and Vedeckis 2005 with permission.

to the DNA at FP12 while GR is present and proposed that the direction of GR gene regulation by the binding of GR at FP11 was determined by which transcription factor, C-MYB or C-ETS, was simultaneously bound to FP12. It was also suggested that the presence of either of these transcription factors may be cell specific and the C-MYB and C-ETS family members may antagonize each other at FP12 to oppositely regulate the response of the hGR1A promoter to hormone in different cell types (Geng and Vedeckis 2004). The “Molecular Switch” model, whereby GR binding to the hGR 1A promoter recruits cell type specific transcription factors to an adjacent DNA sequence, was subsequently confirmed in 2005. It was shown that in the presence of glucocorticoid hormone, C-MYB is recruited to FP12 in T-lymphoblasts (such as Jurkat and CEM-C7 cells) resulting in hGR1A up-regulation and apoptosis. PU.I, a protein of the C-ETS family is recruited to FP12 in IM-9 B cells, cells that do not undergo hormone-mediated apoptosis, and hGR1A is down-regulated in these cells (Geng and Vedeckis 2005) (see figure 1.5). Overexpression of C-Myb in IM-9 cells confers hormone dependent up-regulation to the hGR1A promoter, whereas when members of the C-Ets family were overexpressed in Jurkat T-cells dex mediated up-regulation of the hGR1A promoter was repressed (Geng and Vedeckis 2005). They determined that, in Jurkat T-cells at least, FP11 and FP12 are the critical sequences required for hormone induced up regulation of the hGR1A promoter (Breslin et al. 2001).

Similarly to the up-regulation of 1A3 transcripts in the CEM-C7 cells in response to Dex treatment, up-regulation of transcripts initiating from the 1I promoter in these cells in response to Dex treatment has also recently been observed. It has been suggested that induction of distally located exons (not just exon 1A) may be a mechanism that induces apoptosis in T-cells (Presul et al. 2007). A 250bp sequence located between 1A and 1I is highly conserved between man and rodents (~75%) and contains conserved binding sites for a number of transcription factors including Nfil3 and a predicted GRE (Presul et al. 2007). This further supports a regulatory role for the distal promoter region of the GR gene in

T-cells, as it has previously been shown that NFIL3 is a transactivator of IL-3 in a gibbon T-cell line, but acts as a transcriptional repressor in many other cell lines and is strongly induced by GC in GC sensitive CEM-C7 cells where it is correlated with GICD (Cowell and Hurst 1994; Cowell, et al. 1992; Zhang, et al. 1995; Medh, et al. 2003; Priceman, et al. 2006). Thus this region is a strong candidate for autoregulation by GR and NFIL3.

1.4: Glucocorticoid Induced Cell Death

1.4.1: Gene targets for GICD in murine T-lymphocytes

Specific target genes of activated GR in GICD have not been fully characterised, particularly *in vivo*. Co-application of inhibitors of mRNA or protein synthesis with dex will in fact block apoptosis thus it is clear that the transcriptional actions of GR are important for GICD (Distelhorst 2002; Lepine, et al. 2005; Wang, et al. 2006). The exact subset of genes which are in fact acted upon directly by GR to initiate cell death or to block cell survival is as yet unknown.

Many microarray studies have been performed on *in vitro* treated samples, these studies are mostly focussed on clinical applications of using GCs to treat leukaemias and thus are mostly in ALL T-cell lines, pinpointing genes which have elevated levels of expression at 24 hrs, which is when human cells and some mouse cell lines (WEHI and S49) cultured with dex will go into apoptosis (Wang, et al. 2003). Few microarray studies have been performed on thymus or primary thymocytes. Those that have been performed are on DP T cells following 3hr dex (Bianchini, et al. 2006), total thymocytes following 1.5hr dex treatment (Miller, et al. 2007) and total thymocytes following 4hr dex treatment. The comprehensive study performed by Miller et al., in 2007 (Miller et al. 2007) demonstrated that genes conferring sensitivity to GICD are not the same in malignant cells as they are in primary thymocytes. They determined the time point at which the early apoptosis marker phosphatidyl serine first appeared in response to Dex treatment in various cells lines and in primary thymocytes, and using microarray analysis

Table 1: Summary of GR target genes linked to GICD in mouse thymocytes identified by microarray analysis.

GENE	↑ or ↓	MODEL	PRO or ANTI	REF
AML1	↓	DP thymocytes	PRO	Bianchini et. al., 2006
A20	↑	DP thymocytes	PRO	Bianchini et. al., 2006
Bcl2l11 (Bim)	↑	KO mice Thymocytes DP thymocytes	PRO	Bouillet et., 1999 Erlacher et. al., 2005 Bianchini et. al., 2006 Miller et. al., 2007 Woodward et. al., 2010
Bcl-xL *Bcl-xγ* *Bcl-xΔTM* *Bcl-xβ*	↓ ↓ ↓ ↑	DP thymocytes	ANTI ANTI ANTI PRO	Bianchini et. al., 2006
CDC6	↑	Thymocytes		Miller et. al., 2007
DDIT4	↑	Thymocytes	ANTI	Wang et. al., 2003 Bianchini et. al., 2006 Miller et. al., 2007 Woodward et. al., 2010
Ets2	↓	DP thymocytes	PRO	Bianchini et. al., 2006
E4BP4 (Nfil3)	↑	Thymocytes	PRO	Woodward et. al., 2010
Foxo3A	↑	Lymphoctes, siRNA KO	PRO	Ma et. al., 2008
FKBP5	↑	Thymocytes	ANTI	Bianchini et. al., 2006 Miller et. al., 2007 Woodward et. al., 2010
Id3	↓	DP thymocytes	ANTI	Bianchini et. al., 2006
IL-7	↑	DP thymocytes	ANTI	Bianchini et. al., 2006
NFKB1A	↑	Thymocytes	ANTI	Miller et al., 2007
Notch	↓	DP thymocytes	ANTI	Bianchini et. al., 2006
Sgpp1	↑	DP thymocytes	PRO	Bianchini et. al., 2006
Socs1	↓	DP thymocytes	ANTI	Bianchini et. al., 2006
Tis11d	↑	DP thymocytes	PRO	Bianchini et. al., 2006
TXNIP	↑	Thymocytes	ANTI	Wang et. al., 2006 Bianchini et. al., 2006 Miller et. al., 2007
Zbtb16	↑	DP thymocytes	ANTI	Bianchini et. al., 2006

determined genes regulated by GCs at that particular time point. From the microarray data, only 8 genes were in consensus between the cell lines examined and the primary thymocytes, which may emphasise the importance of these particular genes, which included *BCl2l11 (Bim), Cdc6, Ddit4, Fkbp5, Nfkb1A* and *Txnip* (see Table 1 for more details). Bianchini's study (Bianchini et al. 2006) revealed a large number of regulated genes which may potentially be involved in many aspects of apoptosis, including Bcl-2 family members, Redox regulation genes, genes involved in mRNA and protein stability genes whose normal activity and GC regulation would contribute to pro- or anti-apoptotic effects of GCs. Some genes identified in their study are included in Table 1. In particular they identified some genes in consensus with Miller et al., such as *Bim, Ddit4, Fkbp5* and *Txnip* and some more novel targets such as *Notch, Tnfaip3* and *Id3*. More recent data have shown support for some of these targets; *Notch* signaling was shown to be down-regulated in DP thymocytes, supporting data from experiments in murine DP thymoma 16610D9 cells suggesting that NOTCH1 decreases the GC sensitivity of thymocytes by repressing the SRG3 expression via DELTEX1 (Jang, et al. 2006). Retroviral knock down of *Tnfaip3* in FTOC protected cells from GICD (Woodward, et al. 2010). In addition to *Tnfaip3*, Woodwards study also detected some genes in consensus to Millers; *Ddit4, Bim* and *Fkb51*. Additionally it detected *E4bp4* (*Nfil3*), whose over expression in FTOC resulted in decreased DP T cells indicating a role in thymocyte apoptosis, and an earlier study implicated GC-mediated *Nfil3* up-regulation as playing a potential role in GICD (Priceman et al. 2006). For a more thorough list of GC regulated genes in thymocytes see Table 1, and supplemental data from Bianchini et al., 2006(Bianchini et al. 2006).

1.4.2: Targeting apoptotic pathways

Many mouse studies investigating potential GICD targets have focussed on genes established to be of importance in the apoptotic pathways. Some of these studies have offered conflicting evidence. Mouse models provide evidence

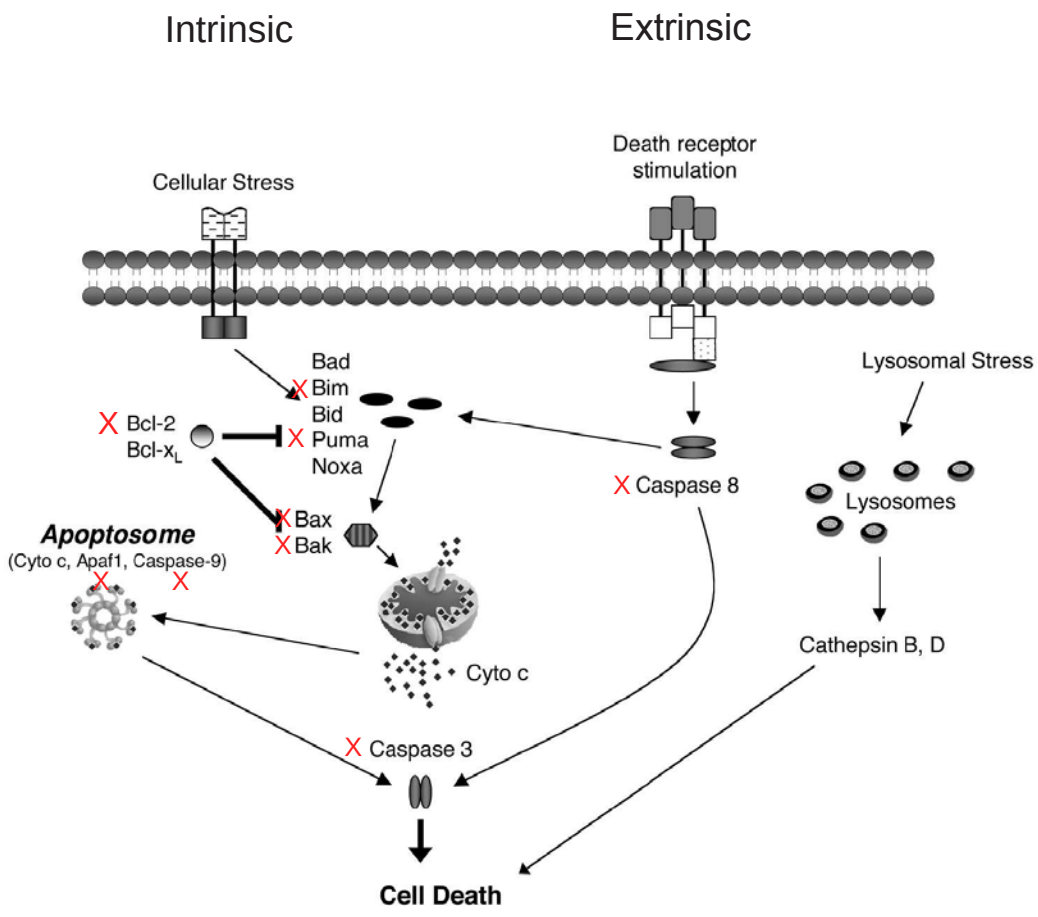


Figure 1.6 Apoptotic pathways

General pathways of apoptosis. The intrinsic pathway involves the formation of the apoptosome and activation of caspase-3. The extrinsic pathway occurs following death receptor stimulation and involves caspase-8. Factors from both pathways have been shown to be regulated by GCs during GICD however no single factor is responsible for GICD. Red crosses indicate genes which have been knocked down in the study of GICD.
Reproduced from Herold, et al. 2006

that GICD involves activation of the intrinsic pathway (See Figure 1.6 for a summary of apoptosis). For example knockdown of apoptotic inhibitor *Bcl-2* results in increased apoptosis (Veis, et al. 1993) and overexpression effectively protected *in vivo* treated thymocytes from GICD (Erlacher, et al. 2005b). Additionally *Bax*$^{-/-}$ *Bak*$^{-/-}$ thymocytes (from mice reconstituted with *Bax*$^{-/-}$ *Bak*$^{-/-}$ hematopoietic cells) are resistant to GICD (Rathmell, et al. 2002). Knockdown of *Bim* or *Puma* provides partial protection from GICD in *in vitro* or *in vivo* treated thymocytes (Bouillet, et al. 1999; Erlacher et al. 2005b; Villunger, et al. 2003; Jeffers, et al. 2003) as do CASPASE-9 and APAF-1 (Hakem, et al. 1998; Kuida, et al. 1998; Yoshida, et al. 1998). Knock down of *Caspase-3* and *-8* (limited to T-cell lineage) in mice does not affect GICD of *in vitro* treated thymocytes (Kuida, et al. 1996; Salmena, et al. 2003), however inhibitors of CASPASE-3 and -8 provided partial protection from GICD in thymocytes (but not in peripheral splenic T-cells) (Wang et al. 2006). The difference observed may be due to the lethality of *Caspase-3* deletion (only 7% survival to birth, and mice die within 1-2 weeks) or may lie in the specificity of the inhibitors of CASPASE-3 and -8 used.

Thus while the evidence from mouse models leans towards the intrinsic pathway, more investigation is required. Clearly *Bim* appears to be a consensus target, however the earlier components signalling to Bim are unknown. Although *Bim* is induced by Dex in murine thymocytes, it is not thought to be a direct GR target; the human *Bim* promoter does not contain a GRE and cyclohexamide blocks induction of *Bim* by Dex (Wang et al. 2003). This in combination with the fact that knockdown of *Bim* only provides partial protection against GICD confirms that other factors are acting in concert and upstream, thus further investigation is required to identify these genes.

1.5: Glucocorticoid actions in the brain in the normal state and during stress

1.5.1: The HPA axis

GC's secreted by the adrenal gland cross the blood brain barrier and exert their effects on receptors located primarily in the hippocampus, cortex and also

the hypothalamus and pituitary gland (components of the Hypothalamic-Pituitary-Adrenal (HPA) axis) where they have a key role in the response to stress. The hippocampus is a region central to neuroendocrine activity, behavioural adaptation and learning thus as acute stressors enhance memory formation this is an important function of GR in the hippocampus (Sapolsky et al. 2000). Two classes of hormone receptors are described in the hippocampus; the MR which is fully occupied and activated by basal levels of corticosteroid, and the GR, which is fully occupied only when corticosteroid levels are elevated, as occurs after acute stress (Lund, et al. 2004). The specific roles of GR and MR in hippocampal function are yet to be fully defined, however a balance in their functions appears essential to functional integrity of the nervous system and rapid stress responses (De Kloet 2004).

Actions mediated by GCs have the ability to alter the electrical properties of neuronal membranes and thus the firing patterns of neurons. Potential targets of GCs and their receptors in the brain include genes coding for proteins that alter membrane potential in neurones, for example voltage gated ion channels, in addition to genes encoding for proteins involved in signalling cascades activated by neurotransmitters, for example G-protein coupled receptors. Another potential target for GCs and their receptors are ion pumps and transporters that maintain the ionic gradients over the cell membrane. However, the specific targets of GR, i.e. genes that contain GREs are largely unknown (Joels 2001).

1.5.2: The stress response

The role of GCs and their receptors in the stress response has been extensively studied, with particular attention to the action of GCs and their receptors in the hypothalamus and the pituitary gland of the HPA axis. As mentioned, GCs are released in response to a stress signal. They are able to activate genes required for energy production in addition to inhibiting transcription of various genes in an effort to conserve energy as well as acting to limit their own production in a negative feedback loop. During stress GCs negatively regulate gene transcription in

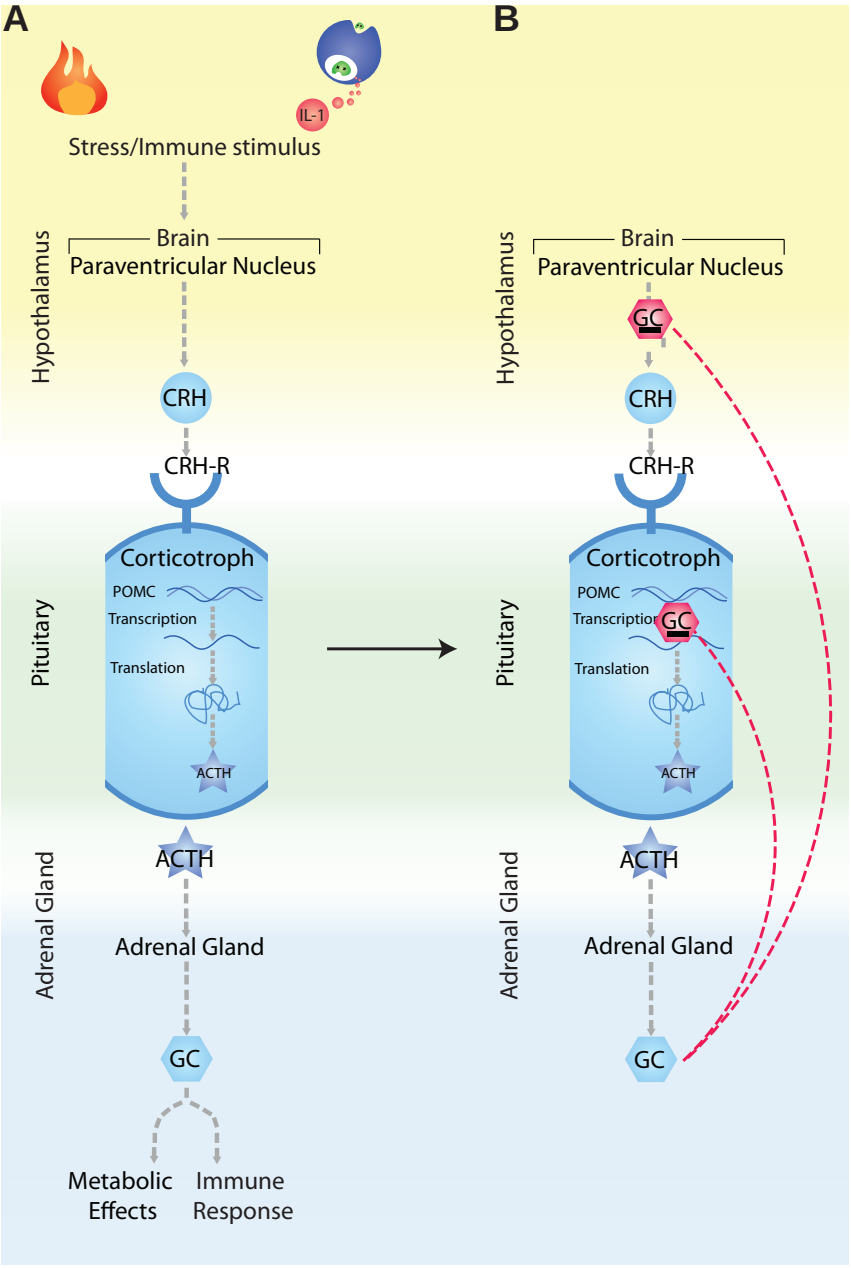


Figure 1.7 The HPA axis

A: In response to a stress signal CRH is released from the paraventricular nucleus of the hypothalamus. It acts on the corticotrophic cells of the anterior pituitary and stimulates the release of ACTH which subsequently acts on the adrenal cortex of the adrenal gland to stimulate synthesis and secretion of glucocorticoidsn (GCs). These have positive effects on metabolism but feedback negatively on the hypothalamus and pituitary as shown in panel (B). This limits their own production and dampens the stress response, enabling a return to the normal state.

CRH: Corticotropin Releasing Hormone. ACTH: Adrenocorticotropin Releasing Hormone.

cells of the HPA axis. Corticotropin Releasing Hormone (CRH) is synthesised and released from the paraventricular nucleus (PVN) of the hypothalamus in response to stress and acts on type I receptors in corticotrophic cells of the anterior pituitary (Smith, et al. 1998; Wong, et al. 1994) (see Figure 1.7).

In humans CRH is able to act synergistically with vasopressin, which acts through type II receptors, to stimulate transcription of the Pro-opiomelanocortin (POMC) gene , whose corresponding protein is the precursor to Adreno-Corticotropin Releasing Hormone (ACTH). ACTH is subsequently released from corticotrophic cells and acts on the adrenal cortex of the adrenal gland to stimulate secretion of GCs (Lamberts, et al. 1984). Investigation of the anterior pituitary in rats has revealed GCs, via the GR, are able to inhibit transcription of the *Pomc* gene in corticotrophic cells during stress via direct binding to DNA (Drouin, et al. 1987; Eberwine and Roberts 1984). In addition, GCs are able to block transcription of the *Crh* gene in a subset of cells of the PVN, the parvocellular paraventricular neurons which project into the median eminence of the hypothalamus, via protein-protein interactions as well as decreasing CRH receptor levels in the anterior pituitary (Hauger, et al. 1987; Lightman and Young 1989; Nicholson, et al. 2004) (see Figure 1.8A and B). These negative feedback mechanisms serve to limit the production of GCs enabling a rapid return to the normal state after a stress event. There are many incidences where deregulated levels of GCs or their receptors within the brain are implicated in disease or abnormal behaviour. For example elevated levels of cortisol is associated with age related dementia (Martignoni, et al. 1992), In addition, mice with increased expression of GR in the forebrain displayed increased emotional lability, which is associated with bi-polar disorder in humans (Wei, et al. 2004). Complete loss of function of the GR in the mouse nervous system, induced after development in a tissue specific manner, resulted in an impaired behavioural response to stress and reduced anxiety (Tronche, et al. 1999). The many fine tuning mechanisms ensuring a rapid return to the normal state after stress appears crucial to a normal stress response.

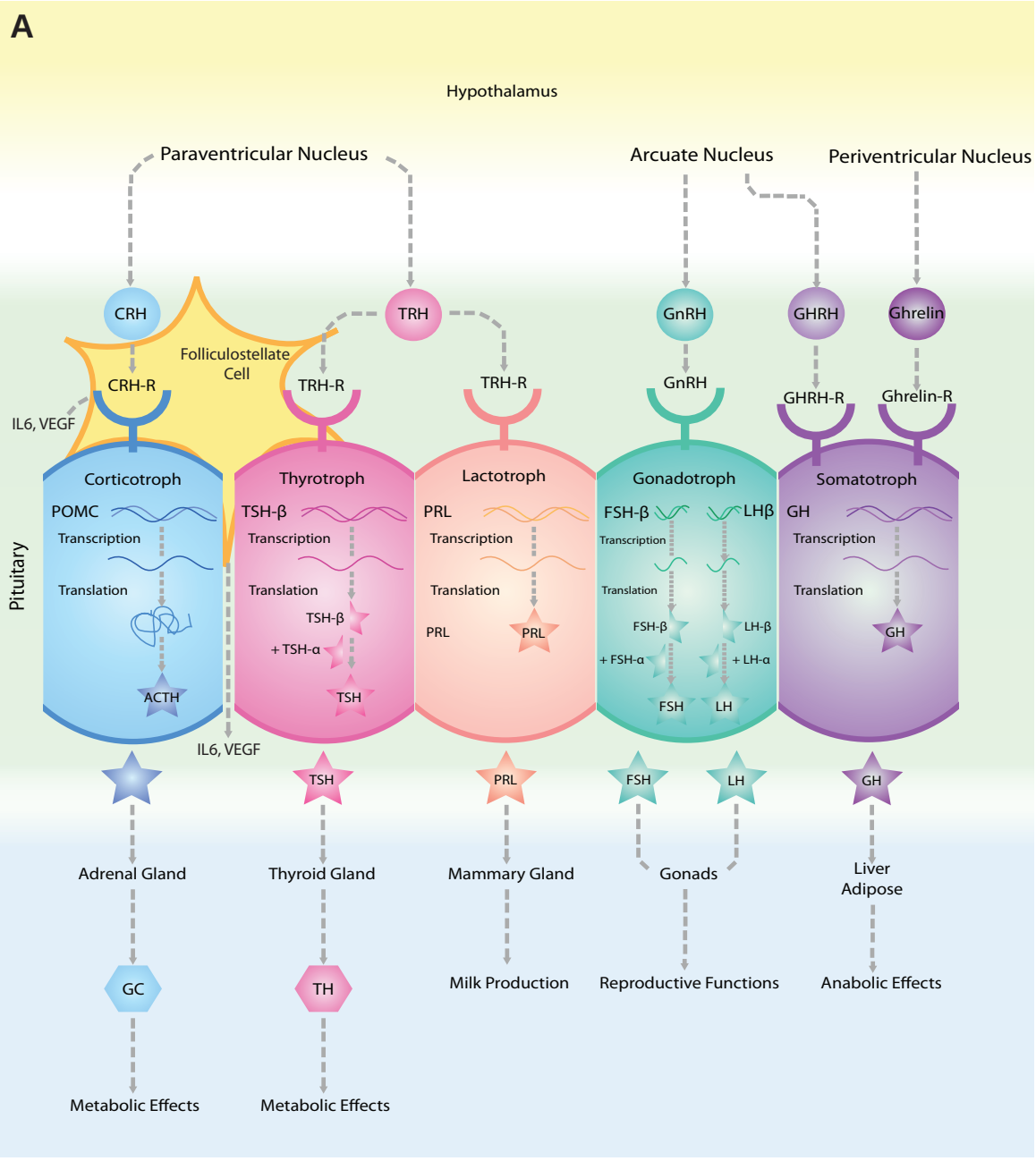
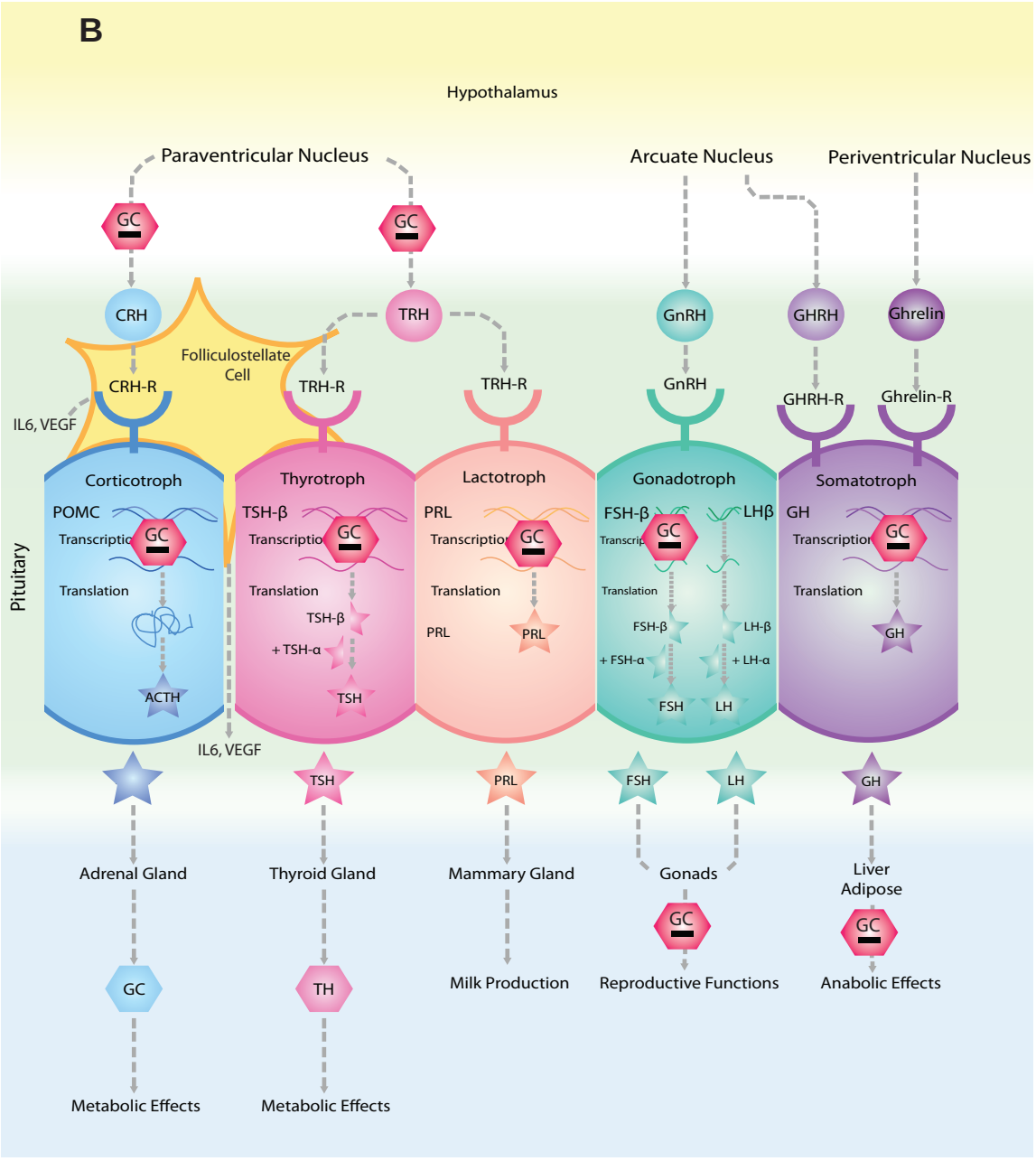


Fig 1.8 Glucocorticoid signalling in the anterior pituitary

A: Hormone production and signalling in the cells of the anterior pituitary in the normal state. In response to hormone signals from the hypothalamus trophic cells of the anterior pituitary secrete hormones with effects on metabolism, reproduction and growth.



B: Negative Feedback of GCs on hormone production in the hypothalamus and anterior pituitary during stress. Importantly, GCs feedback to all cell types of the anterior pituitary limiting production of pituitary hormones and thus limiting metabolism, reproduction and growth GC: Glucocorticoid; POMC: Pro-opiiomelanocortin; CRH: Corticotropin Releasing Hormone; GnRH: Gonadotropin RH; TRH: Thyrotropin RH; GHRH: Growth Hormone RH; ACTH: Adrenocorticotropin Hormone; TSH: Thyroid Stimulating Hormone; PRL: Prolactin; FSH: Follicle Stimulating Hormone; GH: Growth Hormone; LH: Leuteinizing Hormone.

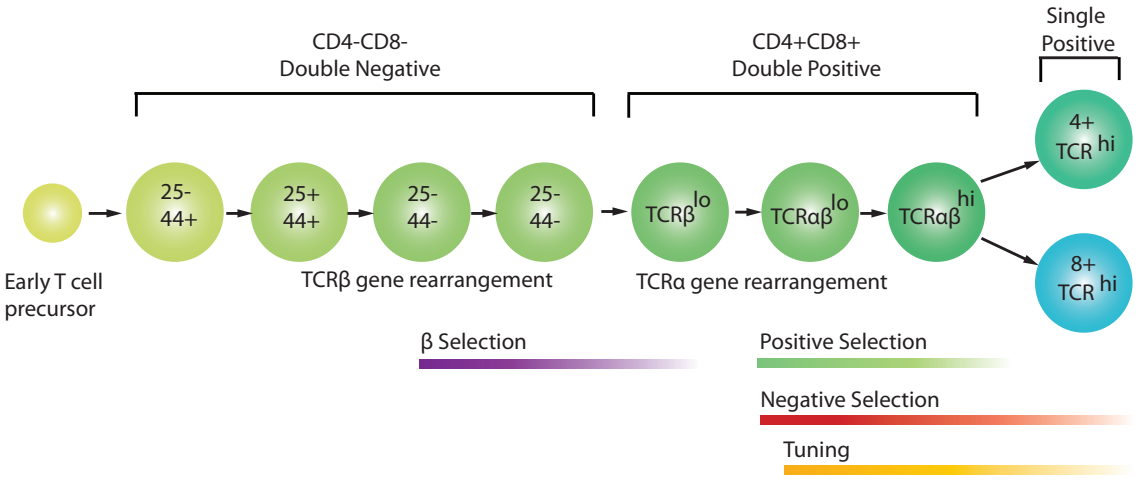


Figure 1.9 T-cell development in the thymus

Early Lymphoid Progenitors (ELPs) enter the thymus and proliferate and differentiate into Early T-cell Precursors (ETPs) upon receiving Notch and cytokine signals, with concomitant upregulation of CD25 on the surface of developing thymocytes. TCRβ chain genes are rearranged and assembled leading to the expression of a TCRβ chain as a pre-TCR. This leads to signals that cause cellular expansion, acquisition of CD4 and CD8 co-receptors and cells undergo *TCRα* gene rearrangement. At this point DP cells are subjected to interaction with peptides presented by MHC molecules and subject positive or negative selection and death by neglect depending on their interactions.

Adapted from Sebzda. et al. 1999

1.6 Glucocorticoids in T-cell Development

1.6.1: T-cell Development and Selection

The process of generating lymphocytes begins with pluripotent Hematopoietic Stem Cells (HSC) in the bone marrow. Upon proliferation, some HSCs differentiate into multipotent Hematopoietic Stem Cells which retain the ability to become Common Myeloid Progenitors or Common Lymphoid Progenitors (CLPs). It is believed that pluri- and multipotent HSCs as well as CLPs have the potential to migrate from the bone marrow, via the blood and enter the thymus at the corticomedullary junction as Early Lymphoid Progenitors (ELPs). Here they receive NOTCH and cytokine signals that result in their proliferation and differentiation into the early T-lineage progenitor (ETP) population, with concomitant up-regulation of CD25 on the surface of developing thymocytes and migration through the thymic cortex toward the capsule. Which of the progenitors settle in the thymus and generate ETPs is unknown (reviewed in (Schwarz and Bhandoola 2006)).

From this stage ETPs undergo a process known as “T-cell selection” to generate mature fully functional, self tolerant T-lymphocytes. The general process is summarized in Figure 1.9.

ETPs are described as being double negative (DN) with respect to CD4 and CD8 co-receptors and during this stage cells must produce an appropriate T-cell receptor (TCR) which is responsible for recognizing antigens displayed by Major Histocompatibility (MHC) molecules. TCRs consist of an α and β subunit which form a dimer (or they may consist of a γ and δ subunit) and each subunit is composed of a constant (C) and variable (V) region. The antigen recognition site of the TCR is formed by the variable regions of the two different subunits. Whereas the exons encoding the constant region are static during development, those encoding the variable region are assembled from variable (V); joining (J); and, in some cases, diversity (D) gene exonic segments. During the DN stage the TCRβ chain genes are rearranged and assembled leading to the expression of a TCRβ chain as a pre-TCR.

This leads to signals that cause cellular expansion, acquisition of CD4 and CD8 co-receptors (Double Positive "DP" stage) and cells will undergo *TCR α* gene rearrangement. For those cells that generate a functional α chain, the pre-TCR is replaced with low levels of the mature αβ TCR (reviewed in (Ashwell, et al. 2000; Sleckman 2005)). The gene rearrangements of the variable region of the TCR mean each T-cell will express a TCR with a unique specificity and that a T-cell repetoire with the ability to recognize a wide variety of antigens is present. However it is important that the TCR responds appropriately, thus at this point DP cells are subjected to positive or negative selection and death by neglect. Thymocytes expressing TCRs that do not recognize self antigens or MHC presented antigens will undergo "death by neglect". Thymocytes that bind strongly to self antigen will undergo activation induced cell death (AICD) or negative selection. Thymocytes bearing TCRs with intermediate avidity for self antigen/MHC are rescued from the default death pathway by positive selection and differentiate into CD4 or CD8 Single Positive (SP) cells and migrate to the periphery. TCRs can be considered defined as MHC class I or II restricted, and in general MHC class I restricted receptors are positively selected to the CD8 lineage, while T cells expressing class II–restricted TCRs are selected to the CD4 lineage (Reviewed in (Ashwell et al. 2000; Sebzda, et al. 1999)).

1.6.2: The Mutual Antagonism Model

There are many models proposed for the process and the molecular mechanisms involved in selection and a consensus has not been completely defined. The involvement of GCs and the GR has been suggested and will be discussed.

As described GCs are known for their lymphocytolytic properties; early on, adrenalectomy was recognised to cause hypertrophy in the thymus, additionally it was noted that administration of ACTH to mice caused a marked reduction in thymus and lymph node mass, and that a purified corticosteroid caused the regression of a lymphosarcoma (reviewed in (Ashwell et al. 2000)). The relative

sensitivity to GICD decreases with developmental maturity of a T-lymphocyte, i.e. DP cells are most sensitive and further differentiated single positive cells are least sensitive, as described this was correlated to the level of GR1A transcripts (Purton, et al. 2002). There is an association between TCR expression, maturity and sensitivity to GICD, less mature DP TCR ^{low} cells were least able to be rescued by TCR stimulation, whereas most SP cells were able to be rescued by TCR stimulation (above 5μg/mL anti-CD3). Peripheral splenic T-cells were also able to be rescued with TCR stimulation, at lower concentrations to that observed in thymocytes (Erlacher, et al. 2005a).

This sensitivity of immature T-cells to GICD is thought to be involved with T-cell selection. In 1990 in studies of T-cell hybridoma cultures, using specific antigens to simulate AICD of T-cells, they discovered that application of either dex or antigen alone induced apoptosis whereas co-application of GCs and antigen did not induce death. This was the birth of the “mutual antagonism” model which postulated that if endogenous glucocorticoids caused thymocyte programmed cell death only those thymocytes that were activated by self antigen would be permitted to survive (positive selection). Too much avidity for self molecules, and thus too strong an activation signal, would overcome the antagonism provided by the GCs, resulting in activation-induced cell death (negative selection) (Zacharchuk, et al. 1990). While the molecular mechanisms have not been fully elucidated, it is though largely to occur via Fas Ligand (FasL), which is known to be repressed by GCs and whose upregulation causes activation induced apoptosis in T-lymphocytes (reviewed in (Ashwell et al. 2000).

1.6.3: Evidence against Mutual Antagonism

Much of the support for the mutual antagonism models comes from *in vitro* thymocyte culture studies using metyrapone; an inhibitor of GC synthesis. In fetal thymus organ culture (FTOC) treated with metyrapone there was a decrease in the proportion of DP cells in the presence of TCR stimulation (Vacchio, et al.

1994) and also in the absence of TCR stimulation (King, et al. 1995) supporting the mutual antagonism hypothesis. It has been since shown that metyrapone caused a similar and significant reduction in DP cell numbers from $GR^{+/+}$, $GR^{+/-}$ and $GR^{-/-}$ mice, demonstrating that this reagent can have inhibitory effects on T cell development that act independently of GR signaling (Purton et al. 2002).

Various models of GR deficient mice have been used and these have given variable results. In one model Antisense GR transcripts under the control of the LCK promoter were used to generate “TKO” mice which have GR protein levels reduced to 43% in T-lymphocytes only. In these mice total thymocyte numbers were decreased to 10% of WT mice, mostly due to the decrease in DP cell numbers, $CD4^{+}CD8^{-}$ cells were also decreased, while the proportion of DN cells was increased. The decrease was thought to be due to a combination of elevated apoptosis and a block in progression between the DN and DP stage which would decrease cell proliferation. Another feature of DP T-lymphocytes in TKO mice was that they were incredibly sensitive to TCR activation induced apoptosis (King et al. 1995). In contrast, another model used the same transgene construct under the control of a human neurofilament promoter which was expressed in cortex, hypothalamus, heart, kidney, adrenal glands and GR levels were decreased in anterior pituitary, hippocampus, liver, thymus and spleen and caused high circulating ACTH and corticosterone. In these mice total T-lymphocyte numbers were slightly increased and the proportion of $CD4^{+}CD8^{-}$ to $CD4^{-}CD8^{+}$ was increased (Morale, et al. 1995). $GR^{dim/dim}$ mice which have a mutation in the transactivation domain and thus have no transactivation potential, whilst maintaining repressive function, were resistant to GICD but were reported to have normal thymocytes in terms of CD4 CD8 profiles, although this data was not shown (Reichardt, et al. 1998). Two studies using similar models of $GR^{-/-}$ mice did not support the involvement of GR in T-cell development (Brewer, et al. 2002; Purton et al. 2002). Brewer’s study (Brewer et al. 2002) used exon 1C-2 deleted $GR^{-/-}$ mice on a C57BL/6 background which was embryonically lethal, while Purton’s study(Purton

et al. 2002) used exon 2 deleted $GR^{-/-}$ on a mixed genetic background (GRMB), which have 10-20% survive to adult stage. In both studies γ-irradiated $GR^{+/+}$ mice were reconstituted with $GR^{-/-}$ or $GR^{+/+}$ fetal liver cells and showed no GR in thymocytes. Total T-lymphocyte numbers in the thymus and peripheral tissues of spleen, lymph node and liver were normal between both GR null models when compared to their wild type counterparts (Brewer et al. 2002; Purton et al. 2002). Additionally, subpopulations of DN, DP or SP, and NKT, memory and activated T-cells were also not significantly different in either model or genotype indicating normal T-cell development and maintenance (Brewer et al. 2002; Purton et al. 2002). Normal TCR Vβ usage and normal numbers of mature SP cells in the periphery indicated positive selection had occurred appropriately, additionally using a culture plate model of negative selection and subsequent apoptosis assays both studies were able to show that $GR^{-/-}$ cells were neither more sensitive or more resistant to TCR mediated deletion (Brewer et al. 2002; Purton et al. 2002). Subsequently a T-cell specific “TGRKO” mouse model has been generated. Lck-Cre mice were mated with GR LoxP exon 2 deleted mice (Brewer at el. 2003), to generate mice which completely lack GR within the T-cell lineage (TGRKO). Lck-Cre Mice have the Cre recombinase under the control of the Lck lymphocyte protein tyrosine kinase promoter which induces Cre expression specifically in the T-cell lineage (Takahama et al. 1998). Lck-Cre homozygous transgenic mice are viable and have no major defects. Resulting mice homozygous for the exon 2 floxed Nr3c1 (GR) gene and harboring the Lck-Cre transgene (TRGKO) are as healthy as Lck-cre negative homozygous floxed Nr3c1 littermates (Brewer et al. 2003). TGRKO mice show normal circadian regulation of plasma corticosterone levels. Corticosterone responses in TGRKO mice are equal to those of controls when given a polyclonal T-cell activation stimulus. Analysis of TGRKO mice revealed normal thymic cellularity and normal subset distribution in thymus, spleen and lymph nodes however the analysis of T-cell subset distribution was not shown, and other specialized T-cells subsets were not examined (Brewer et al. 2003).

These studies provided strong evidence excluding the role of GR in T-cell selection, as they used more reliable models where GR is completely absent from thymocytes and circulating GCs are normal.

Despite these comprehensive studies, research into the involvement of GCs in T-cell development is still being investigated and still continues to produce conflicting results. A more recent study used two models of adrenal insuffiency; the MC2R$^{-/-}$ mouse model which lacks Melanocortin 2 Receptor, the receptor for ACTH hormone in the adrenal cortex of the adrenal gland, and therefore has no detectable corticosterone in the serum; and the CRH$^{-/-}$ which lack Corticotropin Releasing Hormone and therefore does not induce the release of corticosterone. In both models it was found that although the subpopulations of DN, DP and SN cells were normal, the total number of thymocytes and splenic T-cells was proportionally elevated compared to controls and corticosterone administration normalised the thymocyte numbers (Sato, et al. 2010). This study did not determine levels of GCs within the thymus. Similarly, recent studies using adrenalectomized mice and rats showed increased total thymocyte numbers, particularly DP and CD4^{+}CD8^{-} populations, with no change in proliferation but reduced apoptosis (Pazirandeh et al. 2005; Stojic-Vukanic et al. 2009). In Stojic-Vukanic's study (Stojic-Vukanic et al. 2009) less mature thymocyte populations (TCR^{-} or ^{low}) DP cells were increased in proportion, and in more mature (TCR^{high}) cells all subsets were decreased in proportion, indicating increased negative selection or a slower progression of DP TCR^{low} populations to a more mature state. This was supportive of the "Mutual Antagonism" model, however removal of the adrenal gland or adrenally derived GCs is a controversial model as immune development may be affected indirectly, for example via catecholamines whose synthesis is influenced by GCs and which are able to affect T-lymphocyte maturation themselves (Stojic-Vukanic et al. 2009). Additionally adrenalectomy does not account for any possible rescue by thymic GCs, although the sole ability of thymic GCs to control thymopoeisis is still under investigation. In adrenalectomized mice where GR was

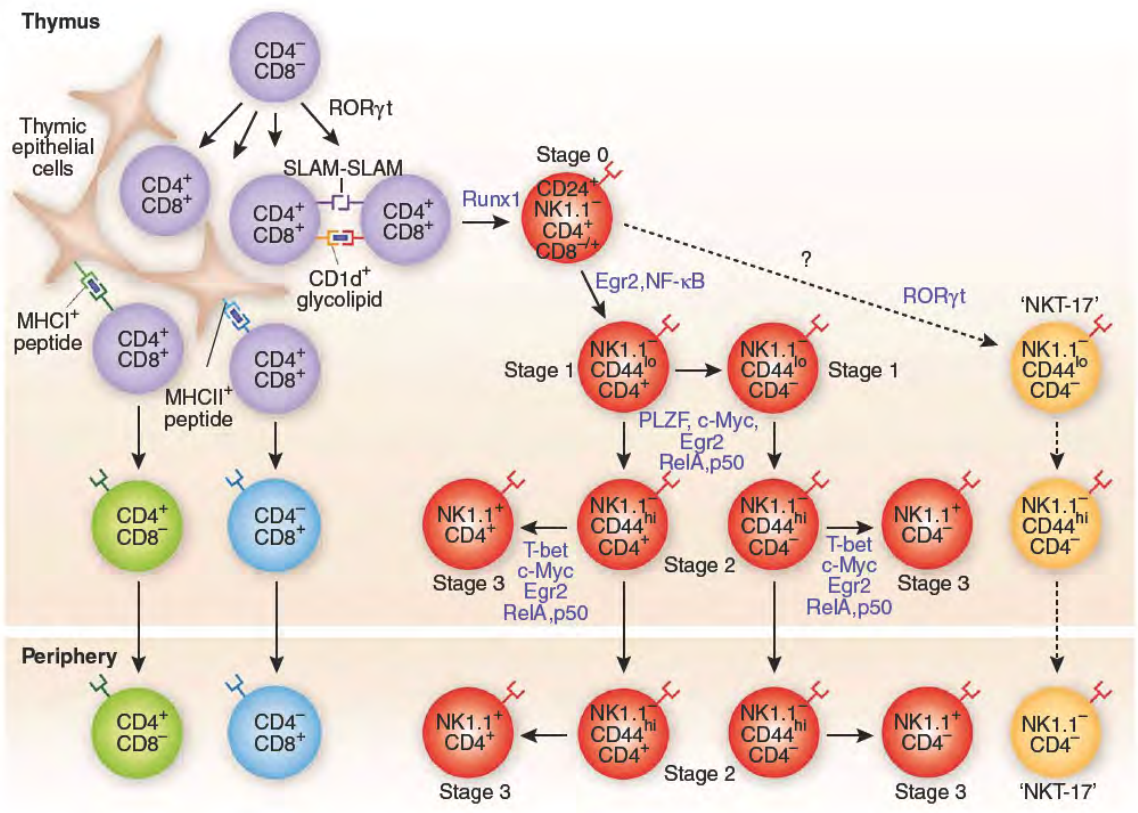


Figure 1.10 NKT cell development

A combination of random events is required to produce NKT cell precursors. Random gene rearrangements must first yield the semi restricted invariant CD1d restricted TcR, if these receptors then interact with glycolipid presented by CD1d molecules, and an interaction with SLAM receptor occurs a specific subset of transcription factors is activated and progression towards mature NKT cells begins. Final maturation can occur in the thymus or periphery.

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overexpressed, thymic GCs induced enhanced T-cell apoptosis but did not affect T-cell sub populations, and cells were rescued from apoptosis by the GR agonist RU486 (Pazirandeh et al. 2005). While this shows that thymic derived GCs are capable of regulating thymic apoptosis, it does not represent a physiological situation and point to the possibility that thymic derived GCs are insufficient to support normal T-cell development (Pruett and Padgett 2004).

Thus the role of GR and GCs in T-cell development and selection still remains controversial. Godfrey et al., (Godfrey, et al. 2000) speculated that GICD and subsequent shut down of T-cell development during immune stress may prevent development of regulatory cells which are tolerant of antigens from invading micro-organisms.

1.6.4: The role of GR in lineage specification in lymphocyte subsets

GCs and GR have long been investigated for their possible regulatory role in development and specification of various T-cell lineages, some subsets are described here with respect to GC involvement.

1.6.4.1: NKT Cells

NKT cells contribute to antimicrobial activity, tumour surveillance and self tolerance. They are able to kill microbes directly or secrete factors to recruit or activate NK cells, Macrophages, Granulocytes, T-cells and B-cells (Brigl and Brenner 2004). NKT cells are quite heterogenous and can be broadly classed as Type 1 or Type 2. Type 1 are the most widely studied and are the subject of this review. In mice Type 1 express an invariant alpha chain T-cell antigen receptor having the composition Vα14Jα18 in mice, along with a restricted TCR beta chain usually Vβ8.2, Vβ7 or Vβ2, which gives them the ability to recognise glycolipid antigens presented by CD1d. Thus NKT cells can be detected in vitro using tetramers of CD1d loaded with α-galactosylceramide. NKT cells may also express NK1.1 and CD4. Development of Type I NKT cells occurs through at least 4 well

defined stages; stage 1 and 2 occur in the thymus and stages 3 and 4 generally occur in the periphery (Figure 1.10). It begins with the random rearrangement of genes yielding the semi-invariant, CD1d-restricted TCR, once these cells interact with a glycolipid presented by a CD1d molecule on a cortical thymocyte, they must also interact with a SLAM surface receptor, these two signals trigger the activation of a series of transcription factors and cells enter the NKT cell lineage (reviewed in (Godfrey, et al. 2010). Although there is no data to suggest NKT cell development is dependent on GR signaling (indeed in GRKO mice NKT cell development was normal (Purton et al. 2002)), many transcription factors involved in NKT cell maturation have been described as being regulated by GCs, some of these are discussed here:

Plzf:

Promyelocytic Leukemia Zinc Finger (Plzf) is essential for NKT cell development, mice lacking *Plzf* show a selective deficiency in NKT cells, with numbers down 90-99% and cells present were stalled at stage 1, consistent with its observed induction following positive selection of NKT cell precursors (Kovalovsky, et al. 2008; Savage, et al. 2008). PLZF is itself a transcription factor which regulates cell cycle progression, it has been shown to be regulated in human endometrial stromal and myometrial smooth muscle cells (Fahnenstich, et al. 2003). Interestingly, PLZF was shown to be protective against GICD in leukemic cell lines (Wasim, et al. 2010).

c-Myc:

Myelocytomatosis oncogene (c-Myc) encodes a transcription factor well known for its role in cell cycle control, and oncogenic potential; it is believed to be present in 20% of human cancers. Additionally it regulates microRNAs and genes involved in protein synthesis, cell adhesion and cytoskeleton, and metabolism (Dang, et al. 2006). C-myc has been shown to be essential to NKT cell development, in CD4 T-cell specific *c-Myc* null mice NKT cell precursors are stalled at

stage 1, indicating that c-MYC is important in the progression beyond this stage. All other T-cell subsets developed normally (Mycko, et al. 2009). GCs have been shown to repress c-Myc in T-cell lymphoma cells and it is believed to be one of the mechanisms via which GCs can mediate GICD (Eastman-Reks and Vedeckis 1986).

Gata-3:

GATA3 is a transcription factor crucial to early stages of T-cell development. Fusion with a LacZ reporter gene showed *Gata3* expression at the onset of positive selection events, i.e., TCRαβ up-regulation and CD69 expression, and expression remains during maturation of CD4 single-positive (SP) cells in the thymus, but is almost absent in CD8 SP cells (Nawijn, et al. 2001). GATA3 deficiency results in defects in the thymic maturation and peripheral maintenance of murine iNKT cell number thought to be due to disrupted maturation and increased apoptosis. Thymic NKT cell numbers from *Gata3* deficient mice were normal however surface TCR levels were decreased, In the liver and spleen, NKT cell numbers were markedly reduced in *Gata3* KO mice, and overall $CD4^+$ NKT cells were decreased (Kim, et al. 2006). GCs have been shown to repress *Gata3* expression and protein synthesis (Liberman, et al. 2009).

As mentioned, there is evidence against the involvement of GR in NKT cell development, thus GC regulation of these genes may be involved in its anti-inflammatory, immunosuppressive function, which is particularly true in the case of *Gata3*. Thus this remains a controversial area that requires further research.

1.6.4.2: Regulatory T-cells

Although the existence and function of Regulatory T-cells was once controversial, it has now been established that "T_{Reg}" cells are critical for immunosuppression of self reactive T-cells that escape T-cell selection, adding an extra line of defence against inappropriate autoimmune responses. T_{Reg} cells reduce the ac-

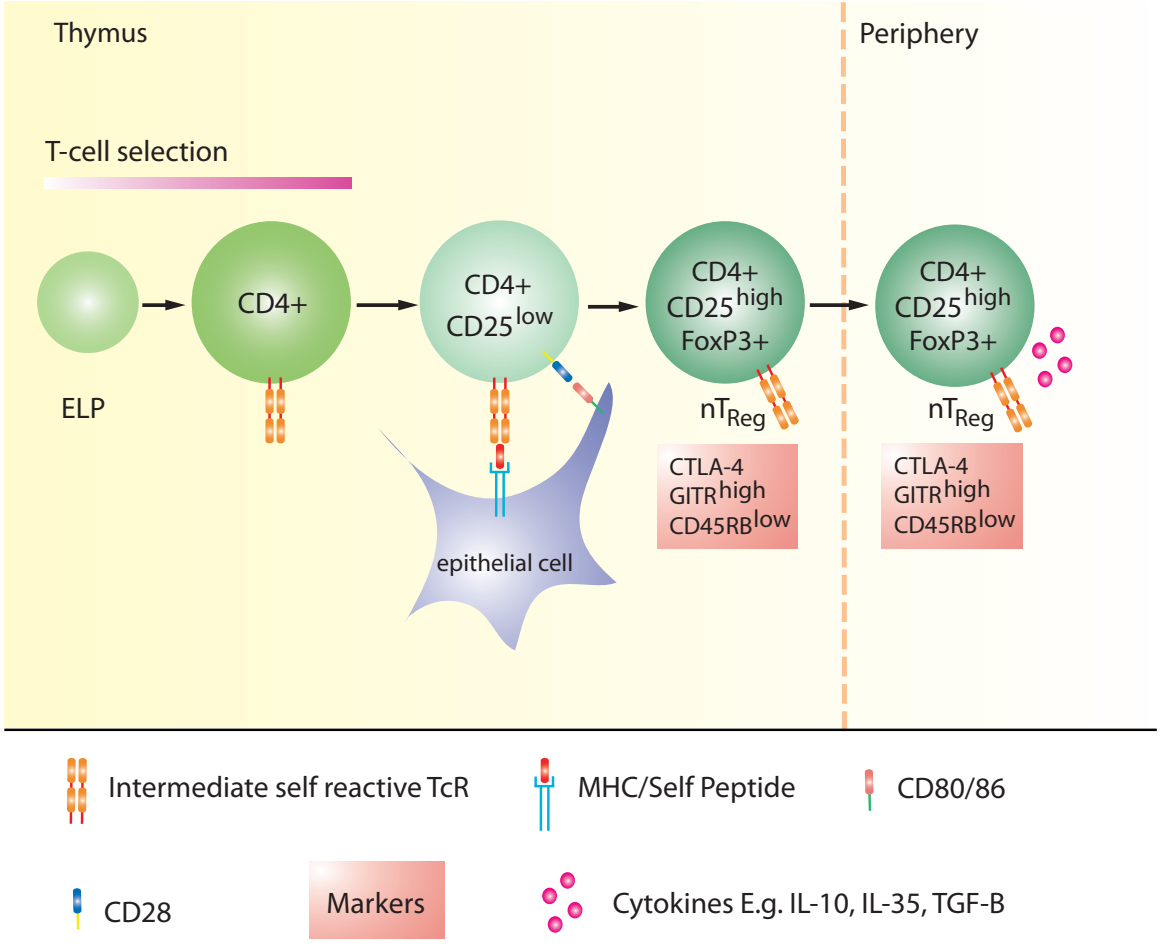

Figure 1.11 Regulatory T-cell development

During T-cell selection a moderate avidity towards self peptide by TcR, plus interaction between CD molecules triggers FoxP3 expression and thus lineage specification towards the T_{Reg} phenotype.

tivation and expansion of these self reactive cells, most likely by a combination of mechanisms mediated by cytokines, direct cell to cell interactions and modulation of antigen presenting cells in addition they are also able to respond to non-self antigens (reviewed in (Sakaguchi 2004)).

There is evidence for many subtypes of T_{Reg} cells expressing different markers and in other tissues of the mouse (reviewed in (Campbell and Koch 2011; Dimeloe, et al. 2010)). Naturally occurring CD4+ regulatory T-cells, "n T_{Reg}s" which are generally characterized by presentation of the markers FOXP3, CD25, CTLA-4, GITRhigh and $CD45RB^{low}$, develop in the thymus and also exist in the periphery. In addition to n T_{Reg}s there are populations of induced – "iT_{Reg}" cells which develop outside of the thymus in response to TCR stimulation and cytokines IL-2 and TGF-β, not all of which express *FoxP3*. There is also evidence that T_{Reg} cells developing in the thymus can begin to express CD44 as they migrate into the periphery. The existence of a regulatory T-cell population was eluded to by early studies where thymectomy of mice and adult rats led to autoimmunity, and when reconstituted with T-cells, particularly CD4+ cells, disease was prevented, demonstrating that self reactive T-cells which escape thymic negative selection persist within the normal immune system and are capable of causing autoimmune diseases (Reviewed in (Sakaguchi 2004)). In order to pinpoint the specific cells responsible for this regulatory function this method was refined to examine specific $CD4^+$ T-cell subtypes sorted by various secondary markers and led to the discovery that $CD25^+$ positive cells were critical to T_{Reg} function (Sakaguchi, et al. 1995). Supporting this was the finding that CD25 deficient mice develop autoimmunity (Malek, et al. 2002).

A spontaneously arising mutant mouse model "Scurfy" was known to contain an x-linked recessive defect that showed severe immune dysfunction which was fatal within a month, with the defect tracked to the *FoxP3* gene (Brunkow, et al. 2001; Godfrey, et al. 1991). Mutation of this same gene in humans was found to be responsible for "IPEX" Immune Dysregulation, Polyendocrinopathy,

Enteropathy and X-linked Syndrome which is characterized by autoimmune disease in multiple organs, IBD, dermatitis and fatal infections (Gambineri, et al. 2003). *FoxP3* was later shown to be necessary and sufficient in the development and suppressive function of T_{Reg} cells; necessary in that the lack of *FoxP3* leads to fatal autoimmune disease, and sufficient in that overexpression of *FoxP3* in transgenic mice resulted in increased numbers of T_{Reg} cells, and retroviral transduction of *FoxP3* into $CD25^{-}CD4^{+}$ cells converted them into T_{Reg}-like cells which expressed markers common to T_{Reg}, and were able to suppress proliferation of other T-cells and suppress autoimmune disease (Hori, et al. 2003). No other factor has been shown to be required to this degree for T_{Reg} cell development.

Lineage specification of T_{Reg}s is believed to occur during TCR selection; cells possessing TCRs with higher affinity for thymic MHC/self-peptide ligands than those of other T cells, but not too strong affinity are recruited to the Treg cell lineage. It is believed this interaction triggers the expression of *FoxP3* which then regulates the expression of factors required for specification although there are many aspects of this process that remain to be fully explained (See Figure 1.11) (Reviewed in (Sakaguchi 2011; Sakaguchi, et al. 2008)).

Of interest in the context of glucocorticoids is the fact that genes for multiple T_{Reg} markers have been shown to be regulated by GCs, for example *Gitr* and *Ctla4* (Chen, et al. 2004), but more significantly *FoxP3* which is so crucial for TReg cell development. In asthmatic patients taking inhaled or systemic glucocorticoids, *FoxP3* expression was increased in T_{Reg} cells, and this was correlated with an increase of the cytokine IL-10 which is an important mediator of the suppressive function of TReg cells, additionally they found that GCs increased the numbers of TReg cells in the peripheral blood. GCs were also able to induce naïve CD4+ T-cells isolated from non-asthmatic control patients to transiently upregulate *FoxP3 in vitro*, and these differentiated cells had the capacity to suppress proliferation of CD4+ cells freshly isolated from the same patient (Karagiannidis, et al. 2004).

In T_{Reg} cells isolated from human blood, FOXP3 positive cell numbers and proportions were decreased 24hr after dex treatment, but after 14 days of recovery FOXP3 positive cells were elevated in the dex-treated samples, mRNA levels of *FoxP3* remained elevated through the 14 day period. However in this study they found that elevated FOXP3 levels did not correlate with suppressive activity, dex induced CD25+ cells were unable to suppress growth of effector cells (Prado, et al. 2011).

In mice, *in vivo* treatment with dex increased $CD25^{+}CD4^{+}$ TReg cell numbers in peripheral organs in response to Dex treatment and reduced the percentage of $CD4^{+}FOXP3^{+}$ T_{Reg} cells (however cell numbers were not given, and cells were not followed up after the 3 day treatment) (Chen, et al. 2006).

Thus the role of GCs in development, regulation and function of T_{Reg} cells is an important topic that requires further attention.

1.7 Glucocorticoids in B-cell Development

B-cells are generated from pluripotent hematopoietic stem cells (HSC) in the fetal liver or in the adult bone marrow. They are the ultimate precursor of B cells, which give rise to multipotent progenitor MPPs and become committed to B cell lineage by the activity of B cell-specific transcription factors (Busslinger 2004). At the Pro B-cell stage, Ig heavy chain VDJ recombination of the B-cell receptor gene occurs and cells begin to undergo positive selection, from here they enter the Pre B-cell stage and VJ recombination at the Ig light chain locus is initiated. During the immature B-cell stage, cells are subjected to negative selection to delete autoreactive cells. Non-reactive immature B cells emigrate to the spleen and are known at this stage as transitional 1 (T1) B-cells, which are subject to negative selection, those which survive become transitional 2 (T2) B-cells and finally by positive selection become mature, immune-competent B cell in the spleen, classified as Follicular Mature (FM - residing in follicles) or Marginal Zone (MZ) cells (reviewed in (Chung, et al. 2003)). FM cells are able to re-circulate and eventually

re-enter the bone marrow and correspond to naïve recirculating mature B cells. The 'immature' cells from the bone marrow are in fact at a similar stage of development as the T1 splenic cells, as they have simply exited the bone marrow and reached the spleen. Thus throughout B-cell development cells are subjected to selection and deletion by apoptosis at most stages.

In the same study from Purton et. al, (Purton et al. 2004) using $GR^{-/-}$ reconstituted mice, GR was shown not to be required for B-cell development. Numbers of Pro B, Pre-B and mature B-cells from $GR^{-/-}$ bone marrow were normal compared to $GR^{+/+}$ mice, and T1, T2 and mature B-cells in the spleen were also present in normal numbers between $GR^{-/-}$ vs $GR^{+/+}$ (Purton et al. 2002). This is contrasted by early data from adrenalectomized mice which therefore lack adrenal-derived corticosterone and show enlarged spleens (Kieffer and Ketchel 1971), and also a study using *Mc2r*$^{-/-}$ mice where total B-cell numbers were increased in spleen compared to wildtype mice and these numbers were corrected upon corticosterone administration. Further investigation into B-cell subpopulations showed T1 B-cells were significantly increased in the spleens of *Mc2r*$^{-/-}$ mice, and in bone marrow pre-B cell numbers were significantly increased in *Mc2r*$^{-/-}$ mice (Sato et al. 2010). They have suggested that GCs may signal via MR in $GR^{-/-}$ mice to allow normal B-cell development to occur in these mice.

The sensitivity of B-lymphocytes to GICD has long been recognised (Levine and Claman 1970; Garvy et al. 1993b; Laakko and Fraker 2002). Studies have shown that B cells are also differentially susceptible to GC treatment according to their stage of development (Andreau et al. 1998). GC treatment resulted in the depletion of B-lymphocytes in the bone marrow, especially pre-B and immature B-cells, whereas mature B cells were shown to be more resistant (Garvy, et al. 1993a; Voetberg, et al. 1994). In a study that examined early B-cell development more closely, Early and Late pro B-cells, Pro B-cells and immature B-cells were sensitive, while PrePro B cells were relatively resistant, and mature cells were resistant and increased in number, possibly reflecting migration of peripheral B-cells

(Laakko and Fraker 2002). This suggests that the hematopoietic stage of development is a factor in determining sensitivity to gGICD in B-cells (Lill-Elghanian, et al. 2002).

Merino et al. (1994) have shown that sensitivity to GICD correlated with decreased *Bcl-2* expression. Mature B-cells from the bone marrow and spleen had high levels of *Bcl-2* expression, and so did Pro-B cells from the bone marrow, while Pre-B and immature B-cells showed low expression. B-cell apoptosis is more prominent in pre-B cells and immature B-cells which are undergoing light chain recombination to generate functional IgH chains and are also subjected to B-cell selection to remove auto-reactive B-cells. Thus a role for Bcl-2 and apoptosis in this process is thought to exist.

B-cells express similar levels of GR1A transcripts to T-lymphocytes (Purton et al. 2004), indicating a similar mechanism to GICD in T-lymphocytes may exist, whereby GR1A content may correlate with increased susceptibility to GICD. In the human pre-B derived 697 cell line, dex induces auto-upregulation of GR mRNA and protein and induces apoptosis. The threshold of GR has been shown to be essential and the optimal window of receptor level is quite narrow in order for apoptosis to occur (Schwartz, et al. 2010). It has been demonstrated in these cells that promoters GR 1A2 and 1A3 are in fact down regulated in response to dex treatment and that transcription of exons C and D are elevated, which is in contrast to T-cells where elevated levels of GR1A correlate with increased sensitivity to GICD (Purton et al. 2004). The authors described a molecular switch model whereby up-regulation of GR co-activator PGC-1 in response to dex is involved in facilitating GR induced up-regulation of exons C and D while PU.I present in 697 cells (which was shown to bind to the GR1A promoter in response to dex treatment) may inhibit transcription from the GR1A promoter (as occurs in developmentally mature IM9 B-lymphoblastoid cells) (Geng and Vedeckis 2005, 2011). Thus the role of GCs in B-cell development as well as the presence of GR1A transcripts in B-lymphocytes remain areas that require further study.

1.8 Project objectives:

The main objectives of this study were to investigate further the molecular mechanisms involved in GICD, to examine more closely the role of GR in the development of T-lymphocytes, and the expression and regulation of the GR1A promoter in mouse tissues previously not examined. This was achieved via four project aims:

(i) Genome wide assessment of acute glucocorticoid regulated genes in the mouse thymus using microarray technology to identify whole genome candidate genes for GICD.

(ii) Identification and analysis of direct GR target genes to determine their in volvement and requirement in GICD of mouse T-lymphocytes.

(iii) Investigation of the requirement of GR in development of murine T-Regulatory and NKT cells through comparison of TGRKO to WT mice.

(iv) To localise and assess GC-mediated regulation of the mGR1A promoter in the mouse brain and T- and B-lymphocytes.

CHAPTER 2:

MATERIALS AND METHODS

Chapter 2: Materials and Methods

The following chapter describes the materials and methods used throughout the project. Materials and methods for submitted papers are described in their respective sections. Specific materials and methods are detailed in Chapter 5 and Appendix 1.

2.1 Animals and housing

Mice used in this study were bred and housed under standard conditions at the animal house and ARL of the Biochemistry Department, Monash University, and Mouseworks, Monash University. All animal experimentation was approved by the School of Biomedical Sciences Animal Ethics Committee, Monash University (Ethics no. 2009/63), and was carried out according to the National Health and Medical Research Council of Australia guidelines for the breeding, care and use of genetically modified and cloned animals for scientific purposes 2007. Lck-Cre mice were mated with GR LoxP exon 2 deleted mice (Brewer at el., 2003, obtained from Dr. L.J. Muglia, Washington University School of Medicine, Saint Louis, Missouri 63110, USA) to generate mice which lack GR within the T-cell lineage (TGRKO). Lck-Cre Mice have the Cre recombinase under the control of the Lck lymphocyte protein tyrosine kinase promoter which induces Cre expression specifically in the T-cell lineage (Takahama et al., 1998). Lck-Cre homozygous transgenic mice are viable and have no major defects. TGRKO mice and Wild Type (WT) mice were treated with Dexamethasone, sacrificed and thymi, spleens and livers and removed for further analysis. Tissues were stored as appropriate for each method. For flow cytometry tissues were placed immediately in FACS buffer (See Appendix 2) and stored on ice until cell isolation was performed as described in 2.3.3. If RNA was to be extracted tissues were snap frozen in liquid nitrogen and stored at -80˚C or RNA was isolated immediately as described in A1.2.8. For Immunohistochemistry tissues were fixed by immersing in 4% PFA (in PBS) immediately after dissection for 2 h and gradually dehydrated as described in A1.2.11.

2.2 Genotyping

To determine genotypes with respect to target genes in transgenic and knockout mice, genotyping PCRs were performed. Genomic DNA was prepared from tail or toe clips using REDExtract (Sigma-Aldrich, Castle Hill, NSW - according to manufacturers instructions) or Tail Digestion Buffer (See Appendix 2). Approximately 4mm of tail was digested with 0.5 mL Tail Digestion Buffer and 10 µL Proteinase K overnight at 55°C. Samples were centrifuged for 1 min at 15,000 rpm in an Eppendorf 5415R. Supernatant was transferred to fresh 1.7 mL tube eliminating as much fur as possible. DNA was precipitated with 1.0mL 95% ethanol containing 1/20th volume 3M NaOAc pH 7.0 at RT for 1.5 h. Sample was centrifuged 10 min at max speed, ethanol removed and pellet air dried and resuspended in sterile water. PCR primer sequences, conditions and cycling are detailed in specifc methods section of each chapter. Resulting amplified DNA was run on 1% Agarose gel in 0.5X TBE against a 1 Kb Plus DNA Ladder (Life Technologies, Mulgrave, Victoria) with Ethidium bromide or GelRed (Life Technologies) detection and visualised using the Gel Dock UV transilluminator.

2.3 Cell preparation from organs

For flow cytometry experiments lymphocytes were isolated from thymus and spleen by first removing any residual fat or connective tissue using fine forceps. Organs were then scored over a petri dish containing FACS Buffer (See Appendix 2) using the frosted ends of 2 glass slides and then gently ground between rough surfaces till cells spilled into the FACS Buffer. Cells were resuspended and transfered to a tube through a 100 µm mesh.

Lymphocytes were isolated from liver using a Percoll gradient. Bile duct was removed from liver and placed FACS Buffer on a 200 µm sieve in 9 cm Petri dish. Liver was cut into small pieces using curved scissors, and pieces pushed through the sieve using the plunger of 3 mL syringe. The sieve was rinsed with FACS Buffer to collect any residual cells. The isolated cell mix was then centrifuged in a 50

mL tube at 1700 rpm (546 x g) for 5 min and the supernatant poured off. At this time the cell pellet was resuspended in 25 mL 33% Percoll (9 mL Percoll, 1 mL 10X PBS, 17 mL 1X PBS) and centrifuged at room temperature at 2000 rpm (755 x *g*) for 12 min. Hepatocyte layer and supernatant were aspirated and cell pellet washed in FACS Buffer and then transferred to a 10 mL tube. Cells were centrifuged at 1700 rpm (546 x *g*) for 5 min and supernatant aspirated. Lymphocyte preparations from spleen and liver were red blood cell lysed in Red Blood Cell Lysing Buffer (Sigma-Aldrich) according to the manufacturer's instructions. Briefly; cell pellets were resuspended in 0.5-3 mL (for Livers) or 5mL (for Spleens) Red Blood Cell Lysing Buffer, underlayed with 0.5 mL FCS and centrifuged at 1700 rpm (546 x *g*) for 5 min. Supernatant was aspirated and cell pellet resuspended in 0.5 mL (liver) or 5mL (Spleen). Cells were counted using a hemocytometer or Coulter Particle counter for the purpose of calculating the amount of antibody required for staining.

2.4 Isolation of total RNA

Total RNA was isolated from mouse tissues, primary cells or cell lines by homogenisation in TRIzol reagent (Life Technologies). After chloroform extraction, RNA was precipitated from the aqueous phase with isopropanol, washed in 70% ethanol and redissolved in nuclease-free sterile water. The concentration and purity of the isolated RNA were measured at OD260 using a spectrophotometer (Eppendorf). A260/280 ratios >1.6 were considered of satisfactory quality. The integrity of total RNA was evaluated by electrophoresis in a 1% agarose gel containing Morpholinopropanesulfonic acid (MOPS) and ethidium bromide. 1µg of RNA was added to: 2µL of 10X MOPS, 1.75µL of Formaldehyde, 5µL of Formamide, 1.25µL of ddH_20. These reagents were incubated at 55˚C for 5 min at which time 1µL of RNA loading dye was added and the total solution was loaded onto the gel. RNA was separated by electrophoresis at 70V for 1 h.

2.5 Synthesis of cDNA

cDNA was generated using the random-primed Promega Two-Step Reverse Transcriptase Polymerase Chain reaction (RT-PCR) kit (Promega), 1µg of total RNA was used to synthesise cDNA according to the manufacturers instructions. cDNA yields were increased by changing the duration of the transcription step from 50 minutes to 90 minutes at 42˚C. The concentration of cDNA was measured at OD260 using a spectrophotometer (Eppendorf)

2.6 Immunohistochemistry

Tissues were fixed by immersing in 4% PFA (in PBS) immediately after dissection for 2 h. Tissue was gradually dehydrated into 70% ethanol and processed into paraffin. 5µm sections were collected onto superfrost plus slides and incubated at 40°C overnight. Prior to experiment, slides were placed at 60°C for 30 min and then dewaxed through 3 changes of xylene, 5 min each. Slides were rehydrated by taking through decreasing ethanol concentrations, finally bringing them to water and placed in 1X PBS for 15 min. Antigen retrievel was performed by boiling slides in 0.01M Sodium Citrate for 20 min and allowed to cool to room temp. Sections were washed in Wash Buffer 3 X 5 min. For Horseradish Peroxidase detection, endogenous peroxidases were blocked by incubating slides in 3% H_2O_2 (in water) for 5-10 min. Slides were washed 3 X 5 min in Wash Buffer before being placed in Block Buffer for at least 30 min at room temperature (RT). Sections were incubated with primary antibody diluted in Block Buffer for 1 hr at RT or 4°C overnight. Primary antibody was washed off with Wash Buffer 3 X 5 min before the addition of the secondary antibody diluted in Block Buffer for 30 - 60 min. Secondary antibody was washed off with Wash Buffer 3 X 5 min, and if necessary a streptavidin conjugated tertiary antibody was applied, diluted in Block Buffer, for 30 – 60 min. Tertiary antibody was washed off with Wash Buffer 3 X 5 min, for HRP detection, DAB solution (1 drop DAB Chromogen /1mL DAB substrate Buffer) was applied to sections until brown staining appeared, after which time

slides were washed 3 X 5 min in 1 X PBS. Slides were then counterstained with Hemotoxylin and mounted using DPX mounting medium.

2.7 Flow Cytometry

Cells were isolated as described in section 2.2.3, and counted using the Coulter particle counter. If antibody staining was required, cells were stained with antibodies outlined in specifc methods sections of respective results chapters. Flow cytometry was performed on the LSRII with 3 lasers (405nm uv laser, Argon 488nm blue laser and a He-Ne 633nm red laser) and data collected with DIVA software or on the FACSCalibur flow cytometer (Argon 488nm blue laser,Becton Dickinson, Franklin Lakes, New Jersey) and data collected using cell quest software. Data analysis was performed using FlowJo v 8.8.6 from TreeStar, Prism and Microsoft Excel.

CHAPTER 3:

NFIL3 IS A GLUCOCORTICOID-REGULATED GENE REQUIRED FOR GLUCOCORTICOID-INDUCED APOPTOSIS IN MURINE T-CELLS

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Project direction, development and writing up of paper, qRT-PCR analysis, ChIP analysis, immunohistochemistry, siRNA transfection and Flow cytometry	70%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Kheng Tan	Microrarray experiments and analysis	5%
Judy Ng	qRT-PCR analysis	
Douglas Liddicoat	Flow cytometry analysis	
Dale Godfrey	Development and writing up of paper	
Timothy Cole	Project direction, development and writing up of paper, qRT-PCR analysis	

Candidate's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia

Signature 1	[redacted]	**Date:** 7/12/2012
Signature 2	[redacted]	9/12/2012
Signature 3	[redacted]	9/12/2012
Signature 4	[redacted]	7/12/2012
Signature 5	[redacted]	7/12/2012

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Nfil3 Is a Glucocorticoid-Regulated Gene Required for Glucocorticoid-Induced Apoptosis in Male Murine T Cells

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Glucocorticoids (GCs) have essential roles in the regulation of development, integrated metabolism, and immune and neurological responses, and act primarily via the glucocorticoid receptor (GR). In most cells, GC treatment results in down-regulation of GR mRNA and protein levels via negative feedback mechanisms. However, in GC-treated thymocytes, GR protein levels are maintained at a high level, increasing sensitivity of thymocytes to GCs, resulting in apoptosis termed glucocorticoid-induced cell death (GICD). $CD4^+CD8^+$ double-positive thymocytes and thymic natural killer T cells in particular are highly sensitive to GICD. Although GICD is exploited via the use of synthetic GC analogues in the treatment of hematopoietic malignancies, the intracellular molecular pathway of GICD is not well understood. To explore GICD in thymocytes, the authors performed whole genome expression microarray analysis in mouse GR exon 2 null vs wild-type thymus RNA 3 hours after dexamethasone treatment. Identified and validated direct GR targets included *P21* and *Bim*, in addition to an important transcriptional regulator *Nfil3*, which previously has been associated with GICD and is essential for natural killer cell development in vivo. Immunostaining of NFIL3 in whole thymus localized NFIL3 primarily to the medullary region, and double labeling colocalized NFIL3 to apoptotic cells. In silico analysis revealed a putative GC response element 5 kb upstream of the *Nfil3* promoter that is strongly conserved in the rat genome and was confirmed to bind GR by chromatin immunoprecipitation. The knockdown of *Nfil3* mRNA levels to 20% of normal using specific small interfering RNAs abrogated GICD, indicating that NFIL3 is required for normal GICD in CTLL-2 T cells. **(*Endocrinology* 154: 0000–0000, 2013)**

Glucocorticoids (GCs) play an essential role in the regulation of the immune response and are widely prescribed as immunosuppressive and anti-inflammatory drugs to control autoimmune diseases and airway inflammation in respiratory asthma (1). In addition, the lymphocytolytic actions of GCs are used to treat lymphocytic leukemias and lymphomas, such as childhood acute lymphoblastic leukemia (2). The actions of GCs are mediated via the glucocorticoid receptor (GR) a member of the superfamily of intracellular nuclear receptors (1). Upon ligand binding, homodimerization and nuclear import of the active GR dimer can occur, enabling modulation of gene transcription through interactions with GC response elements (GREs), which can be located more than 10 kb away from promoter regions of target genes, or via non–DNA-binding, protein-protein interactions with other transcription factors that have already activated the gene (1, 3–8). For example, the major anti-inflammatory effect of GCs occurs via interactions between the activated GR and nuclear factor-κB or activator protein 1 transcription factors that promote expression of proinflammatory genes (1, 9, 10).

In most cells, GC treatment results in down-regulation of GR mRNA and protein via negative feedback mecha-

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Abbreviations: AV, annexin V; ChIP, chromatin immunoprecipitation; Dex, dexamethasone; DP, double positive; GC, glucocorticoid; GICD, glucocorticoid-induced cell death; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GRKO, GR exon 2 null; qRT-PCR, quantitative RT-PCR; siRNA, small interfering RNA; TGRKO; T-cell–specific GR; TSS, transcription start site; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

nisms. In GC-treated T-cell lines, such as CEM-C7 cells, human GR mRNA and protein are increased, and active GR remains stable, so GR protein levels are high, increasing sensitivity of T cells to GCs and allowing a threshold of GR protein to be reached, which triggers apoptosis termed glucocorticoid-induced cell death (GICD) (11). In mouse GC-treated T lymphocytes, elevated levels of GR transcripts originating from an alternate upstream GR1A promoter of the GR gene are implicated in increasing sensitivity to GICD (12). Although GICD is exploited in the use of GC analogues as a chemotherapy agent for hematopoietic malignancies, the normal physiological role of GICD is not well understood (reviewed in Ref. 13). In mice it has been identified that of all the T-cell subsets, $CD4^+CD8^+$ double-positive (DP) thymocytes are most sensitive to GICD (12), which supports the idea that GICD might play an important role in intrathymic T-cell selection (reviewed in Ref. 14; however, studies performed with GR null mice, which had normal T-cell development and thymocyte populations, have questioned the significance of GC signaling in the thymus (15–17). The possibility remains that sensitivity of DP cells to GICD reflects more subtle effects on T-cell development or limits this process during times of stress (18).

Specific target genes of activated GR in GICD are not well characterized, particularly in vivo. Analysis of individual proapoptotic proteins Bim, Puma, Caspase-9, and Apaf-1 via knockdown has revealed that these components affect sensitivity to GICD; in a similar manner, knockdown of the antiapoptotic protein Bcl-2 results in increased GICD. However, no individual protein has been demonstrated as essential for GICD (19–27). Microarray studies of various GICD-sensitive human and murine leukemic cell lines have been performed to identify a related subset of genes that may be involved in GICD (28–30). More recently, microarray studies have been performed in vitro with primary mouse thymocytes treated with dexamethasone (Dex) for 1.5 and 4 hours or sorted DP thymocytes treated for 3 hours (31–33). However, no microarray studies have examined acute changes in gene expression in response to Dex treatment in vivo. Our study aimed to further investigate the normal function and mechanism of GICD in mouse T cells.

To identify novel GC-regulated genes involved in GICD, we have performed whole genome expression microarray analysis using GR exon 2 null (GRKO) mice, which have normal T-cell development (15). Most GRKO mice die at birth of lung atelectasis; however, a small proportion of GRKO mice survive to adulthood. Microarray analysis was performed on thymus RNA from adult GRKO mice and wild-type (WT) mice 3 hours after Dex treatment, and GR-regulated target genes were identified. One such gene was the b-zip transcriptional repressor *Nfil3*, whose levels were significantly induced after GC treatment. Nfil3 previously has been shown to be responsive to Dex induction in human leukemic lymphoid CEM-C7 GICD-sensitive cells (34), and elevated levels of Nfil3 were shown to correlate with GICD in these cells (32). This study has further characterized the GC regulation of *Nfil3* in the mouse thymus and thymocytes, where it plays a role in apoptosis. Investigation of the upstream enhancer region of the *Nfil3* gene identified a GRE, which was confirmed by chromatin immunoprecipitation (ChIP) to bind the GR. Using small interfering RNA (siRNA) knockdown technology, we have demonstrated that Nfil3 is necessary for GC-induced apoptosis in the mouse CTLL-2 T-cell line.

Materials and Methods

Mice

All animal experimentation was approved by the School of Biomedical Sciences Animal Ethics Committee, Monash University (Ethics no. 2009/63), and was carried out according to the National Health and Medical Research Council of Australia guidelines for the breeding, care, and use of genetically modified and cloned animals for scientific purposes. For microarray analysis, GRKO mice generated by gene targeting were used (35). On a nonisogenic 129sv-C57BL/6 background, because of a partially penetrant lung phenotype, a small percentage (~10%) of GRKO mice survive and develop normally into adulthood. Male GRKO mice were used for subsequent gene array studies and compared with WT mice (n = 3). Mice were injected ip with a single 100-μL dose of 4 μg/μL Dex and then killed at 3 hours. To generate mice that lack GR within the T-cell lineage Lck-Cre mice that have the Cre recombinase under the control of the Lck lymphocyte protein tyrosine kinase promoter were mated with GRLoxP mice that contain loxP sites flanking exon 1C to downstream of exon 2 of the GR gene (36). Male T-cell–specific GR (TGRKO) floxed mice and WT mice were treated with a single 100-μL dose of 4 μg/μL Dex, killed at 3 hours, and thymi removed for additional analysis.

RNA isolation

Total RNA was isolated from whole mouse thymus, mouse thymocyte cell suspension, and mouse CTLL-2 T cells by homogenization in TRIzol reagent (Invitrogen, Carlsbad, California) as described previously (37). For microarray analysis, RNA was further purified using an RNeasy cleanup kit (Qiagen, Valencia, California).

Whole mouse genome gene expression microarray analysis

Thymus samples were isolated from male GRKO and WT adult mice (n = 3) 3 hours after treatment of mice with Dex and the total RNA purified. RNA preparation, the microarray experiment, and analyses were performed as described previously (38). The microarray experiment was performed by Australian

Genome Research Facility (WEHI, Melbourne, Australia). The normalized data were subjected to ANOVA model using the Bonferroni method (39). The false discovery rate (40) was calculated based on the *P* value (≤.01) from ANOVA. Genes that experienced a change of 1.5-fold or more in signal intensity and false discovery rate of less than 0.05 were considered to be differentially expressed.

All Affymetrix-microarray data presented in this manuscript is MIAME (Minimum Information About a Microarray Experiment) compliant and has been submitted to the European Bioinformatics Institute ArrayExpress repository (Cambridge, United Kingdom). The data can be viewed by accessing the following web site: http://www.ebi.ac.uk/microarray-as/aer/login and using the following information: log-in, Reviewer_E-MEXP-1295; password, 9achuSeC; Accession number, E-MEXP-1295.

Isolation and culture of primary thymocytes

For quantitative RT-PCR (qRT-PCR) experiments examining gene expression of microarray target genes, male mice were injected ip with a single 100-μL dose of 4 μg/μL Dex, then killed at 3 hours and thymus cells isolated. For ChIP experiments, thymi were removed from untreated male mice, and thymocytes were isolated and treated in vitro. Cells were cultured in RPMI 1640 media with 5% fetal calf serum (FCS) and 1 μM Dex for 1 hour and then fixed in 4% paraformaldehyde.

Synthesis of cDNA and RT-PCR

Microarray data were verified using qRT-PCR. Total RNA from WT and GRKO mouse thymus was transcribed into cDNA using random hexamers and M-MLV reverse transcriptase, RNase H Minus, Point Mutant (Promega, Madison, Wisconsin). Primer pairs were designed using Primer3 software (38). Primers for qRT-PCR were *Angptl7*; Fwd-TGACTGTTCTTCCCTGTACCA, Rev-TGTCACAGAACACCTCTAGCTC, *Ptgr1*; Fwd-TAAGCTCAAGGGCTGCAAA, Rev-GTTAAAGGCCACGTCGAATC *Nfil3;* Fwd-AGGGAGCAGAACCACGATAA, Rev-TTCAGCCTCTCATC CATCAA, *Zfp553*; Fwd-CCATCGGTACAGGGACTGAG, Rev-CCTTGATCTCTGGGCCATC, *Zfp704*; Fwd-GATGTCCCTCCTGCAAGAAA, Rev-GACCAAAGGGCTGGTTGAC, *Errfi1*; Fwd-GGGAATGAAAGCTACTGGTTG, Rev-CAGCAACTCC TGCTGTTGAC, *Bcl2l11*; Fwd-AGAACCGCAAGCTTCCA TAC, Rev-GTTGAACTCGTCTCCGATCC, Rev-AAGATGCGGCCGTCTCTGGAACAG, *P21*; Fwd–CCTGACAGATTTCTATCACTCCA, Rev-CTGACCCACAGCAGAAGAG, *Il9r*; Fwd-GACTTCCAGAGTTGGACAGG, Rev-CCCAGATGCTGGA CTCTGA, *Gapdh*; Fwd-AACTTTGGCATTGTGGAAGG, Rev-GGATGCAGGGATGATGTTCT, *Gilz*; Fwd-GGTGGCCCTAGACAACAAGA, Rev- TCTTCTCAAGCAGCTCACGA, *c-myc*; Fwd-GCGACTCTGAAGAAGAGCAA, Rev- TCTCCACAGACACCA CATCA. Assays (in triplicate) were repeated at least twice with the *18S* rRNA, whose expression levels are stable between samples of the same type and are not influenced by GCs, used as an internal control between samples. Cycling was performed using Platinum SYBR Greener qPCR SuperMix (Invitrogen) on a Rotor-Gene 3000 (Corbett Research, Sydney, New South Wales, Australia). qRT-PCR data were analyzed using Rotor-Gene 6.0 software (Corbett Research), and differential expression was determined using the comparative ΔΔCT method (41).

Bioinformatics

Functional annotation of the microarray data set was done using the freely available Database for Annotation, Visualization and Integrated Discovery (DAVID) software (http://david.abcc.ncifcrf.gov/home.jsp). *Mus musculus* was chosen as the background list. A combination of the Functional Annotation Chart and Clustering tools were used to analyze the list and identify trends. For statistical analysis, a modified Fisher exact test was performed by the DAVID software to produce an EASE score. In silico promoter analysis was performed using the freely available TFSearch software (http://www.cbrc.jp/research/db/TFSEARCH.html) to look for putative transcription factor binding sites for GR or NFIL3. Sequence data 10 kb upstream from the beginning of exon 1 for each gene of interest was obtained from Ensembl (http://asia.ensembl.org/index.html). The vertebrate matrix and the default threshold score of 85.0 were applied.

ChIP assay

Dex-treated primary thymocytes were used for ChIP assays using a GR antibody M-20x (Santa Cruz Biotechnology, Santa Cruz, California) and the EZ-CHIP kit (Millipore, Billerica, Massachusetts) according to the manufacturers' instructions. Ten microliters DNA was collected before antibody precipitation as input control. The primer sequences were as follows: *Nfil3* GRE; Fwd-TACCTTGGCAGGGACTGAAG, Rev-ATATGGCTGCCAACCAGAAG, *Nfil3* GRE +1kb; Fwd-TGTCTGCTCTTCTGCTCTGC, Rev-GCTGTCCTGGCAT TAGCTCT, *Gilz* GRE; Fwd-GGCCTCAAACTCCAAGA TCA, Rev-TGCCAACCTCTGGACATTT, *Gapdh* TATA; Fwd-CTGCGCCCTTGAGCTAGGACTGGA, Rev – CTGTT CCAGAGACGGCCGCATCTT.

Immunohistochemistry

Tissues were prepared for immunostaining as described previously (42). For fluorescence detection, slides were incubated with 10 mg/mL sodium borohydride in 0.1 M PBS for 40 minutes to block autofluorescence, washed three times for 5 minutes and placed in block buffer (Tris-buffered saline with Tween 20 plus 5% serum derived from the host of secondary antibody) for 30 minutes at room temperature. Sections were incubated with primary antibody (rabbit antimouse NFIL3; Abcam, Cambridge, United Kingdom) diluted in block buffer, or block buffer alone for no primary antibody control, for 1 hour at room temperature or 4°C overnight and detected using an horseradish peroxidase-conjugated or fluorescently conjugated anti-rabbit Ig. Bright field images were taken on an Olympus CKX41 (Tokyo, Japan) using NIS-Elements software F3.0, Fluorescent images were captured on an Olympus IX71 using Spot Advanced software version 4.6.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stain

After dewaxing and antigen retrieval, as described, sections were stained by TUNEL staining, as described previously (42). For double labeling experiments, NFIL3 antibody staining was performed, as described above, starting from the addition of primary antibody. Fluorescent images were captured with an Olympus IX71 microscope.

In vitro culture and treatment of CTLL-2 cells

Mouse CTLL-2 cells were grown in RPMI 1640 media (Invitrogen) containing 10% FCS (Thermoscientific, Waltham, Massachusetts), 2 mM L-glutamine (Sigma, St Louis, Missouri), 2 mM sodium pyruvate (Sigma), penicillin (100 units/ml), streptomycin (0.1 mg/ml) (Sigma) and 0.5 to 1.0 ng/mL IL-2 (R&D Systems, Minneapolis, Minnesota) and switched to charcoal-dextran stripped serum 18 hours before the addition of Dex. Cells were sensitive to GICD at 0.25 ng/mL IL-2 in trial apoptosis assays, thus cell media gradually were changed from 0.5 ng/mL IL-2 to 0.25 ng/mL IL-2 before apoptosis assays. Dose-dependent and time course experiments were performed with 10 nM and 1 μM doses of Dex over a 0-, 1-, 2-, and 3-hour time period for qRT-PCR analysis. Cells were treated with 10 nM Dex for 0, 3, and 6 hours for apoptosis assays.

Nfil3 siRNA transfection

Three MISSION siRNA Oligos (Sigma) targeted to various sites of the *Nfil3* mRNA were used to knockdown Nfil3 expression. Optimization was performed using MISSION Positive Control siRNA targeted to *Gapdh* (Sigma). *Nfil3* levels were measured following transfection of nonspecific negative control siRNA (Sigma) to check for nonspecific off-target effects. Optimal transfection was achieved using Lipofectamine 2000 (Invitrogen) at a ratio of 1:20 000 cells, with 50 nM of each siRNA oligo, and was evident 40 to 48 hours after transfection. Lipofectamine 2000 transfection was performed according to the manufacturer's instructions. Cells were diluted in filtered complete growth medium without antibiotics to 2×10^5 cells/mL and added to siRNA-Lipofectamine 2000 complex to give ~13 000 cells/mL. Cells were incubated at 37°C.

Annexin V (AV) Apoptosis Assay

Cells were changed into full media containing charcoal dextran stripped FCS and seeded to a density of 1×10^5 cells/mL 18 hours before Dex treatment. Forty-two hours after *Nfil3* siRNA transfection, normal and *Nfil3* deficient cells were treated for 0, 3, and 6 hours with 10 nM Dex, with or without 20 μM EGTA. Dex was freshly dissolved in ethanol at 1000 times the working concentration such that ethanol concentration never exceeded 0.1% in cell treatments. Appropriate ethanol alone (sham) controls were run in parallel. The ApoAlert AV (Clontech, Madison, Wisconsin) assay kit was used to monitor early apoptosis by translocation of phosphatidyl serine on CTLL-2 cells according to the manufacturer's protocol. Samples were analyzed using a 488-nm Argon laser on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey). Data analysis was performed using Cell Quest software. The appropriate cell population based on forward and side scatter was gated and calculations performed on the gated population.

Statistical analysis

Statistical analysis was performed using Excel (Microsoft, Redmond, Washington) and using an ANOVA statistical test where appropriate. P values $<.05$ were considered statistically significant.

Results

Whole genome thymus gene expression microarray analysis of GR null mice

To identify novel GR-regulated genes, total thymic RNA was isolated from WT and GRKO mice (n = 3) 3 hours after Dex treatment and analyzed using Affymetrix mouse whole-genome exon microarrays that contained approximately 1 000 000 exon clusters representing nearly all genes expressed from the mouse genome. Final bioinformatic analysis identified a total of 85 genes that were differentially regulated in thymus of GRKO mice with a P value of $<.01$ and a cutoff fold change value of $\pm$ 2.0. Of these, 82 were elevated and 3 were suppressed by Dex. A shortened list detailing the top 20 positive GR-regulated genes is shown in Table 1. Analysis of the most positively regulated genes in WT compared with GRKO thymus revealed a number of known GR target genes, such as *Nfil3* and *Angptl7*, as well as a number of novel targets, such as *Zfp553* and *Zfp704*. The up-regulation of such genes as *Bim* (+1.76), *Ddit4* (+1.86), *Fkbp5* (+2.03), *Nfkbia* (+1.49), and *Txnip* (+1.68) in response to Dex treatment was similar to the results of previous microarray studies of mouse primary thymocytes (31–33).

The DAVID bioinformatics database was used to analyze a less stringently selected subset of genes in the microarray gene list (a P value of $<.05$ and a cutoff fold change value of $\pm$ 1.5) to detect more subtle gene expression changes and potentially identify genes acting synergistically in pathways. Functional cluster analysis revealed a number of genes associated with a variety of cellular processes, with a strong bias toward apoptosis, cellular growth and proliferation, and immune response, and a large number of membrane-associated proteins were identified (See Table 2).

To validate the microarray results, several of the most strongly differentially expressed target genes were analyzed using qRT-PCR with RNA from both GRKO and WT adult mice (Figure 1). These target genes were *Angptl7, Pgtr1, Nfil3, Zfp553, Zfp704*, and *Errfi1*. *Bim* and *p21*, which were characterized previously as GR gene targets involved in apoptosis, were included as positive controls. Expression levels of these targets were significantly lower in thymuses from GRKO mice than in WT controls in response to Dex treatment (Figure 1A). In thymuses from WT sham vs Dex-treated mice, there was a dramatic induction of these specific mRNAs, consistent with the microarray data, with all genes having significantly high levels 3 hours after Dex treatment (Figure 1B). A smaller number of genes was examined in isolated thymocytes from TGRKO mice (Figure 1C), with the expression of most the genes examined not increased by Dex treatment

Table 1. Selected List of Genes Differentially Regulated by Glucocorticoids in Thymus

Accession No.	Name	*P* Value	Fold Change	Gene Function
NM_001039554	Angiopoietinlike 7 (Angptl7)	3.95E-05	7.92	Overexpressed in glaucoma
ENSMUST00000110512	AA4647197	0.00033	7.87	Unknown
NM_025968	Leukotriene B4 12-hydroxydehydrogenase (Ptgr1)	1.86E-05	7.58	Anti-inflammatory activity
NM_017373	Nuclear factor, interleukin 3, regulated (Nfil3)	7.33E-05	5.97	Transcriptional repressor
NM_029858	Stonin 1 (Ston1)	7.69E-04	5.68	Endocytosis
NM_011580	Thrombospondin 1 (Thbs1)	1.17E-04	5.40	Adhesive glycoprotein
NM-026931	RIKEN cDNA 1810011O10 gene (1810011O10Rik)	1.88E-04	5.01	Unknown
NM_010516	Cysteine rich protein 61 (Cyr61)	4.85E-04	4.42	Promotes cell proliferation
NM_146201	Zinc finger protein 553 (Zfp553)	1.28E-04	3.98	Unknown
NM_008152	G-protein coupled receptor 65 (Gpr65)	1.34E-03	3.43	Glycosphingolipid receptor
NM_027460	Solute carrier family 25, member 33 (Slc25a33)	6.97E-05	3.39	Mitochondrial transporter
NM_133218	Zinc finger protein 704 (Zfp704)	1.50E-05	3.38	Unknown
NM_010287	G protein-coupled receptor 83 (Gpr83)	1.02E-03	3.36	Orphan receptor
NM_133753	ERBB receptor feedback inhibitor (Errfi1)	2.64E-05	3.24	Regulates EGFR signaling
NM_028658	KLRAQ motif containing 1 (Klraq1)	3.54E-05	3.10	Unknown
NM_019688	Rap guanine nucleotide exchange factor (GEF) 4 (Rapgef4)	1.23E-03	2.92	Guanine nucleotide exchange factor
NM_013642	Dual specificity phosphatase 1 (Dusp1)	1.24E-03	2.91	Phosphatase induced during oxidative and heat stress
NM_030180	Ubiquitin specific peptidase 54 (Usp54)	3.65E-05	2.84	Ubiquitin binding
NM_007974	Coagulation factor II (thrombin) receptor-like 1 (F2rl1)	4.99E-03	2.84	Regulation of vascular tone
	Solute carrier family 43, member 1 (Slc43a1)			Amino acid transporter

Affymetrix whole genome expression microarray analysis was used to identify genes regulated by GCs in thymus taken from GRKO and WT mice 3 hours after induction by a single 100 μL dose of 3 μg/μL Dex. Accession numbers for a subset of genes that experienced 2-fold change or more in signal intensity with a *P* value of $<.01$ and were of interest in the literature. *P* values were determined by ANOVA between the mean values of WT and GRKO normalized for signal intensities. The fold change is the relative level of expression in the WT vs GRKO animals after Dex treatment, measured as a ratio of normalized signal spot intensities of WT/GRKO (GR up-regulated) or GRKO/WT (GR down-regulated).

in TGRKO thymocytes. Interestingly, Errfi1 gene expression was unchanged in response to Dex treatment in TGRKO thymocytes, which indicates the induction observed in total thymus may be specific to thymic stromal cells and not thymocytes. Among the genes analyzed, *Nfil3* was consistently found to be significantly induced in response to Dex treatment of WT mice. *Nfil3* is a bZIP family transcriptional repressor that has a DNA binding specificity that overlaps that of the CREB-ATF family of transcription factors (43, 44). NFIL3 may play a role in the GC repression of some GC target genes, *Cox-2*, *iNOS*, and *cPLA2* (45), all of which contain NFIL3-like DNA binding elements located in their promoters. We therefore further examined *Nfil3* as a GC-responsive gene and its importance in GICD.

Nfil3 gene expression is induced by GCs via an upstream GRE

Analysis by qRT-PCR of *Nfil3* mRNA levels in uninduced GRKO primary thymocytes relative to those of WT (Figure 2A) showed that *Nfil3* mRNA levels are significantly lower in GRKO thymocytes, suggesting physiological GR signaling is important for maintenance of basal *Nfil3* mRNA levels. In a Dex-induction time course experiment on primary thymocytes, *Nfil3* mRNA was strongly and rapidly elevated (Figure 2B), providing additional evidence to support *Nfil3* as a direct, rapidly induced GR target. To understand the molecular mechanism by which GR regulates *Nfil3* gene expression, we analyzed the promoter and 5′ upstream region of the mouse *Nfil3* gene to look for putative GREs. The consensus GRE is the partially palindromic 15-bp sequence GGTACAnnnTGTTCT (1, 4). Analysis of a start site 30 kb upstream of the *Nfil3* gene transcription identified a potential GRE ~5 kb upstream from the *Nfil3* transcription start site (TSS) with a match score of 86.8 (Figure 2,Ci and Cii). We confirmed binding of GR to this GRE in primary thymocytes 1 hour after treatment with Dex using ChIP analysis compared with a proximal control region of the *Nfil3* enhancer region containing no obvious GRE sequence. The mouse *Gilz* gene GRE was used as a positive control for

Table 2. Functional Annotation Analysis of Genes Differentially Regulated by GCs in the Thymus

Function or Type of Protein Group	No. Genes in Cluster	*P* Value[a]
Phospho protein	174	8.8E-4
Membrane	149	6.2E-3
Intrinsic to membrane	139	6.5E-2
Transmembrane region	117	4.0E-2
Glycoprotein	101	1.2E-2
Glycosylation site: N-linked	98	6.2E-2
Topological domain: Cytoplasmic	90	3.4E-3
Plasma membrane	75	4.2E-2
Topological domain: Extracellular	71	8.6E-3
Disulfide bond	71	2.5E-2
Nucleotide binding	60	6.5E-2
Purine nucleotide binding	52	7.4E-2
Adenyl nucleotide binding	43	9.6E-2
Transcription regulator activity	35	9.4E-2
Mutagenesis site	32	1.7E-3
Endoplasmic reticulum	27	3.3E-2
Regulation of cell proliferation	24	3.2E-3
Golgi apparatus	24	1.9E-2
Regulation of apoptosis	21	3.0E-2
Regulation of programmed cell death	21	3.4E-2
Regulation of cell death	21	3.6E-2
Response to wounding	19	1.1E-3
Response to organic substance	19	4.4E-2
Transmembrane protein	17	1.0E-2
Immune response	17	7.6E-2

The DAVID bioinformatics database was used to analyze the microarray gene list. The Functional Annotation tool examined a list of genes with a fold change of ±1.5 and a *P* value of <.05.

[a] *P* values were determined by Fisher exact test.

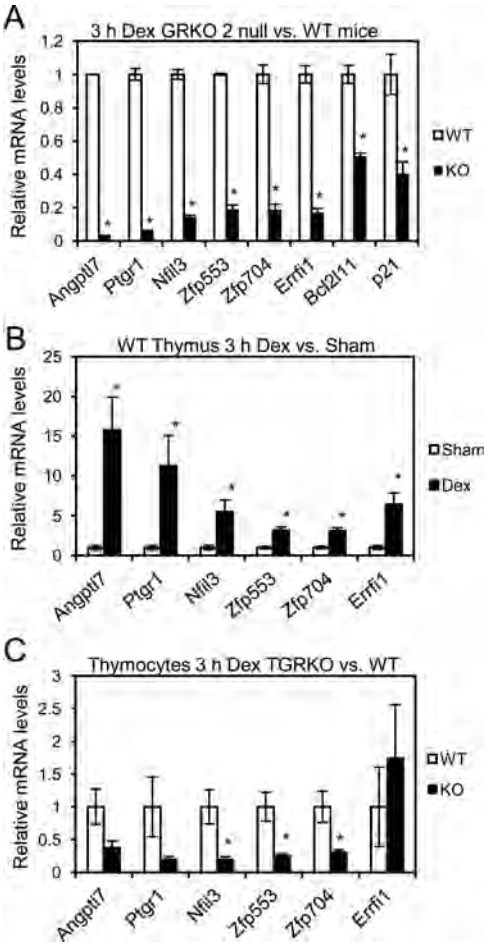

Figure 1. Validation of target genes differentially regulated by GCs. A, Gene expression changes in thymus from GRKO and WT mice (n = 4 mice) treated 3 hours with 20 mg/kg Dex. B, Gene expression changes in thymus from WT sham mice and mice treated for 3 hours with 20 mg/kg Dex (n = 3 mice). C, Gene expression changes in thymocytes isolated from WT vs TGRKO mice treated for 3 hours with 20 mg/kg Dex (n = 6 mice). Results are representative of single induction experiments, with qRT-PCR analysis repeated at least once for each target and a representative graph displayed. Bar graphs indicate mean ± SEM. Statistical analysis was performed using a Student *t* test. *Significant differences in transcript levels between vehicle-treated and Dex-treated mice ($P < .05$).

GR binding in these cells (46), whereas RNA Pol II binding to the TATA box of the *Gapdh* gene promoter was used as an experimental ChIP control (Figure 2D). Analysis of the rat genome revealed a conserved GRE (14/15 nucleotide match; Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) ~20 kb upstream of the rat Nfil3 gene, but a similar GRE sequence could not be found as much as 30 kb from the human Nfil3 gene (data not shown).

NFIL3 is localized in thymocytes undergoing apoptosis

NFIL3 expression in the thymus was analyzed by immunohistochemistry. In bright field sections, NFIL3 protein was widely detected in the thymus (Figure 3A). To further characterize the potential involvement of Nfil3 in GICD, double labeling was performed between NFIL3 and TUNEL stain on thymic sections after Dex treatment to mark apoptotic cells (Figure 3B). In overlaid images of NFIL3 and TUNEL staining (Figure 3B, iv and ix), NFIL3 protein is seen colocalized to apoptotic cells. A strong increase in the number of apoptotic cells was observed after 3-h incubation with 1 μM Dex relative to the sham control (Figure 3B, iv vs ix).

Nfil3 is required for normal GICD

We next investigated just how crucial *Nfil3* was during the initial events of apoptosis by knocking down *Nfil3* mRNA levels in the CTLL-2 cytotoxic T-lymphocyte cell line using siRNAs. CTLL-2 cells previously have been shown to be sensitive to GICD (47–49). We first demonstrated that *Nfil3* is present and responsive to Dex in these cells (Figure 4A). In time-course Dex-induction experiments, at 10 n M and 1 μM Dex, *Nfil3* mRNA levels were rapidly increased after 2- and 3-hour exposures to Dex (Figure 4A). These results show *Nfil3* is induced in CTLL-2 cells in a manner similar to that of mouse thymocytes, and therefore these cells provide a good model to examine the role of *Nfil3* in GICD. In trial experiments,

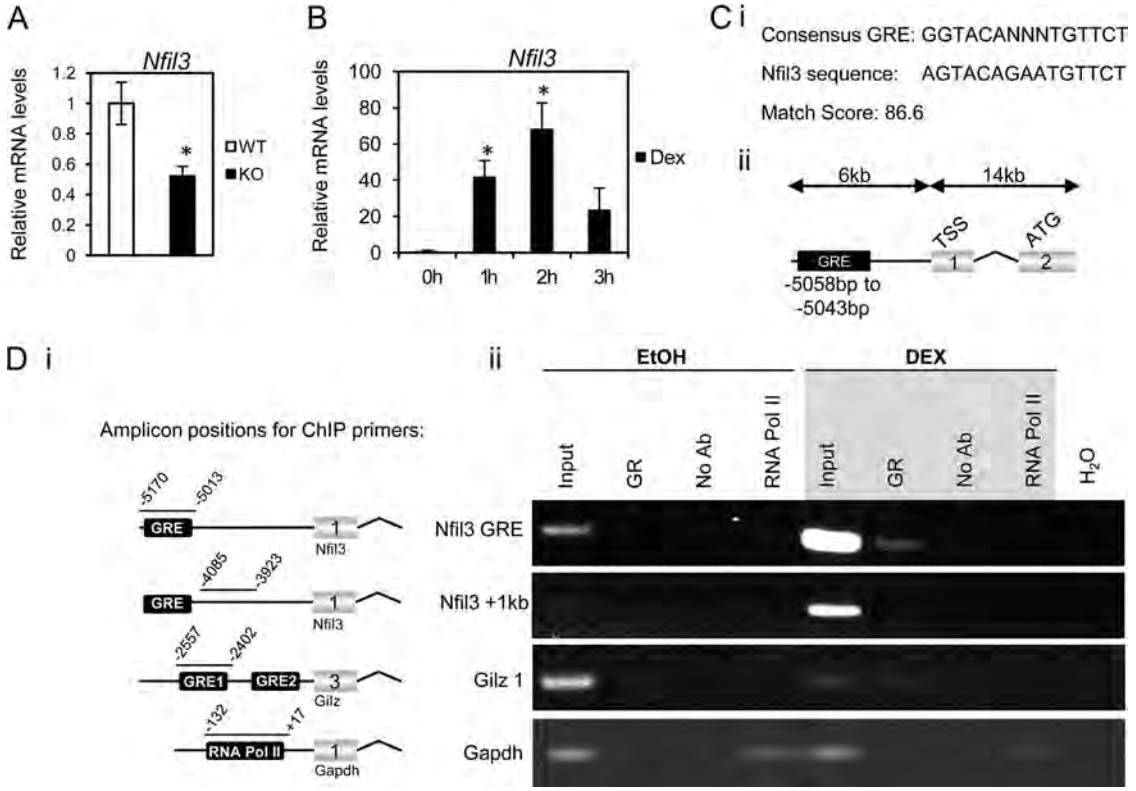

Figure 2. A, Basal Nfil3 expression levels in thymocytes of TGRKO mice vs WT mice. B, *Nfil3* gene expression changes in thymocytes isolated from C57BL/6 mice and treated individually in vitro over 3 hours with 1 μM Dex (n = 3 mice). Results represent a single experiment. Bar graphs represent mean ± SEM. Statistical analysis was performed using a Student *t* test (A) and ANOVA (B). Significant differences in transcript levels between WT and knockout mice (KO) or vehicle-treated and Dex-treated thymocytes are indicated by * ($P < .05$). C, (i) Alignment between consensus GRE sequence and putative GRE in Nfil3 distal promoter. (ii) Schematic representation of potential GRE along the Nfil3 gene distal promoter with reference to the TSS. D, (i) Schematic representation of amplicons targeted by PCR primers. Numbering is relative to TSS. (ii) ChIP analysis confirmed binding of GR to a GRE ~5 kb upstream of exon 1 (5058 bp upstream from the TSS) in primary thymocytes 1 hour after treatment with Dex compared with a proximal control region of the Nfil3 promoter containing no GRE. The mouse GILZ gene GRE (1) (46) was used as a positive control for GR binding in these cells, whereas the RNA Pol II binding site of the *Gapdh* gene was used as an experimental control. Results are representative of two independent experiments.

knockdown of *Nfil3* was optimized in comparison with a *Gapdh*-positive control siRNA oligonucleotide. Supplemental Figure 1 shows the percentage knockdown achieved (Ai) and cell viability (Aii). The same conditions were used during experiments; *Nfil3* mRNA levels were knocked down by specific *Nfil3* siRNAs to ~20% of normal (Figure 4Bi) by 48 hours, and cell viability was not affected (Figure 4Bii). Figure 4Biii shows percentage gene expression of some other targets after siRNA transfection of a negative control siRNA oligo acquired from Sigma, indicating there were no off-target effects. In addition, mRNA levels of c-myc, a putative Nfil3 target gene, were measured and found to be elevated (20-fold) 48 hours after transfection of Nfil3 siRNAs (Figure 4C). Forty-two hours after transfection, normal and *Nfil3*-deficient cells were treated for 0, 3, and 6 hours with 10 nM Dex and compared with vehicle control cells. AV staining is used to assess early apoptotic events in these cells and was monitored by flow cytometry. In preliminary experiments examining AV labeling from 0.5 to 24 hours at regular intervals, 2 to 6 hours after Dex administration was the optimal time frame to observe AV staining in CTLL-2 cells (data not shown). Figure 5A shows representative dot plots of the AV assay. In normal and *Nfil3*-knockdown CTLL-2 cells at time zero, the large majority of gated cells (>90%) are negative for both propidium iodide and AV staining (Figure 5, Ai and Aiii), indicating cells are viable at time zero. After 6 hours of Dex treatment in normal CTLL-2 cells, there is a clear shift in fluorescence toward AV positivity, indicating cells are going through early apoptosis (Figure 5Aii). This is in contrast to *Nfil3*-knockdown cells, in which this shift was not seen (Figure 5Aiv). In normal CTLL-2 cells after 6 hours of treatment with 10 nM Dex, it is evident that there is a significant increase in cell death, with an average of 45% to 50% being positive for AV and undergoing apoptosis compared with 10% at time zero (Figure 5, B and C). The number of *Nfil3*-knockdown cells positive for AV after 6 hours of Dex treatment was significantly less than that of their normal cell counterparts at this time point. These results further support

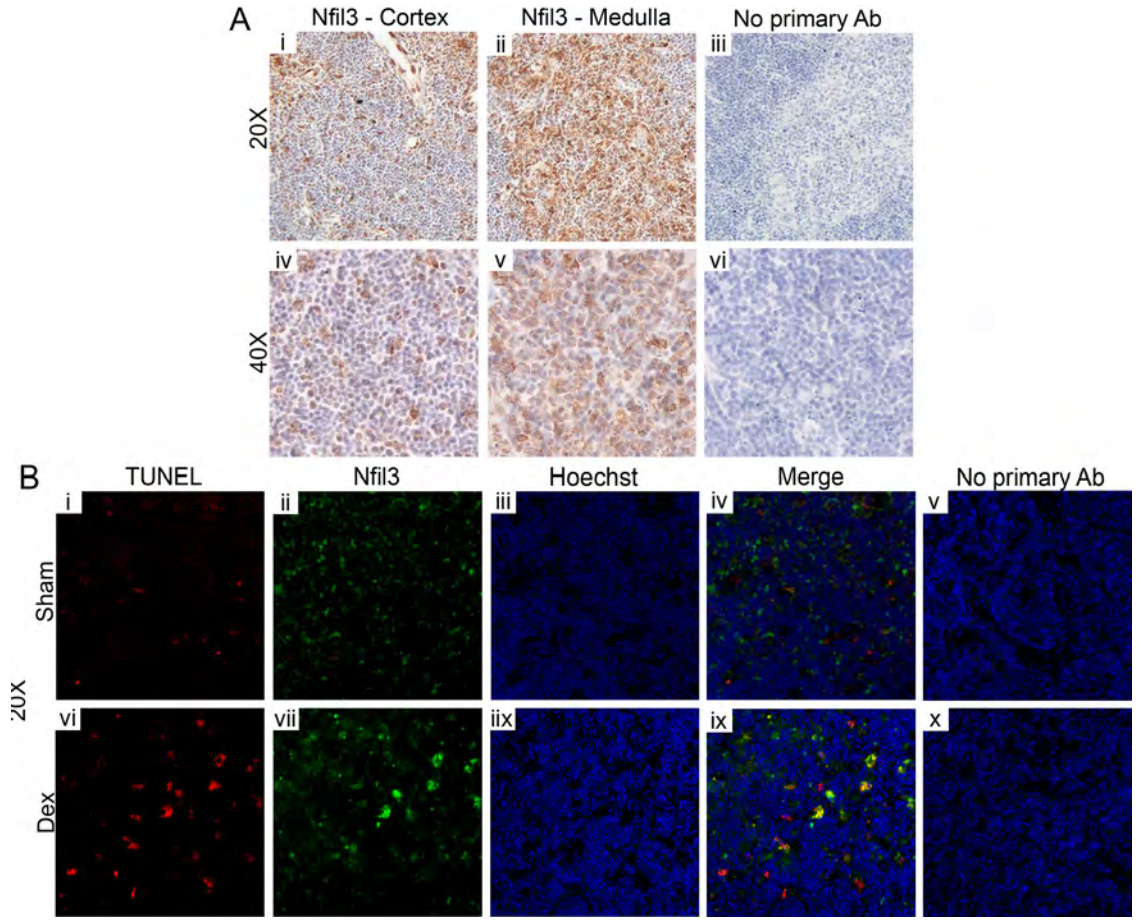

Figure 3. NFIL3 protein localization within the thymus. A, Bright field images, magnification ×20 (i–iii) and ×40 (iv–vi), of immunohistochemical staining for NFIL3 protein in 5-μm thymus sections from C57BL/6 mice 3 hours after treatment with 20 mg/kg Dex. B, Fluorescent images, magnification ×20 (i–v) and ×40 (vi–x), of immunohistochemical stain for NFIL3 protein and TUNEL stain showing apoptotic cells in 5-μm thymus sections from WT sham mice (i–v) or mice treated for 3 hours with 20 mg/kg Dex (vi–x). Results are representative of three independent experiments, with tissue from different mice used for each experiment.

that *Nfil3* plays a significant role in mediating the mechanism of GICD in T cells. Priceman et al (50) speculated that GC-mediated NFIL3 up-regulation may induce transcriptional changes, including gene repression, which can drive a cell toward apoptosis. In addition, it has been suggested previously that NFIL3 may mediate GC-induced gene repression of indirect GR targets, with NFIL3 binding sites found in the promoters of a number of genes known to be down-regulated by GR (45). Analysis of the promoters of a subset of negatively regulated genes identified in our microarray list for NFIL3 consensus binding sites [(G/A)T(G/T)A(C/T)GTAA(C/T) (43)], using the functional annotation clustering component of the DAVID bioinformatics database, generated a list of prosurvival/antiapoptotic genes that may be potential NFIL3 targets (Supplemental Table 2). Some interesting prosurvival target genes with NFIL3 binding sites in their promoters included C-Myc and Ccnd3. C-myc expression levels were also examined and found to have a 20-fold elevation in mRNA levels 48 hours after transfection with the Nfil3 siRNA (Figure 4C). Future studies will explore the role of these factors in NFIL3-mediated thymocyte apoptosis.

Discussion

This study focused on the role of a strongly Dex-induced target gene called *Nfil3* during thymocyte GICD. Studies of thymocytes from GR^{dim} mice, which are defective in DNA-binding–dependent transactivation of target genes, show the thymocytes are relatively resistant to GICD, thereby implying that GR-induced gene transcription is essential for GCID (51). Using ChIP in primary mouse thymocytes, we have demonstrated that GR binds directly to a region upstream of the *Nfil3* promoter and that this most likely accounts for the rapid induction of *Nfil3* mRNA after Dex treatment. NFIL3 was colocalized to apoptotic cells in the thymus, demonstrating its association with apoptotic thymocytes in vivo, and the induction of *Nfil3* in the cytotoxic T-lymphocyte cell line, CTLL-2,

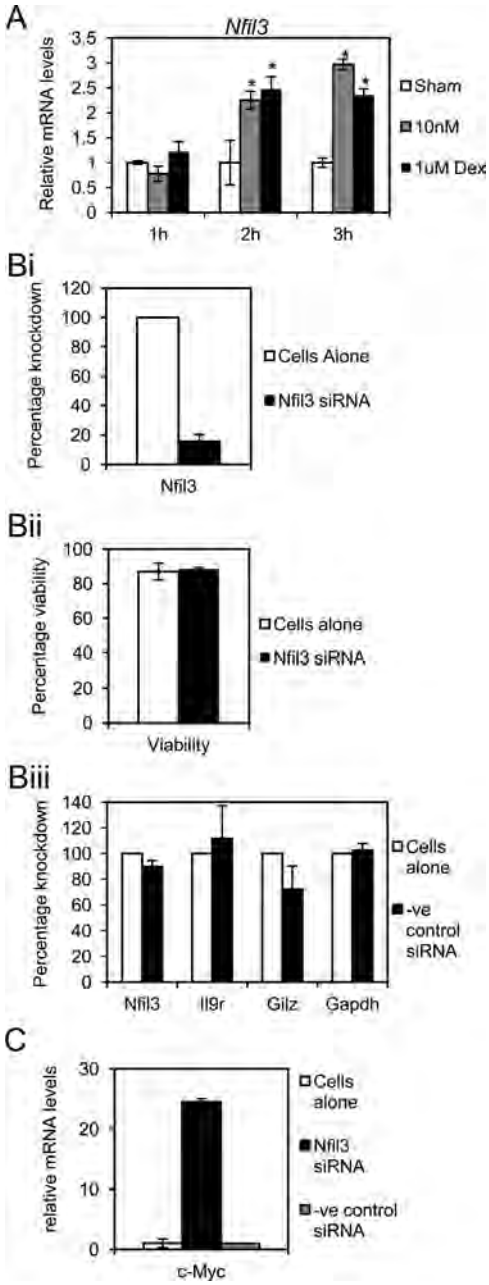

Figure 4. Nfil3 is regulated by Dex in CTLL-2 cells. A, *Nfil3* gene expression changes over 3 hours in CTLL-2 cells treated with 10 nM or 1 μM Dex; treatments were performed in triplicate, and results are representative of two independent experiments. B, (i) Percentage mRNA levels of *Nfil3* in CTLL-2 cells after siRNA transfection targeted to *Nfil3*. Results represent the mean percentage of two independent experiments. (ii) Percentage viability of cells after siRNA transfection. (iii) Percentage mRNA levels of various gene targets in CTLL-2 cells after transfection of a negative control siRNA oligonucleotide. Results represent the mean percentage of two independent experiments. C, c-myc Expression level in Nfil3-deficient CTLL-2 cells. mRNA levels of *c-Myc* after siRNA transfection targeted to *Nfil3* or transfection of a negative control siRNA oligonucleotide. Results represent the mean percentage of three independent experiments. Bar graphs show mean ± SEM. Statistical analysis was performed using ANOVA. *Significant differences in mRNA levels between sham and Dex-treated cells ($P < .05$).

has been characterized, demonstrating that the response is comparable to that of thymocytes in vivo. By decreasing expression of *Nfil3* in CTLL-2 T cells using siRNA technology, we have demonstrated that *Nfil3* is necessary for the normal GC-induced apoptosis response.

Our analysis of Dex-induced genes in the mouse thymus using whole genome expression microarrays has discovered a large subset of acutely GC-regulated genes. Functional cluster analysis showed many of these induced genes were linked to cell proliferation or apoptosis, giving a number of interesting candidates for mediating GICD in mouse thymocytes. A recent study has demonstrated that genes conferring sensitivity to GICD are not necessarily the same in malignant cells as they are in primary thymocytes. They identified only eight genes that were in consensus between various cancer cell lines and thymocytes, including *BCl2l11 (Bim), Cdc6, Ddit4, Fkbp5, Nfkb1a*, and *Txnip*. Five of these genes, *Bim*, *Ddit4*, *Fkbp5, Nfkbia*, and *Txnip,* also were induced in our microarray study and in other published reports (31–33). Interestingly, all these targets except *Bim* are thought to be antiapoptotic, so their early induction may be an effort to protect cells from apoptosis.

The role of NFIL3 as a potential proapoptotic protein has been described previously, where overexpression of NFIL3 facilitated the induction of p53-mediated apoptosis in murine myeloid leukemia cells (52, 53). In addition, a microarray analysis performed by Woodward et al (33) observed *Nfil3* as an up-regulated GR target in primary mouse thymocytes. Nfil3 has been shown to have many functions in the immune system; it is essential to intrathymic natural killer cell development (54) and cD8a$^+$ cDC development (55), and it regulates cytokine production in multiple T-cell subsets (56) and has a role in macrophage function. In a study investigating the kinetics of apoptosis induction in mouse thymocytes, TUNEL labeling was shown to increase from 2 to 7 hours (57); thus, it may be expected that cells positive for Nfil3 may later become positive for TUNEL.

To investigate if *Nfil3* is necessary for normal GICD, we have strongly reduced *Nfil3* mRNA levels by 80% with siRNA knockdown using a combination of three *Nfil3*-targeted siRNA oligonucleotides. Our results showed that after 6 hours of Dex treatment, there was a significant reduction to the extent of apoptotic cell death in CTLL-2 T cells. Thus, we have demonstrated that *Nfil3* is directly linked to GC-mediated apoptosis in thymocytes and plays an essential role in this process in CTLL-2 T cells. Priceman et al (50) reported that in human CEM-C7-14 cells, Ca^{2+} was necessary for Dex-induced up-regulation of *Nfil3* and subsequent apoptosis of cells. We observed a significant increase in AV-positive apoptotic cells between

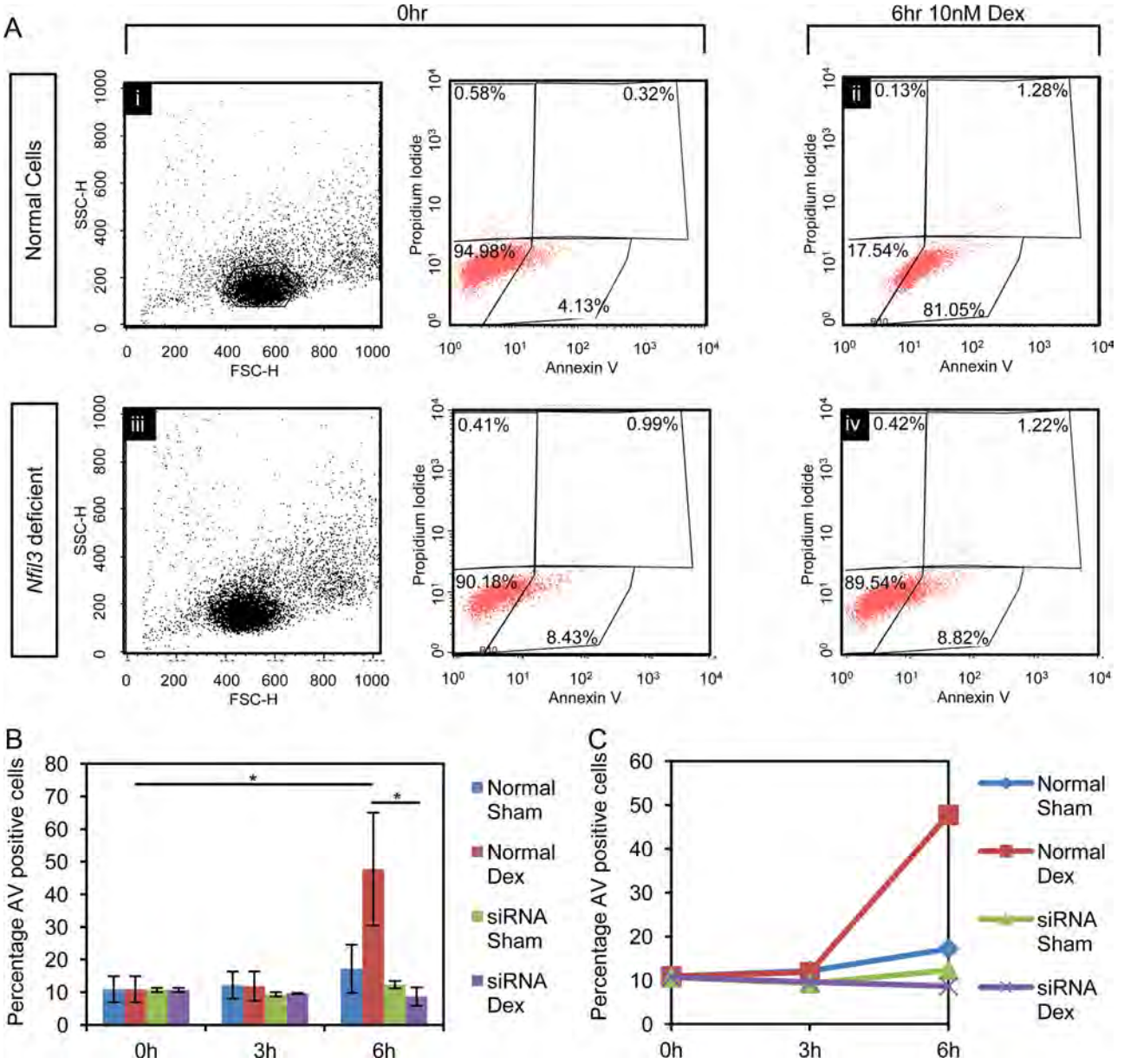

Figure 5. *Nfil3* is required for GC-induced apoptosis in CTLL-2 cells. A, Representative dot plots of AV staining in CTLL-2 cells before treatment (i and iii) and after 6 hours of treatment with 1 μM Dex (ii and iv) in normal (i and ii) and Nfil3 siRNA-transfected cells (iii and iv). B and C, Bar and line graphs represent the mean percentage of cells positive for AV and AV + propidium iodide staining in normal and *Nfil3*-deficient cells over a period of 6 hours for sham cells and those treated with 10 nM Dex and 10 nM Dex + EGTA from three independent experiments. Bar graphs show mean ± SE. Statistical analysis was performed using ANOVA. *Significant differences in percentage of apoptotic cells ($P < .05$).

zero-hour CTLL-2 cells and those treated for 6 hours with 10 nM Dex and 20 μM of the calcium chelator EGTA and only a slight decrease (5%) in the number of AV-positive apoptotic cells between cells treated with 10 nM Dex alone and those treated with 10 nM Dex and 20 μM EGTA for 6 hours (data not shown). Priceman et al (50) observed 20% more viable cells in cells treated for 4 days with 100 nM Dex and 20 μM EGTA than in ethanol-treated control cells as monitored by Trypan blue exclusion. However, given that the experimental design of our study differs greatly from that of Priceman et al and that the human cells used respond much more slowly to Dex in terms of apoptotic induction, the cellular response and timeline of treatment of the two studies are not comparable, and neither is the method of cell viability determination.

A novel GRE 5 kb upstream of the mouse Nfil3 gene TSS was described that binds the GR, and analysis of the rat genome revealed a similar conserved GRE (14/15 nucleotide match; Supplemental Table 2) 20 kb upstream of the rat Nfil3 gene. This agrees with recent genome-wide GR-binding studies that show that most GR-binding sites

can reside more than 10 kb from regulated genes (8, 58). A search of the human genome 30 kb either side of the human Nfil3 gene failed to locate a similar GRE, which may explain why an identical GRE was not detected in studies with human cell lines (8), although it may lie further from the TSS and have a greater sequence variation. Recent GR ChIP-seq analysis using the mouse 3134 mammary adenocarcinoma and mouse AtT-20 pituitary cell lines did not detect a GR-binding site with the AtT-20 pituitary cell line (8). In 3134 mammary cells, a binding site was localized close by, and a DNase I hypersensitive site was mapped directly over the position of the Nfil3 GRE before and after Dex treatment, indicating this is an open region of chromatin that could be accessible to GR binding. In addition, expression analysis in mouse 3134 cells after Dex treatment detected 265 up-regulated genes, with Nfil3 ranked 30th in fold change after 2 hours. This highlights the cell-type specific differences in GR activity and binding to DNA. Therefore, the Nfil3 GRE is most likely cell-type specific in function, may require the binding of other factors, and in our study may be specific for the activation of GCID in immune cells.

NFIL3 has been characterized as an active transcriptional repressor, binding to response elements in the promoters of target genes and inhibiting their transcription directly, not by inhibiting binding of an activator by competitive binding to DNA or by protein-protein interactions with an activator (43, 44). Active transcriptional repressors are presumed to cause repression by interfering with the formation of preinitiation complex (44). NFIL3 has been shown to interact directly with DNA topoisomerase 1 (Dr1), which has the ability to down-regulate both basal and activated transcription at a variety of promoters (59). It has been suggested that NFIL3 may play a role in mediating GC repression of the GC target genes *Cox-2, iNOS*, and *cPLA2* (45), all of which contain NFIL3-like response elements located in their promoters. From our microarray data, we have identified genes that are down-regulated in mouse thymus after 3 hours of Dex treatment, have been previously associated with apoptosis, and have putative NFIL3 response elements within their promoters. Two genes of particular interest are c-Myc and Ccnd3. Previous studies in other cell lines have indicated c-Myc and Ccnd3 are down-regulated by GCs in various murine lymphoma cell lines (29, 60–62). Repression of these genes does appear essential for cell cycle arrest in human CCRF-CEM leukemic cells; however, their specific involvement in apoptosis and GICD individually or together is quite varied among cell lines and requires additional investigation (60–62).

When investigating the c-myc 5′UTR ,our analysis identified two potential NFIL3 binding elements 1103 bp and 9588 bp upstream from the beginning of exon 1. C-myc expression decreases rapidly in response to GCs (62), suggesting direct GR action upon the c-Myc promoter; however, the mechanisms of GC suppression of c-myc have not been fully elucidated (63, 64). Preliminary experiments revealed an increase in c-Myc expression after Nfil3 knockdown in CTLL-2 cells, indicating a possible mechanism for the protective effect against GICD. In silico promoter analysis identified two NFIL3 binding elements in the promoter of the Ccnd3 gene; however, GC regulation of Ccnd3 has been shown to occur largely via RNA-protein interactions occurring in the 3′UTR (65).

Our study sheds light on rapidly induced GR target genes in thymocytes and in particular characterizes a rapidly induced GR target gene, *Nfil3*, a transcriptional repressor. *Nfil3* shows a similar pattern of Dex induction in CTLL-2 T cells and *Nfil3* is necessary for normal GICD in these cells. Identification and analysis of target genes directly repressed by NFIL3 and similar analysis in vivo using Nfil3 knockout mice will further elucidate the intracellular pathway and role of steroid-induced GICD within the immune system.

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CHAPTER 4:

DIFFERENTIAL ACTIVITY OF THE GR GENE 1A PROMOTER IN THE PITUITARY AND B CELL LINEAGES OF THE MOUSE

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Project direction, development and writing up of paper, qRT-PCR analysis, in situ hybridization	70%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Viola Lobert	B-lymphocyte flow cytometry, qRT-PCR analysis	
Douglas Liddicoat	B-lymphocyte flow cytometry,	
Jared Purton*	B-lymphocyte flow cytometry anlysis	
Timothy Cole	Project direction, development and writing up of paper	

Candidate's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

Signature 1	[redacted]	**Date: 7/12/2012**
Signature 2	[redacted]	**9/12/2012**
Signature 3	[redacted]	**7/12/2012**
Signature 4	[redacted]	**7/12/2012**

(* signed on behalf as deceased)

Differential activity of the glucocorticoid receptor gene 1A promoter in the pituitary and B lymphocyte lineage of the mouse

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Abstract

The glucocorticoid receptor (GR) is an important endocrine mediator of the response to stress. Expression of the GR gene in rodents and humans is controlled by multiple promoters, including a far upstream promoter designated GR1A. Previous studies have localised GR1A transcripts to the cortex of the brain and to subsets of the T-lymphocyte lineage. The physiological role of GR1A promoter activity in the brain is unknown. Using qRT-PCR and *in situ* hybridisation we have detected GR1A transcripts in the anterior pituitary and to distinct populations of the developing B-lymphocyte lineage. Following dexamethasone treatment a 2.5 fold increase in GR1A promoter usage was observed in the pituitary. An increase in GR1A promoter activity from pro-B to mature B-cells indicates increased sensitivity to glucocorticoid-induced cell death. Increased GR1A promoter activity in specific cells during stress may enhance cell sensitivity to glucocorticoids, promoting a return to a normal homeostatic state after stress.

(149 words)

Key words: glucocorticoids, glucocorticoid receptor, pituitary, B-lymphocytes, glucocorticoid-induced cell death

1. Introduction

Glucocorticoids (GCs) play essential roles in the regulation of biological processes such as growth, development, metabolism, behavior and apoptosis, and for the biological response to stress. The immunosuppressive and anti-inflammatory actions of GCs make them a widely prescribed class of drug to control autoimmune diseases and airway inflammation in respiratory diseases such as asthma, and their lymphocytolytic actions are used for the treatment of acute lymphoblastic leukaemias (ALLs) (Sapolsky, et al. 2000). The actions of GCs are mediated via the glucocorticoid receptor (GR), a transcription factor which belongs to the superfamily of nuclear receptors that also includes receptors for mineralocorticoids, thyroid and sex hormones, vitamin D and retinoic acid (Pujols, et al. 2004).

In the brain GCs act primarily on GRs localized within the hippocampus, the cortex and also the hypothalamus and pituitary where they have a key role in the biological response to stress. In response to an immune challenge or stress signal, corticotropin releasing hormone (CRH) is secreted from the hypothalamus (Charmandari, et al. 2004) and subsequently acts on the anterior pituitary, stimulating the secretion of adrenocorticotropin hormone (ACTH), which in turn promotes the synthesis and secretion of GCs, such as cortisol (in humans) or corticosterone (in rodents), from the adrenal cortex. GCs are carried to target cells through the bloodstream and diffuse freely across the cell membrane to their receptors (Zhou and Cidlowski 2005) causing dimerisation, activation and nuclear import where they can act upon target genes which contain glucocorticoid response elements (GREs) within their promoters. Negative feedback via ACTH and CRH serve to limit the production of GCs enabling a rapid return to the normal state after a stress event (Sapolsky et al. 2000).

The GR gene is composed of 9 exons, with exons 2-9 encoding the GR. Exon 1 is untranslated and is alternately spliced to yield at least 13 transcripts in humans and at least 10 in mice (Chen, et al. 1999; Turner and Muller 2005). It is believed that each of the alternate transcripts is under the direction of their own promoter conferring a complex tissue-specific distribution to each of the individual RNA transcripts through interaction with unique combinations of regulatory transcription factors. Transcripts initiating from the GR1A promoter in particular has previously been localized in the mouse to T- and B-lymphocytes, and the cortex of the brain (Purton, et al. 2004a), and very recently have been detected in the pituitary, hippocampus and hypothalamus (Bockmuhl, et al. 2011). The relative level of expression of the

GR1A exon compared to the remaining exons 1 within a T-lymphocyte population was found to directly correlate with susceptibility to GC induced cell death (GICD), with immature, $CD4^+CD8^+$ DP cells the most sensitive to GICD (Purton, et al. 2004b). It was proposed that increased activity of the GR gene 1A promoter may serve to make thymocytes more sensitive to increased levels of GCs. Pedersen et al., (2004) have proposed that increased levels of human GR mRNA and increased expression of the GR-B translational isoform via an increase in exon 1A3 (homologous exon in humans)-containing transcripts, together with no decrease in the stability of GR protein in the presence of GCs, contribute to the increased sensitivity of human T-lymphocytes to GCs and thus results in a strong apoptotic response.

The expression of the GR gene 1A promoter in the cortex of the brain may also serve a similar role to that in thymocytes, to make cells more sensitive to GC levels, thus ensuring that cells of the cerebral cortex can respond to rising stress-induced levels of GC (Purton et al. 2004b) and this may also apply to other cell-types in the brain. Studies have shown that B cells are also differentially susceptible to GC treatment according to their stage of development (Andreau, et al. 1998). GC treatment resulted in the depletion of lymphocytes, especially precursor B cells (pro-B, pre-B and immature B cells), whereas mature B cells were shown to be more resistant (Voetberg, et al. 1994). This suggests that the hematopoietic stage of development is a factor in determining sensitivity to glucocorticoid-induced cell death in B-cells (Lill-Elghanian, et al. 2002). Our study focused on GR1A transcripts in the brain and the immune system. We have detected of GR1A transcripts in the pituitary, further defining localization to cells in the anterior pituitary and have examined the regulation of the GR1A promoter in response to dexamethasone treatment. We have further examined the B-lymphocyte lineage for expression of GR1A-containing transcripts and determined that a similar correlation does not exist between developmental maturity and GR1A levels, as it does with T-lymphocytes.

2. Materials and Methods

2.1 Mice: All animal experimentation was approved by the School of Biomedical Sciences Animal Ethics Committee, Monash University (Ethics no. SOBSB/2009/63) and was carried out according to the National Health and Medical Research Council of Australia guidelines for the breeding, care and use of genetically modified and cloned animals for scientific purposes 2007. C57/B6 mice were treated with 20mg/kg dexamethasone, sacrificed and thymi removed and placed into 5% PBS-FCS for further analysis. Spleen and bone marrow were removed from 6 week old C57B10/J mice and placed into 2% PBS-FCS for further analysis. To allow the isolation of bone marrow, the leg was cut under the petulla and at the hip joint, releasing an intact femur.

2.2 Preparation of cell suspensions: For thymocyte isolation, thymi were removed from untreated C57/B6 mice, cells isolated and treated *in vitro*. In each case, thymi were immediately collected into 5% PBS-FCS, and then placed in a petri dish containing 5% PBS-FCS. For bone marrow isolation the petulla was removed and bone marrow was flushed into 2% PBS-FCS to a total volume of 2mL. Spleen cell suspensions were made using 5ml 2% PBS-FCS. The spleen was cut once longitudinally and several times width-wise at similar intervals with the end of a glass slide, allowing the cells to enter the solution by capillary action when crushed between the frosted parts of two glass slides. Cell suspensions were filtered through a 100μm nylon mesh. Erythrocyte lysis was performed on bone marrow and spleen suspensions by incubation with 4ml red cell lysis buffer for 4 min at room temperature. The cell solution was then underlayed with 0.5 ml 100% FCS and centrifuged at 217Xg for 4 min. The supernatant was removed and the pellet resuspended in 2 ml 2% PBS-FCS. Cells were counted using a Beckman Z2 Coulter Counter (for both spleen and bone marrow) using 10μL of cell suspension, added to 10 ml PBS.

2.3 Cell staining: Cell staining was performed on spleen and bone marrow cell suspensions prior to cell sorting. $5X10^7$ cells were incubated with monoclonal antibodies on ice for 20 minutes in 5% PBS-FBS, for the spleen cells and bone marrow cells, respectively. Two types of antibodies were used; directly conjugated to the fluorochrome and biotinylated monoclonal antibodies. All antibodies were purchased from Pharmingen. Primary antibodies included Anti-B220 PE, Anti-CD24 PE, Anti-CD25 APC, Anti-CD23 FITC, Anti-CD21 Biotinylated and Anti-IgM FITC. Directly conjugated antibodies were all added and incubated simultaneously, however the biotinylated antibodies required a further incubation step with Streptavidin-$PECy^7$. Fc Receptor

(FcR) block was added to each antibody incubation (dilution 1:100) to reduce unspecific binding of the antibody via its constant unspecific region FcR. Cells were washed with 2% PBS-FBS and centrifuged at 202Xg for 3 minutes after each incubation.

2.4 Fluorescence Activated Cell Sorting (FACS): Sorting of B-cell populations from spleen and bone marrow was performed using the FACSAria 7-colour flow cytometer. Data was collected using the software program FACS Diva. Selective gating of cells was made according to the expression of cell surface markers specific to different developmental stages of B cell lineage.

2.5 In vitro cell culture: For qRT-PCR assay primary thymocytes were cultured in RPMI media with 5% FCS and 1uM Dex for 0-, 1-, 2- and 3h. mouse AtT-20 and LBT2 cells and rat GH3 cells were grown in DMEM (Invitrogen) containing 10% FCS (Thermoscientific), 1x Penicillin/Streptomycin (Sigma) and 2mM L-Glutamine (Sigma) in a humidified atmosphere of 95% air and 5% CO_2.

2.6 Isolation of Total RNA: Total RNA was isolated from mouse tissues and pituitary cell lines by homogenisation in TRIzol reagent (Invitrogen) as previously described (Cole, et al. 1999). Total cellular RNA was isolated from FACS-sorted B cell populations using the RNeasy Mini, RNA isolation kit (Qiagen, Australia) according to the manufacturer's specifications. Total RNA was eluted from the RNeasy Mini columns with 30 µL of nuclease-free water. Sorted B cells required a further step of purity to ensure no DNA contamination. DNaseI treatment was done using the DNase kit (Qiagen, Australia) according to the manufacturer's specifications.

2.7 Synthesis of cDNA and qRT-PCR: Gene expression levels were determined using Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Total RNA from mouse tissues and cell lines was transcribed into cDNA using random hexamers and M-MLV reverse transcriptase, RNase H Minus, Point Mutant (Promega,Madison, WI). Primer pairs were designed using Primer3 software (Rozen et al., 2000). Primers for quantitative real-time PCR (qRT-PCR) are mGR1A/2 Fwd 5'- CGTTAAGATGTCTGGGAGGAAGTT -3', REV 5'-GGTTTTATACAAGTCCATCACGCTT-3'. Assays (in triplicate) were repeated at least twice with the 18S rRNA, whose expression levels are stable between samples of the same type and are not influenced by GCs, used as an internal control between samples. Cycling was performed using Platinum SYBR Greener qPCR SuperMix (Invitrogen, Carlsbad, CA) on a Rotor- Gene 3000 (Corbett Research, Sydney, New South Wales, Australia). qRT-PCR data were analyzed using RotorGene 6.0 software (Corbett Research), and differential expression was determined

using the comparative ΔΔCT method (Pfaffl et al., 2001). For qRT-PCR experiments comparing only two groups (such as WT vs KO or Sham vs Dex) statistical analysis was performed using a students t-test. For qRT-PCR experiments analysis by ANOVA was used for studies involving more than two groups.

2.8 In situ hybridization on Paraffin embedded tissue sections: Tissues were fixed in 4% PFA immediately after dissection then gradually dehydrated into 70% ethanol and processed into paraffin. 12μm sections were collected onto superfrost plus slides and incubated at 40°C overnight. Prior to experiment, slides were placed at 60°C for 30 min and then dewaxed through 3 changes of xylene, 5 min each, then rehydrated through ethanol to water and placed into PBS. Slides were incubated in active DEPC in PBS, then for 30 min in 0.3% Triton x-100 in PBS and washed in PBT. For fluroescent detection slides were incubated with 10mg/mL sodium borohydride in PBS for 40 min to block autofluorescence. Sections were digested with 1ug/mL Proteinase K in PBS for 20 min after which time Proteinase K was deactivated with 0.2% Glycine. Slides were washed in PBT and post fixed for 10 min in 4% PFA and washed in PBT prior to Pre Hybridization in Hybridization buffer (4 X SSC, 20% Dextran Sulphate, 50% Deionised Formamide, 0.25mg/mL PolyA, 0.25mg/ml ssDNA, 0.25mg/ml tRNA, 0.5x Denhardts; make up with DEPC ddH_2O) for 2 h at 55°C in a humidified chamber. Hybridization was performed with a 230bp antisense Digoxigenin (DIG) labelled riboprobe diluted in Hybridization buffer at a concentration of 1mg/mL overnight at 55°C in a humidified chamber followed by high stringency SSC/formamide washes at 55°C. Slides were treated with 20ng/mL RNase A in TBST for 5 min at 37°C to decrease background, washed in TBST and finally washed in MABT at room temperature. Slides were blocked in Blocking Solution (1.1% Blocking Reagent, 10% Sheep serum in MABT), then anti-DIG-Alkaline Phosphatase diluted 1:1000 in Blocking Solution was applied to slides overnight at 4°C. Primary Antibody was washed off in MABT. BM Purple substrate (Roche) was then applied to slides and left to develop overnight. Slides were washed in PBS and counterstained with nuclear fast red or hemotoxylin, dehydrated and mounted with DPX mounting medium. For negative control, sense probes were applied.

2.9 Immunohistochemical Labelling; Fluorescence on paraffin embedded tissue sections: For co-localization experiments the method described for *in situ* hybridization was followed up to the point of washing off excess BM Purple precipitating substrate. At this time, sections were

incubated with primary antibody (mouse anti LH (1/1000, a kind gift of Javed Iqbal, Dept Physiology Monash University, Clayton), Rabbit anti mouse ACTH (1/1000, Abcam), Rabbit anti mouse PRL (1/50, Santa Cruz), Rabbit Anti mouse s100 (1/50, Santa cruz), diluted in Block Buffer (TBST plus 5% serum from host of secondary antibody) for 1hr at RT or 4°C overnight. Slides were washed 3 x 5min in TBST before the addition of secondary antibody (Biotin anti mouse IgG (1/250,, Goat anti rabbit Alexa Fluor 488 (1/1000, Invitrogen), Goat anti Rabbit Alexa Fluor 568 (1/1000, Invitrogen) or Goat anti Rat Alexa Fluor 555 (1/500, Invitrogen) diluted in Block Buffer for 30 - 60 min. Slides were washed 3 x 5min, and if necessary a streptavidin 488 conjugated tertiary antibody was applied (1/1000,) diluted in Block Buffer for 30 – 60 min. Slides were washed 3 x 5min. Fluorescent slides were immediately counterstained with Hoechst (1/2000 in PBS) washed 3 x 5 min in PBS, quickly washed in dH_2O and mounted using fluorescent mounting medium (Dako). Brightfield images were taken on an Olympus CKX41 using NIS-Elements software F3.0, Fluorescent images were captured on an Olympus IX71 using Spot Advanced software v4.6. Image J was used to artificially colour and overlay fluorescent images.

2.10 Statistical analysis: Statistical analysis was performed using Microsoft excel. Specific statistical tests are described under each method. p values < 0.05 were considered statistically significant.

3. Results

3.1 Analysis of GR1A promoter activity in mouse tissues and cell lines.

To further investigate the presence and localization of transcripts initiating from the GR1A promoter, qRT-PCR and in situ hybridization were used with samples from different regions of the mouse brain. Primers specific for the GR exon 1A/exon 2 boundary were used to detect GR1Acontaining transcripts by qRT-PCR. Relative levels of total GR mRNA were determined using primers spanning the GR exon 2/3 boundary and detected GR transcripts initiating from all GR gene promoters. The mouse brain was dissected into the following regions; cerebellum, brain stem, midbrain, cortex, hypothalamus and pituitary, and represented major regions expressing high levels of GR. In addition to the thymus, transcripts from the GR1A promoter were detected in the brain cortex, pituitary, midbrain and at low levels in the spleen (Fig. 1A). The percentage of GR1A usage (Fig. 1C) is a measure of the relative GR1A promoter usage compared to the other GR promoters and is calculated by dividing the raw values for GR1A transcript levels by the total GR transcript levels (Fig. 1B). In summary, GR1A promoter activity was highest in the pituitary and cortex of the brain, with GR1A promoter usage as a percentage of total GR promoter activity, highest in the pituitary. *In situ* hybridization for GR1A transcripts was performed in brain cortex, pituitary, thymus and kidney (Fig. 2). GR1A transcripts were detected in brain cortex, where staining appeared consistent with a pattern in specific regions containing cortical neurons (Fig. 2A). Specific localized staining was detected in regions of the pituitary (Fig. 2B). Staining was also observed in the thymus (Fig. 2C), with staining distributed evenly between the cortex and the medulla. No staining was observed in section of the mouse kidney (Fig. 2D).

3.2 GR1A-encoded transcripts are regulated by dexamethasone in the pituitary.

Auto-regulation of the GR1A promoter by GCs has been well characterized in mouse thymus and T-cell lines of human and mouse origin (Breslin, et al. 2001; Geng and Vedeckis 2004; Pedersen, et al. 2004; Purton et al. 2004b). Therefore, regulation of mGR1A promoter activity by GCs was examined in regions of the brain found to have high levels of GR1A promoter activity. Male C57/B6 mice were injected with 20mg/kg BW of dexamethasome-21-phosphate, or PBS as a control, and tissues harvested after three hours. Relative GR1A transcript levels from the cortex and pituitary of mice were then analyzed by qRT-PCR. In both the pituitary and cortex, dexamethasone treatment did not affect the absolute level of GR1A-encoded mRNAs relative to

control treatment (Fig. 3A). In the pituitary however dexamethasone treatment decreased levels of total GR transcripts (Fig. 3B). In the pituitary GR1A promoter usage was therefore increased dramatically after dexamethasone treatment to 23% of total, a 2.6 fold increase over the controls (Fig. 3C). This reflects the fact that the GR1A transcripts levels were maintained following treatment with dexamethasone, while total GR levels were decreased. There was little variation in GR1A promoter usage in the cortex of the brain between treatment groups.

3.3 Cells localized with GR1A transcripts are surrounded by folliculostellate cells in the pituitary

Quantitative-RT-PCR was used to detect GR1A transcripts in dissected regions of the pituitary and to assess expression of the GR1A promoter in pituitary cell lines which included mouse AtT20 corticotrophic cells, mouse LBT2 gonadotroph cells, and rat GH3 somatotroph cells (Fig. 4). As shown in Figure 4A, except for thymus, the GR1A transcript levels were highest in the anterior pituitary and virtually absent in all other tissues or cell lines tested. The percentage of GR1A promoter activity followed this trend (Fig. 4C). The pituitary is primarily composed of five major hormone-secreting cells, which are characterized by the hormones they produce and are easily detected by immunohistochemical staining of the protein components of those hormones; these include corticotrophs, which secrete ACTH; lactotrophs, which secrete prolactin (PRL); somatotrophs, which secrete growth hormone (GH); gonadotrophs, which secrete leuteinizing hormone (LH) and follicle stimulating hormone (FSH); and thyrotrophs, which secrete TSH. The other main cell type is the folliculostellate (FS) cell which are non-hormone secreting, but can be detected by immunostaining for the s100 protein. To determine whether GR1A transcripts were present in other cell types of the pituitary we performed double label in situ hybridization and immunohistochemistry (Fig. 5). As expected from qRT-PCR results, GR1A transcripts are not present in corticotrophic cells, detected by immunostaining to ACTH (Fig. 5A). GR1A transcripts were also not present in lactotrophs, marked by PRL immunostaining (Fig. 5B), or gonadotrophs, marked by LH staining (Fig. 5C). Although s100 protein and GR1A transcripts were not completely overlaid it does appear that GR1A positive cells are closely surrounded by FS cells (Fig. 5D).

3.4 GR1A transcript levels increase with development of the mouse B-cell lineage.

Mature B-cells are generated from pluripotent hematopoietic stem cells (HSC) in the fetal liver or in the adult bone marrow (Busslinger 2004). At the Pro B-cell stage, Ig heavy chain recombination occurs and cells begin to undergo positive selection, and from here they enter the Pre B-cell stage and recombination at the Ig light chain locus is initiated. During the immature B-cell stage, cells are subjected to negative selection to delete autoreactive cells. Non-reactive immature B cells emigrate to the spleen and are known as transitional 1 (T1) B-cells, which are subjected to negative selection. Those which survive selection become transitional 2 (T2) B-cells and finally after positive selection become mature, immunocompetent B cells in the spleen, classified as Follicular Mature (residing in follicles) or Marginal Zone (MZ) cells (reviewed in (Chung, et al. 2003). FM cells are able to re-circulate and eventually re-enter the bone marrow and correspond to naïve recirculating mature B cells. The 'immature' cells from the bone marrow are in fact at a similar stage of development as the T1 splenic cells, as they have simply exited the bone marrow and reached the spleen. Thus throughout B-cell development cells are subjected to selection and deletion by apoptosis at most stages. To determine GR1A transcript levels in the developmental lineage of B-cells, the level of GR 1A promoter activity was assessed in FACS-sorted pro-, pre-, immature and mature B cells from the bone marrow as well as transitional T1 & T2 cells, follicular mature (FM) and marginal zone (MZ) B cells from the spleen, using qRT-PCR. B lymphocyte populations from bone marrow were sorted according to the surface expression markers CD25, B220 and sIgM. Splenic B lymphocytes were sorted according to the differential expression of the surface expression markers CD21, CD23 and CD24. mRNA levels are depicted relative to the highest expressing population which was set to 1 (Fig. 6). GR1A transcripts are highest in the T2 B cell population (Fig. 6A). From the pro-B stage to immature B cells, GR1A mRNA levels increase steadily. Pre-B cells have a 2-fold increase compared to pro-B cells, and immature cells have a 3.4-fold increase compared to pre-B cells. T1 cells have slightly less transcripts containing exon GR1A; however T2 cells had the highest amount of GR1A mRNAs (9.25-fold increase compared to pro-B cells). FM cells show a 1.8-fold decrease compared to T2 cells. Finally, MZ cells show a GR1A activity similar to that of mature cells. Total GR mRNA transcript levels are shown in Fig. 6B and are quite similar across the cell populations, with pre-B cells and MZ cells having the lowest levels and T1 cells the highest. Percentage of GR1A usage is presented in panel C, and corresponds to the relative

GR1A promoter usage compared to that of the other exon 1 promoters (1B-E). Values range from 1.3% (pro-B cells) of GR1A promoter usage to nearly 12% (MZ cells) and follow a similar trend to the GR1A/2 mRNA transcripts. As a general trend GR1A transcript levels increase with the relative maturity of the B-cells population.

Finally we investigated the regulation of GR1A transcripts in three different human B-cell lines by dexamethasone. The Reh (Pre-pro), Namalwa (pre-B)and Raji (mature B) cell lines all express GR and express all three forms of the human GR1A promoter termed 1A1, 1A2 and 1A3. Three hours of incubation with dexamethasone caused a similar change in the three B cell lines. Most of the exon 1-containing transcripts (1A1, 1A2, 1B and 1C) showed little or no response, with some decrease in mRNA levels observed, which was only significant for 1A2- and 1C-containing transcripts in Reh and Namalwa B cell lines respectively (Fig. 7A and B). In contrast, the GR1A3-containing mRNA levels were strongly and significantly down-regulated in all three B cell lines (Fig. 7A, B and C). GR1A3-containing transcripts showed a significant 1.9-fold decrease in Reh B cell RNA, a 2.5-fold decrease in Namalwa B cell RNA, and a 2.8-fold decrease in Raji B cell RNA (Fig. 7A, B and C) respectively.

4. Discussion

Detection of GR1A transcripts in total brain RNA was first made in 2004 and localized initially to the brain cortex where it was demonstrated that this was not due to the presence of lymphocytes using an analysis of Rag-/- mice (Purton et al. 2004b). Using qRT-PCR and *in situ* hybridization we have confirmed this finding in addition to identifying GR1A transcripts in the anterior pituitary and low levels of GR1A mRNA in the midbrain and spleen. The presence of GR1A transcripts in brain cortex, pituitary and thymus were also confirmed by qRT-PCR and *in situ* hybridization. Bockmuhl et al., (2011) have recently detected GR1A transcripts in the pituitary and pre frontal cortex and contrary to our results also showed relatively high levels in hippocampus and low levels in hypothalamus. These differences may be explained by the method of quantification (we have used relative quantification by normalization to the housekeeping gene 18SrRNA, whereas they have performed absolute quantification using plasmid DNA as an external calibration curve). Also it is not clear which mouse strain their tissues were derived from for PCR analysis.

With the knowledge that the GR1A promoter is regulated by dexamethasone treatment in primary thymocytes and T-cell lines (Breslin et al. 2001; Geng and Vedeckis 2004, 2005; Pedersen et al. 2004; Purton et al. 2004b) we examined regulation of the GR1A promoter *in vivo* in the cortex of the brain and pituitary. In both the pituitary and cortex three hours of dexamethasone treatment did not greatly affect the levels of GR1A transcripts, relative to vehicle-treated control mice. In the pituitary dexamethasone treatment led to a decrease in levels of total GR transcripts. When GR1A transcripts are measured as a percentage of total GR activity we can see that there is a 2.6 fold increase over the sham control to 23%. This demonstrates that the GR1A transcripts levels are maintained following dexamethasone treatment, while total GR levels are decreased. The overall decrease in GR mRNA levels at this time point may reflect negative feedback responses which occur to limit GR production and therefore the stress response and it appears that the other GR promoters (that is, excluding 1A) may be responsive in the pituitary. GCs feedback to all cells of the anterior pituitary to limit production of unnecessary proteins during stress and also in particular to the corticotrophic cells; studies in rats have revealed that during stress GCs feedback to these cells where they act via GR to directly inhibit transcription of the POMC gene (Drouin, et al. 1987; Eberwine and Roberts 1984), thereby decreasing production of ACTH and thus GCs. Thus the presence of GR in the pituitary

is important to mediate these repressive actions. The role of elevated GR1A may maintain required GR levels, but also allow the sensitivity to rapidly changing conditions. Maintenance of GR protein stability has been associated with elevated GR1A transcripts, as well as higher levels of the GR-B translational isoform in particular, which has higher transactivation potential than the GR-A isoform and is conserved in mouse (Lu and Cidlowski 2005; Yudt and Cidlowski 2001). Additionally alternate translational isoforms of GR have been shown to activate distinct but partially overlapping subsets of genes (Lu and Cidlowski 2005), thus contributing to the variable response of different cells to GCs. However levels of the GR-B isoform have not yet been examined in the brain, nor has the regulation of GR exon promoters by GCs within these cell types. Interestingly, little variation in GR1A promoter usage between treatment groups was observed in the cortex of the brain; evidence does exist for differential regulation of the GR1A promoter in a range of cell types. It is believed that each exon 1 has its own individual promoter region, as observed in mice, which may explain tissue specific expression of the individual GR isoforms (Turner and Muller 2005) as each of the promoters can have an individual subset of response elements in that promoter, and these can in turn be used variably depending on the cell type and transcription factors present (Breslin et al. 2001; Chen, et al. 1999b) (Geng and Vedeckis 2004, 2005).

The presence of GR1A transcript levels in the pituitary is an important observation and it was therefore of interest to identify the particular pituitary cell types with an active GR1A promoter. qRT-PCR results from dissected pituitary identified the anterior pituitary and not the posterior pituitary as having detectable high GR1A mRNA levels. Using combined *in situ* hybridization and immunohistochemistry GR1A transcripts were detected in very close association with follicuostellate (FS) cells. It is possible that the precipitated BM purple substrate may have masked some fluorescence from the s100 immunolabelled cells.

FS cells have multiple functions reminiscent of immune cells; they have phagocytic activity, are known to surround immature glandular cells, and produce numerous signaling molecules, and are believed to create a signaling network within the pituitary with evidence of electrical and paracrine signaling within the FS cell population and also between FS cells, and other glandular cells via junctions (Allaerts, et al. 1990; Inoue, et al. 1999) (Claudius, et al. 2006; Fauquier, et al. 2001). IL-6 which is produced by immune cells as well as FS cells promotes lymphocytic proliferation and differentiation and stimulates the HPA axis. FS cells have been shown to

release IL-6 in response to immune (such as LPS or TFa/b) and neuroendocrine stress signals (such as PACAP and VIP), and are thought to be the exclusive producers of intra-pituitary IL-6 which acts locally to boost immune and stress responses (Allaerts, et al. 1997; Kageyama and Suda 2009; Renner, et al. 1998). IL-6 has been demonstrated to increase secretion of GH, PRL, LH and FSH (*in vitro* studies on rat cells) and ACTH (*in vivo* in rat and mouse and *in vitro* studies) (reviewed in (Arzt, et al. 1999) in response to stress or immune signals and its role in inducing ACTH in particular is an important mechanism to elevate GCs independently of CRH (Bethin, et al. 2000; Renner et al. 1998). IL-6 also stimulates the release of VEGF by FS cells (Gloddek, et al. 1999), which can promote blood vessel permeability and thus possibly enhance transport of hormones and regulatory molecules in and out of the pituitary (Renner et al., 2009). It is known that GCs are able to inhibit production of IL-6 and VEGF from the AP, which would therefore decrease ACTH production and transport, inhibiting GC production (Gloddek et al. 1999; Renner, et al. 2009). Data from co-culture experiments of AtT20 (corticotrophic) and TtT/GF (Folliculostellate) cell lines has also provided evidence for a paracrine mechanism of ACTH attenuation by GCs (Tierney, et al. 2003). Thus the possibility exists that high GR1A mRNA levels in FS cells may maintain GR levels to allow an extra level of attenuation of the release of ACTH and thus GC hormones during a response to stress. If GR1A transcripts are not present in FS cells, the possibility exists that GR1A transcripts occur in the pituitary in a subset of peripheral T-lymphocytes and various CD4+ or CD8+ markers could be used to investigate this possibility further.

With the observation of elevated levels of GR1A transcripts in mouse B-cells, spleen, bone marrow and in B cells of human origin (Presul, et al. 2007; Purton et al. 2004b; Russcher, et al. 2007) we examined the developmental lineage of mouse B-lymphocytes for GR1A transcript levels. As a general trend, in B-cells from mouse bone marrow (BM) GR1A transcripts levels increased with the level of B-cell maturation. This is in contrast to T-lymphocytes where GR1A levels decrease with developmental maturity (Purton et al. 2004b). Mouse primary pre-B and immature B cells from the bone marrow were shown to be sensitive to GICD triggered by a variety of GCs, whereas pro-B-cells and mature B-cells were more resistant (Merino, et al. 1994; Voetberg et al. 1994). GR1A transcripts were low in GICD resistant pro-B cells, and increased in GICD sensitive Pre-B and immature B-cells. However GR1A transcripts levels remained high in relatively GICD resistant, mature BM B-cells. GR1A levels were quite high in T1 and FM B-

cells and were particularly high in T2 and MZ mature B-cell subpopulations, however sensitivity of the transitional and mature B-cell subpopulations from spleen to GICD has not been fully examined. Total splenocytes (Miyake, et al. 1994) or T-cell depleted total IgM positive B-lymphocytes from spleen have been demonstrated to be sensitive to GICD (Andreau et al. 1998; Souvannavong, et al. 1998). Thus a clear correlation between sensitivity to GICD and GR1A transcript levels cannot be established for the B-cell lineage. (Merino et al. 1994) have shown that sensitivity to GICD correlated with decreased Bcl-2 expression. B-cell apoptosis is more prominent in pre-B cells and mature B-cells which are undergoing light chain recombination to generate functional IgH chains and are also subjected to B-cell selection to remove autoreactive B-cells. Thus a role for Bcl-2 and apoptosis in this process is thought to exist. In the human pre-B derived 697 cell line, dexamethasone induces auto-upregulation of GR mRNA and protein, and induces apoptosis. The threshold of GR has been shown to be essential and the optimal window of GR levels is actually quite narrow in order for apoptosis to occur (Schwartz, et al. 2010). It has been demonstrated in these cells that promoters GR 1A2 and 1A3 are in fact down regulated in response to dexamethasone treatment and that transcription of exons C and D are elevated, which is in contrast to T-cells where elevated levels of GR1A correlate with increased sensitivity to GICD (Purton et al. 2004b). The authors described a molecular switch model whereby up-regulation of GR co-activator PGC-1 in response to dexamethasone is involved in facilitating GR induced up-regulation of exons C and D while PU.I present in 697 cells (which was shown to bind to the GR1A promoter in response to dexamethasone treatment) may inhibit transcription from the GR1A promoter (as occurs in developmentally mature IM9 B-lymphoblastoid cells) (Geng and Vedeckis 2005, 2011).

Thus the role for GR1A-expressed transcripts and differential GR1A promoter activity is most likely very different in B-cell development compared to the brain during the stress response and the mechanisms appear complex, although the interplay of cell specific transcription factors is clearly important. What is clear is that the alternate GR gene exons 1s, in particular GR exon 1A, do provide an important opportunity for differential regulation of the GR, which is essential to its numerous functions in mediating glucocorticoid responses in different cell types.

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Figure Legends

Fig. 1

GR1A promoter activity in the mouse brain region.

A: Relative mRNA levels transcribed from the GR1A promoter in various mouse tissues. mRNA levels are graphed relative to thymus which has been arbitrarily set to 1. B: Relative levels of total GR mRNA in various mouse tissues. C: Percentage of 1A promoter usage, calculated by dividing the raw values for GR1A transcript levels by the total GR transcript levels, corresponding to the relative activity of the GR1A promoter in these tissues. Results are representative of n = 3, with qRT-PCR analysis repeated at least once for each target and a representative graph displayed. Bar graphs represent mean ± SEM.

Fig. 2

***In situ* hybridization of GR1A transcripts in mouse brain and thymus.**

Tissue sections of mouse brain cortex, pituitary, thymus and kidney were stained for the presence of GR1A exon-containing RNA transcripts by non-radioactive in-situ hybridization. Dense purple staining indicated presence of GR1A transcripts in A) Cortex, B) Pituitary or C) Thymus. D) Kidney was used as a negative control. Images were i) 10x magnification of tissue sections stained with an antisense GR1A nucleotide probe, ii) 20x magnification, and iii) 20x magnification with a negative control GR1A nucleotide sense probe.

Fig. 3

Regulation of GR1A transcripts in the pituitary by dexamethasone.

Changes in GR transcript levels in brain cortex and pituitary from 3 h 20mg/kg Dex induced C57/B6 mice (n = 3 mice). Sham control samples were set to 1 and Dex-treated samples are presented relative to control samples. A) Changes in relative levels of GR1A mRNA in brain cortex and pituitary. B) Changes in relative levels of total GR mRNA in brain cortex and pituitary. C) Percentage of GR1A promoter usage, calculated by dividing the raw values for GR1A transcript levels by the total GR transcript levels, corresponding to the relative activity of the GR1A promoter in these tissues. Results are representative of single induction experiments, with qRT-PCR analysis repeated at least twice for each target. Bar graphs indicate mean ± SEM. Statistical analysis was performed using a student's t-test. Significant differences in transcript levels between vehicle-treated and Dex-treated mice are indicated by * ($p < 0.05$).

Fig. 4

GR1A transcripts are present in the anterior pituitary.

A) Relative mRNA levels transcribed from the GR1A promoter in the anterior and posterior pituitary and various pituitary cell lines; AtT20, GH3 and LBT2 (n = 3). RNA levels are depicted relative to thymus which was arbitrarily set to 1. B) Relative levels of total GR mRNA in these tissues and cell lines. C) Percentage of GR1A promoter usage calculated by dividing the raw

values for GR1A transcript levels by the total GR transcript levels, corresponding to the relative activity of the GR1A promoter in samples Bar graphs indicate mean ± SEM.

Fig. 5

GR1A transcripts are associated with folliculostellate cells.

Ai) Brightfield image of GR1A staining in the pituitary. Aii) 20x magnification fluorescent image showing overlay of inverted brightfield *in situ* hybridization image (i) (white) and adrenocorticotropin hormone protein (green) iii) 40x. B: ii) 20x fluorescent overlay image of inverted brightfield *in situ* hybridization image (Bi) (white) and prolactin protein (green) iii) 40x. C: ii) 20x fluorescent overlay image of inverted brightfield *in situ* hybridization image (Ci) (white) and s100 protein (red). iii) 40x. D: ii) 20x fluorescent overlay image of inverted brightfield *in situ* hybridization image (Di) (white) and leuteinizing hormone protein (green) iii) 40x.

Fig. 6

GR 1A mRNA in mouse B cell subsets from pro-B to mature B cells.

A. Relative mRNA levels transcribed from the GR1A promoter in various stages of mouse B-cell development B) Relative levels of total GR mRNA in mouse B-cells at various stages of development. C) Percentage of 1A promoter usage, calculated by dividing the raw values for GR1A transcript levels by the total GR transcript levels, corresponding to the relative activity of the GR1A promoter in these cells. Results are representative of n = 2 for two independent FACS analyses. Bar graphs represent mean ± SEM. Kidney is a positive control for GR exon 2/3, but a negative control for the 1A promoter.

Fig. 7

Dexamethasone regulation of human GR exon 1 promoters in B-lymphocyte cell lines.

(A) hGR exon 1 promoter activity in Reh prepro-B cells. (B) hGR exon 1 promoter activity in Namalwa pre-B cells. (C) hGR exon 1 promoter activity in Raji mature B cells. Bar graphs represent mean ± SEM (n=3). Significant differences in transcript levels between vehicle-treated and dexamethasone-treated cells are indicated by ** ($p<0.01$) and * ($p<0.05$).

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Figure

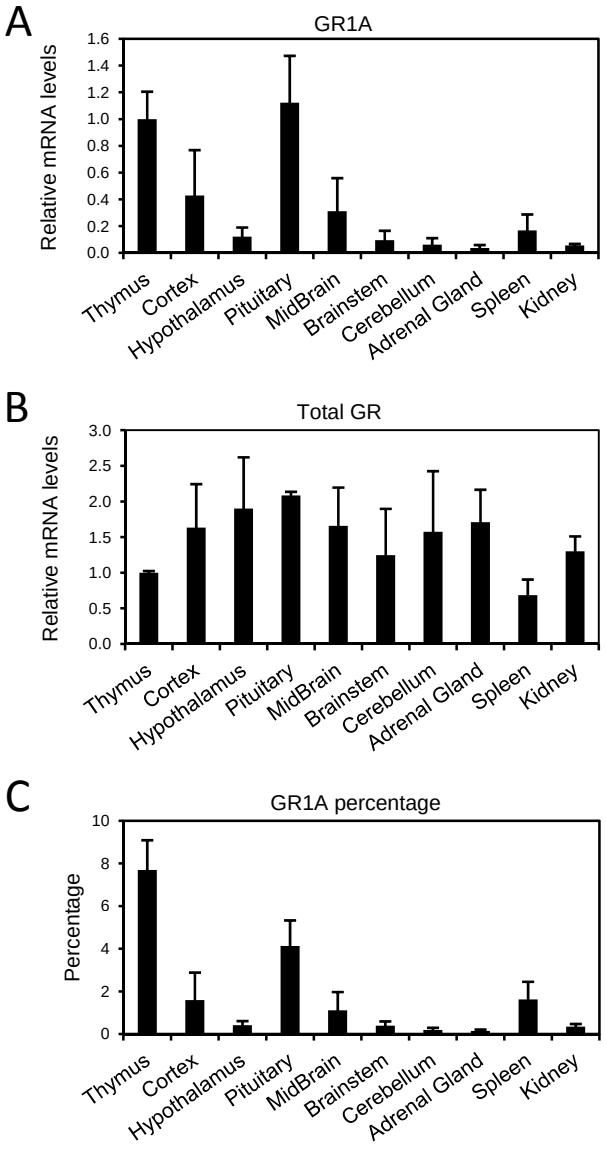

Figure 1

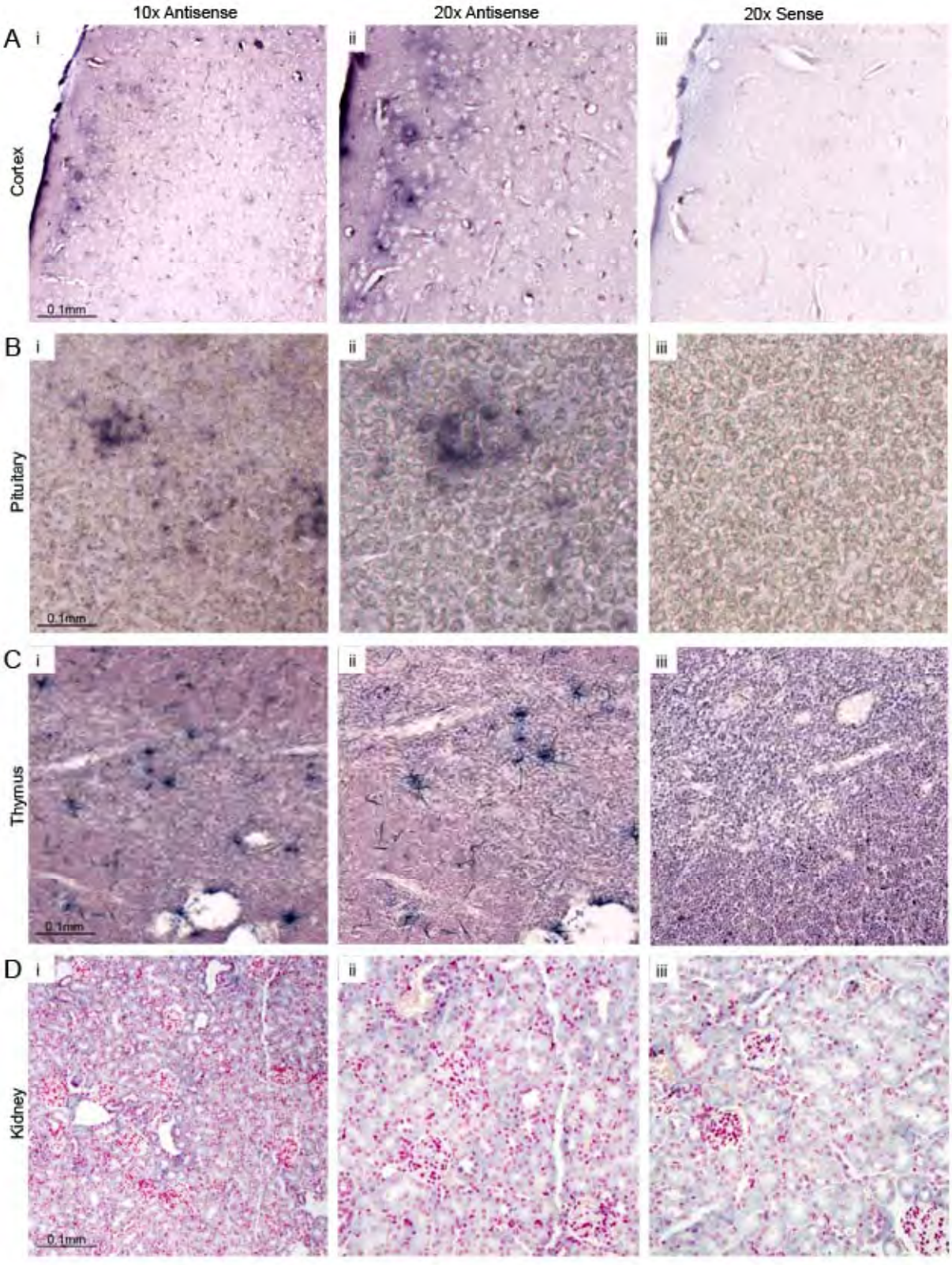
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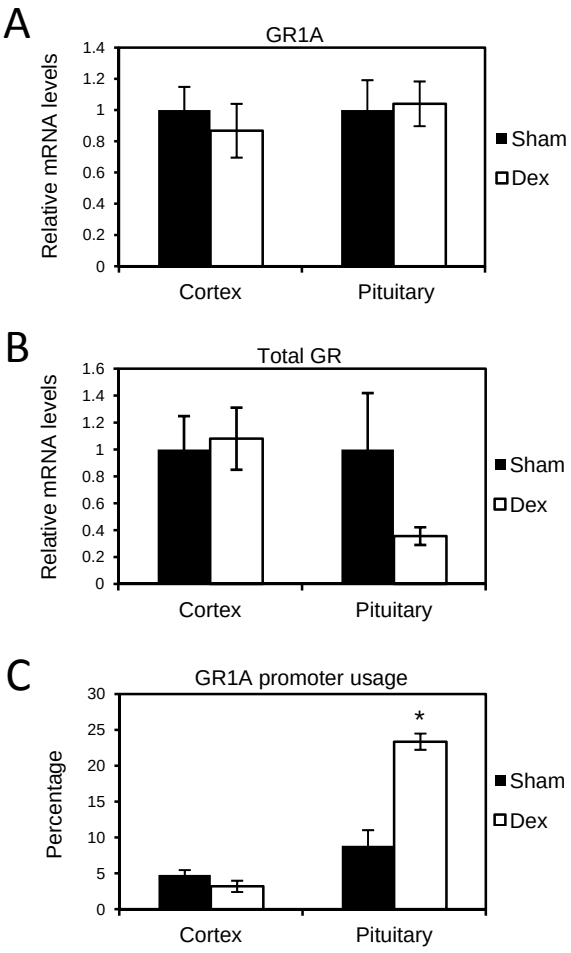
Figure 2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0
*
Cortex
Pituitary
Sham
Dex

Figure 3

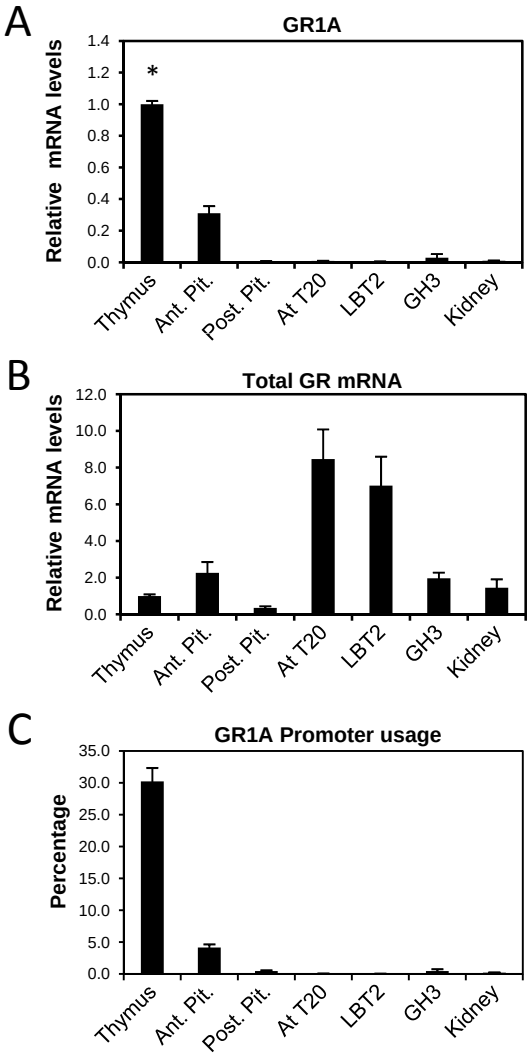

A
GR1A
Relative mRNA levels
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Thymus
Ant. Pit.
Post. Pit.
At T20
LBT2
GH3
Kidney
B
Total GR mRNA
Relative mRNA levels
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Thymus
Ant. Pit.
Post. Pit.
At T20
LBT2
GH3
Kidney
C
GR1A Promoter usage
Percentage
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Thymus
Ant. Pit.
Post. Pit.
At T20
LBT2
GH3
Kidney

Figure 4

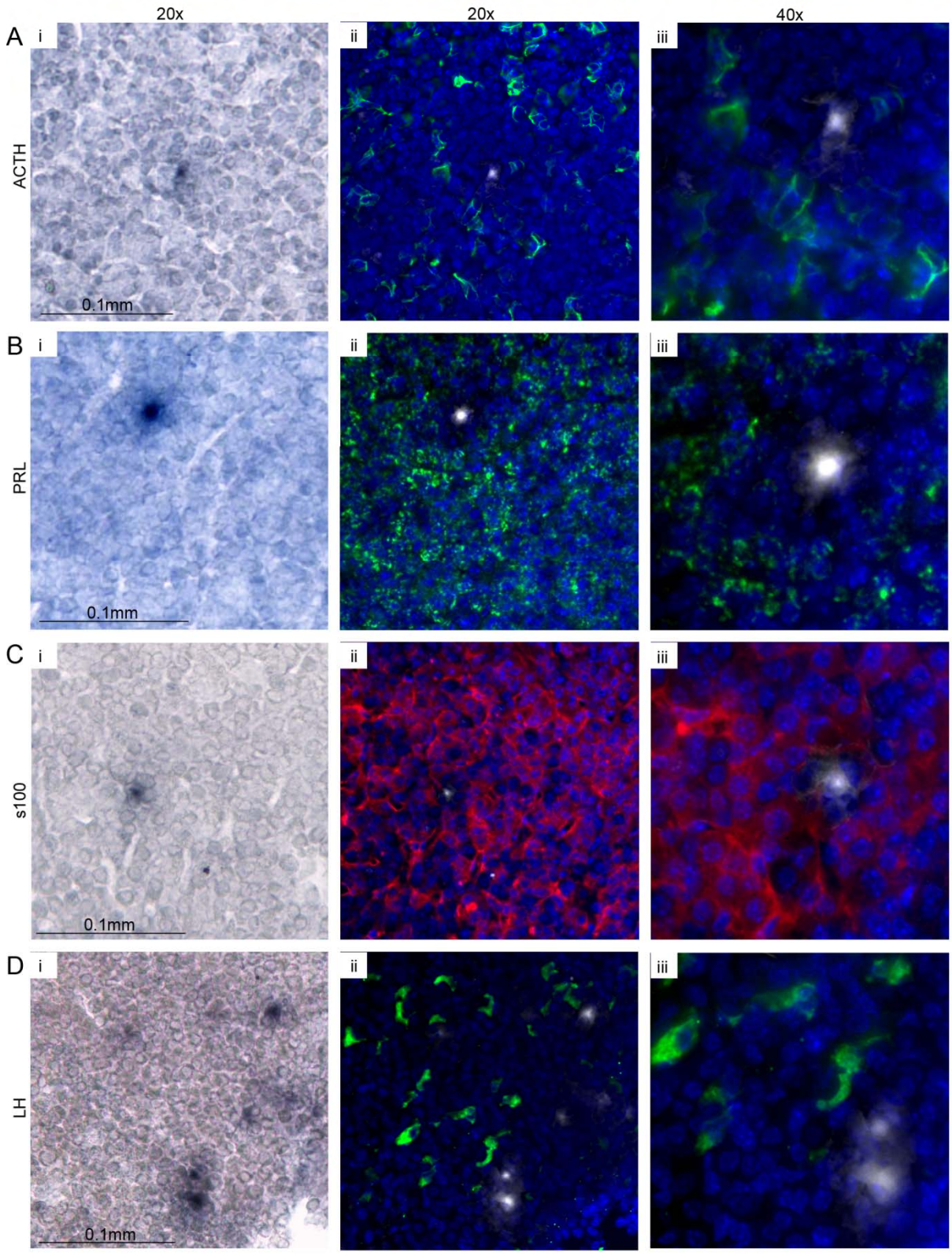
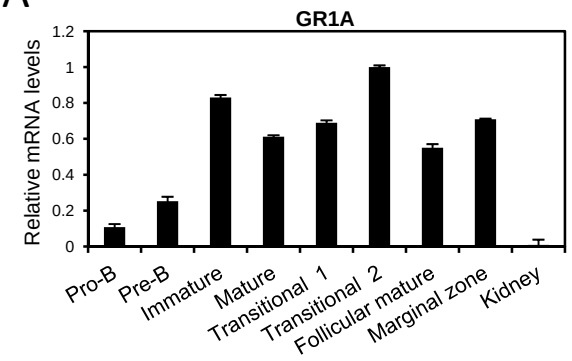
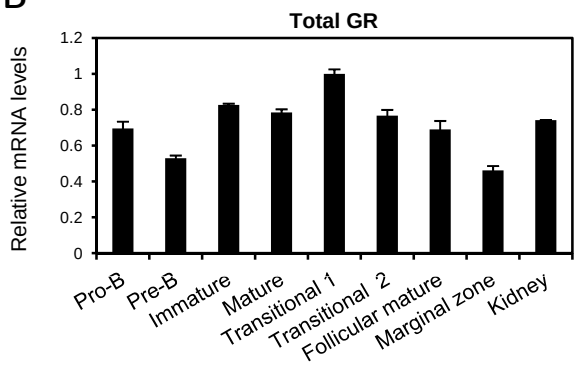

20x
20x
40x
A
i
ii
iii
ACTH
0.1mm
B
i
ii
iii
PRL
0.1mm
C
i
ii
iii
s100
0.1mm
D
i
ii
iii
LH
0.1mm

Figure 5

A


GR1A
Relative mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
Pro-B
Pre-B
Immature
Mature
Transitional 1
Transitional 2
Follicular mature
Marginal zone
Kidney

B


Total GR
Relative mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
Pro-B
Pre-B
Immature
Mature
Transitional 1
Transitional 2
Follicular mature
Marginal zone
Kidney

C

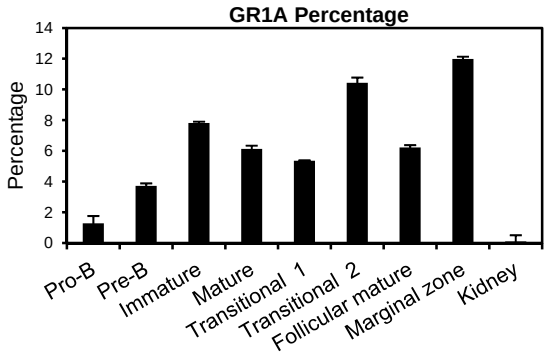

GR1A Percentage
Percentage
14
12
10
8
6
4
2
0
Pro-B
Pre-B
Immature
Mature
Transitional 1
Transitional 2
Follicular mature
Marginal zone
Kidney

Figure 6

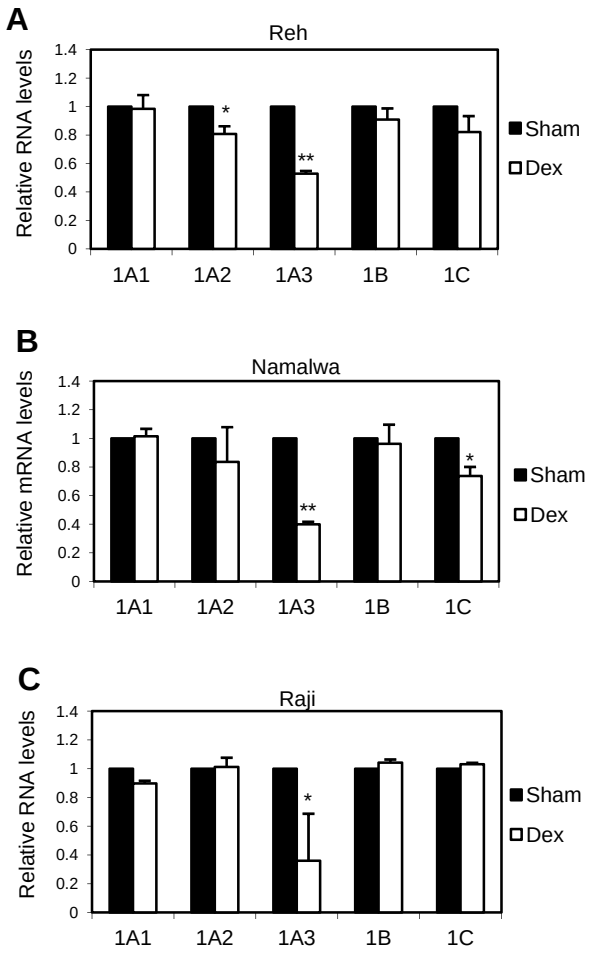

A
Reh
Relative RNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
1A1
1A2
1A3
1B
1C
Sham
Dex
B
Namalwa
Relative mRNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
*
1A1
1A2
1A3
1B
1C
Sham
Dex
C
Raji
Relative RNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
1A1
1A2
1A3
1B
1C
Sham
Dex

Figure 7

CHAPTER 5:

REQUIREMENT OF THE GLUCOCORTICOID RECEPTOR FOR NORMAL T-LYMPHOCYTE DEVELOPMENT IN MICE

Chapter 5:

5.1: Introduction

The production of an appropriately reactive T-cell repertoire is crucial to normal immune function. Within the thymus, Early T-lineage Progenitors (ETP) which have migrated from the bone marrow and express CD25 are subject to “T-cell selection” to generate mature fully functional, self tolerant T-lymphocytes. ETPs are “DN” with respect to CD4 and CD8 co-receptors and during this stage cells must produce an appropriate T-cell receptor (TCR) which is responsible for recognizing antigens displayed by Major Histocompatibility (MHC) molecules. During the DN stage the TCRβ chain genes are rearranged and assembled leading to the expression of a TCRβ chain as a pre-TCR. This leads to signals that cause cellular expansion, acquisition of CD4 and CD8 co receptors (Double Positive “DP” stage) and cells will undergo TCRα gene rearrangement. For those cells that generate a functional α chain, the pre-TCR is replaced with low levels of the mature αβ TCR (reviewed in Ashwell, et al. 2000; Sleckman 2005). The gene rearrangements of the variable region of the TCR mean each T-cell will express a TCR with a unique specificity and that a T-cell repertoire with the ability to recognize a wide variety of antigens is present. However it is important that the TCR responds appropriately, thus at this point DP cells are subjected to positive or negative selection and death by neglect. Thymocytes expressing TCRs that do not recognize self antigens or MHC presented antigens undergo “death by neglect”. Thymocytes that bind strongly to self antigen undergo activation induced cell death (AICD) or negative selection. Thymocytes bearing TCRs with intermediate avidity for self antigen/ MHC are rescued from the default death pathway by positive selection and differentiate into CD4 or CD8 Single Positive (SP) cells, and migrate to the periphery. In general MHC class I-restricted receptors are positively selected to the CD8 lineage, while T cells expressing class II–restricted TCRs are selected to the CD4 lineage (Reviewed in Ashwell et al. 2000; Sebzda, et al. 1999). Following selection to single positive cells they begin to receive signals to further define their functional role in immune surveillance.

5.1.1: The Mutual Antagonism Model and Glucocorticoids

One of the models proposed for the T-cell selection process involves glucocorticoid signaling and is called the mutual antagonism model. Briefly; the model proposes endogenous glucocorticoids can cause thymocyte programmed cell death and only those thymocytes that were activated by self antigen would be permitted to survive (positive selection). Too much avidity for self molecules, and thus too strong an activation signal, would overcome the antagonism provided by the GCs, resulting in activation-induced cell death (negative selection) (Zacharchuk, et al. 1990). The evidence for the involvement of GCs comes from studies in culture using specific antigens to simulate AICD of T-cells, where application of either Dex or antigen alone induced apoptotis whereas co-application of GCs and antigen did not induce death (Zacharchuk et al. 1990).

There is much evidence against the mutual antagonism model for T-cell selection, the earliest comes from studies of two similar mouse models of $GR^{-/-}$ mice which did not support the involvement of GR in T-cell development (Brewer, et al. 2002; Purton, et al. 2002). Brewer's study used exon 1C-2 deleted $GR^{-/-}$ mice on a C57BL/6 background which was embryonically lethal, while Purton's study used exon 2 deleted $GR^{-/-}$ on a mixed genetic background (GRMB), which have 10-20% survive to adult stage. Total T-lymphocyte numbers in the thymus and peripheral tissues of spleen, lymph node and liver were normal between both GR null models when compared to their wild type counterparts (Brewer et al. 2002; Purton et al. 2002). Additionally, subpopulations of DN, DP or SP, and NKT, memory and activated T-cells were also not significantly different in either model or genotype indicating normal T-cell development and maintenance (Brewer et al. 2002; Purton et al. 2002). Subsequently a T-cell specific "TGRKO" mouse model generated by crossing GR exon 2 floxed alleles with Lck-cre mice revealed normal thymic cellularity and normal subset distribution in thymus, spleen and lymph nodes (Brewer, et al. 2003). These studies provided strong evidence excluding the role of GR in T-cell selection, as they used more reliable models where GR is

completely absent from thymocytes and circulating GCs are normal, unlike some adrenalectomized models; models where GC release is inhibited or models where GR activity is only partially reduced (Pazirandeh, et al. 2005; Reichardt, et al. 1998; Sato, et al. 2010; Stojic-Vukanic, et al. 2009). In Brewer's study of TGRKO mice, although thymocyte cellularity was described as normal, the analysis of T-cell subset distribution was not shown, and other specialized T-cells subsets were not examined (Brewer et al. 2003).

Thus there are aspects of the involvement GR and GCs in T-cell development and selection that remain controversial. Following T-selection and differentiation into CD8 or CD4 cells, cells receive a multitude of signals specifying them toward their function. With GCs known to regulate so many target genes the potential for GCs to impact this development is enormous. We have studied two particular T-cell subtypes further, NKT cells and T_{Reg} cells.

5.1.2: NKT cells

NKT cells contribute to antimicrobial activity, tumour surveillance and self tolerance. They are able to kill antimicrobials directly or secrete factors to recruit or activate NK cells, Macrophages, Granulocytes, T-cells and B-cells (Brigl and Brenner 2004). NKT cells are quite heterogeneous and can be broadly classed as Type 1 or Type 2. Our study focused on Type I or invariant NKT cells, which in mice express an invariant TCRα, consisting of the rearrangement Vα14Jα18, and a limited TCRβ which may contain Vβ8.2, Vβ7 or Vβ2, they may be CD4+ or CD4- and are reactive to glycolipids presented by CD1d, in C57BL/6 mice they may also express NK1.1 (reviewed in Godfrey et al. 2010). Thus NKT cells can be detected using tetramers of CD1d loaded with marine sponge derived α-galactosylceramide (α-GalCer). Development of Type I NKT cells occurs through at least 4 well defined stage; stage 1 and 2 occur in the thymus and stages 3 and 4 generally occur in the periphery. It begins with the random rearrangement of genes yielding the semi-invariant, CD1d-restricted TCR, these cells enter the

NKT cell lineage and differentiate under the control of a series of transcription factors some of which have been shown to be regulated by GR (reviewed in Godfrey et al. 2010).

5.1.3: T_{Reg} cells

It has become apparent that not all cells "successfully" completing T-cell selection are in fact completely self tolerant, some will induce auto immune reactions and a special subset of T-cells known as Regulatory T-cells "T_{Reg}s" serve to control this response. T_{Reg} cells reduce the activation and expansion of self reactive cells, most likely by a combination of mechanisms mediated by cytokines, direct cell to cell interactions and modulation of antigen presenting cells (reviewed in (Sakaguchi 2004)) additionally they are also able to respond to non self antigens. The GC regulated gene *FoxP3* in particular is crucial to TReg development, deletion of this gene in mice is fatal within weeks and mice suffer severe autoimmune defects. The large majority of regulatory T cells express *FoxP3*, CD4 and high levels of the interleukin-2 receptor alpha chain, CD25, although CD25 may be absent (Sakaguchi 2004), thus subpopulations were examined according to these markers.

5.2: Aims

The purpose of this study was to further investigate the involvement of GR in development of T_{Reg} cells, which have not previously been studied in this context and to examine more closely T-cell development of NKT and NK cells in the absence of GR. Thus we used Lck Cre T-cell specific GR null "TGRKO" mice which we believe to an excellent model for this study as it allows mice to develop normally in the context of GCs and means no other organs are affected by loss of GR. This study will allow the definitive determination of the role of GR in development of these T-cell subsets.

5.3 Methods

5.3.1 Animals and housing

Mice used in this study were bred and housed under standard conditions at the animal house and ARL of the Biochemistry Department, Monash University, and Mouseworks, Monash University. All animal experimentation was approved by the School of Biomedical Sciences Animal Ethics Committee, Monash University (Ethics no. 2009/63), and was carried out according to the National Health and Medical Research Council of Australia guidelines for the breeding, care and use of genetically modified and cloned animals for scientific purposes 2007. Lck-Cre mice were mated with GR LoxP exon 2 deleted mice (Brewer at el., 2003, obtained from Dr. L.J. Muglia, Washington University School of Medicine, Saint Louis, Missouri 63110, USA) to generate mice which lack GR within the T-cell lineage (TGRKO). GR LoxP exon 2 deleted mice were originally generated on a C57BL/6 X 129/Sv background, however they were backcrossed with C57BL/6 mice by Dr. Muglia, and additionally backcrossed for a further 8 generations with C57BL/6 mice once we received them. Lck-Cre Mice have the Cre recombinase under the control of the Lck lymphocyte protein tyrosine kinase promoter which induces Cre expression specifically in the T-cell lineage (Takahama et al., 1998). Lck-Cre homozygous transgenic mice are viable and have no major defects. TGRKO mice and Wild Type (WT) mice were treated with Dexamethasone, sacrificed and thymi, spleens and livers and removed for further analysis. Tissues were placed immediately in FACS buffer (See Appendix 2) and stored on ice until cell isolation was performed as described in 5.3.3. For total RNA isolation tissues were snap frozen in liquid nitrogen and stored at -80˚C or RNA was isolated immediately as described in Chapter 2, Section 2.4. For Immunohistochemistry tissues were fixed by immersing in 4% PFA (in PBS) immediately after dissection for 2 h and gradually dehydrated as described in Chapter 2, Section 2.6.

5.3.2 Genotyping

To determine genotypes with respect to target genes in transgenic and knockout mice, genotyping PCRs were performed. Genomic DNA was prepared from tail or toe clips using REDExtract (Sigma-Aldrich, Castle Hill, NSW - according to manufacturers instructions) or Tail Digestion Buffer (See Appendix 2). Approximately 4mm of tail was digested with 0.5 mL Tail Digestion Buffer and 10 µL Proteinase K overnight at 55°C. Samples were centrifuged for 1 min at 15,000 rpm in an Eppendorf 5415R. Supernatant was transferred to fresh 1.7 mL tube eliminating as much fur as possible. DNA was precipitated with 1.0 mL 95% ethanol containing 1/20th volume 3M NaOAc pH 7.0 at RT for 1.5 h. Sample was centrifuged 10 min at max speed, ethanol removed and pellet air dried and resuspended in sterile water. Genotyping of TGRKO mice was performed using 1 µl of tail DNA isolated using REDExtract according to the manufacturers instructions, with 200 µM sense and antisense primers (Sigma-Aldrich, Castle Hill, NSW) in a total volume of 20 µl. The Primer sequences were as follows: LoxP Fwd 5'TGCGAGCTGTACTGATAACTTC3', GR1 Fwd 5'TTGCAAATAACTCAGTACAAATGG3', GR2 Rev 5' TACTACTTCCAGTTCTTAACCCTCTC3'.

32 cycles of PCR were performed at the following temperatures:

Initial Denaturation: 1 minute at 95˚C

Denaturation: 1 minute at 95˚C

Annealing: 1 minute at 63˚C

Extension: 1 minute at 72˚C

Final extension: 5 minutes at 72˚C

Resulting amplified DNA was run on 1% Agarose gel in 0.5X TBE against a 1 Kb Plus DNA Ladder (Life Technologies, Mulgrave, Victoria) with Ethidium bromide or GelRed (Life Technologies) detection and visualised using the Gel Dock UV transilluminator. In GR LoxP exon 2 deleted mice two bands were present; a 4292 bp band resulting from amplification between LoxP Fwd and GR2 Fwd in addition to a 460 bp band resulting from amplification between GR1 Fwd and GR2 Rev. This

region contains the loxp-PGKneo-loxp cassette and thus contains an additional 100 bp when compared to the wildtype GR sequence. In TGRKO mice a 190 bp band was present as a result of amplifcation between LoxP Fwd and GR2 Rev. The smaller amplification product confirms the deletion of exon 2. In GR wildtype mice a 361 bp band appeared as a result of amplification between GR1 Fwd and GR2 Rev.

5.3.3 Cell preparation from organs

For flow cytometry experiments lymphocytes were isolated from thymus and spleen by first removing any residual fat or connective tissue using fine forceps. Organs were then scored over a petri dish containing FACS Buffer (See Appendix 2) using the frosted ends of 2 glass slides and then gently ground between rough surfaces till cells spilled into the FACS Buffer. Cells were resuspended and transfered to a tube through a 100 µm mesh.

Lymphocytes were isolated from liver using a Percoll gradient. Bile duct was removed from liver and placed FACS Buffer on a 200 µm sieve in 9 cm Petri dish. Liver was cut into small pieces using curved scissors, and pieces pushed through the sieve using the plunger of 3 mL syringe. The sieve was rinsed with FACS Buffer to collect any residual cells. The isolated cell mix was then centrifuged in a 50 mL tube at 1700 rpm (546 x *g*) for 5 min and the supernatant poured off. At this time the cell pellet was resuspended in 25 mL 33% Percoll (9 mL Percoll, 1 mL 10X PBS, 17 mL 1X PBS) and centrifuged at room temperature at 2000 rpm (755 x *g*) for 12 min. Hepatocyte layer and supernatant were aspirated and cell pellet washed in FACS Buffer and then transferred to a 10 mL tube. Cells were centrifuged at 1700 rpm (546 x *g*) for 5 min and supernatant aspirated. Lymphocyte preparations from spleen and liver were red blood cell lysed in Red Blood Cell Lysing Buffer (Sigma-Aldrich) according to the manufacturer's instructions. Briefly; cell pellets were resuspended in 0.5-3 mL (for Livers) or 5mL (for Spleens) Red Blood Cell Lysing Buffer, underlayed with 0.5 mL FCS and centrifuged at 1700

rpm (546 x *g*) for 5 min. Supernatant was aspirated and cell pellet resuspended in 0.5 mL (liver) or 5mL (Spleen). Cells were counted using a hemocytometer or Coulter Particle counter for the purpose of calculating the amount of antibody required for staining.

5.3.4 Flow Cytometry

Cells were isolated as described in section 5.3.3, and counted using the Coulter particle counter. Cells were stained with antibodies outlined in section 5.3.6. Flow cytometry was performed on the LSRII with 3 lasers (405 nm uv laser, Argon 488 nm blue laser and a He-Ne 633 nm red laser) and data collected with DIVA software. Data analysis was performed using FlowJo v 8.8.6 from TreeStar, and Prism.

5.3.5 Statistics

Statistical analysis was performed using Prism (GraphPad Software, San Diego, California) and using a student's t-test or Mann-Whitney U test where appropriate. P values <0.05 were considered statistically significant.

5.3.6 Antibody Information

Specificity	Conjugate	Company
Flow Cytometry		
TCRβ	FITC, APC	BD Biosciences
NK1.1	PE, PE-Ly7	Biolegends, BD Biosciences
CD4	FITC, PerCP	BD Biosciences
CD8	PerCP	BD Biosciences
CD44	FITC	BD Biosciences
CD62L	PE	BD Biosciences
CD3e	PE-Ly7	BD Biosciences
CD25	APC	BD Biosciences
FoxP3	PE	BD Biosciences

5.4: Results

5.4.1: CD8 CD4 T-cell subpopulations are altered in TGRKO Spleen and Liver

Lck-Cre mice were mated with GR LoxP mice to generate TGRKO mice which lack GR within the T-cell lineage. Lck-Cre Mice have the Cre recombinase under the control of the Lck lymphocyte protein tyrosine kinase promoter which induces Cre expression specifically in the T-cell lineage. Lck-Cre TGRKO homozygous transgenic mice are viable and have no major defects (Brewer et al. 2003). Cells were isolated from thymus, liver and spleen of WT vs TGRKO male mice according to section 5.3.3 and analysed by flow cytometry in order to examine any differences (n = 4). Cells were stained for CD8 and CD4 and also TcRβ in spleen and liver (Antibodies are presented in table 5.3.6). Representative dot plots are shown in Panel A (WT) and B (KO) for each figure (Figure 5.1-5.3). Total cellularity and CD8 CD4 subpopulations were largely normal between thymuses of TGRKO vs WT mice (Figure 5.1C, D, E). There was a non significant trend towards a decrease in total thymocyte number, and this reduction was reflected in the cellularity of some subpopulations, with $CD8^{+}CD4^{-}$ (~$2x10^{6}$ fewer cells) and $CD8^{-}CD4^{+}$ (~$6x10^{6}$ fewer cells) numbers significantly deceased (based on Mann-Whitney U test, $p < 0.05$). Proportions of these cells remained normal, most likely due to a decrease in total thymic cellularity. DN cells were increased by 2% (Figure 5.1E). Total cellularity in the spleen was decreased in TGRKO mice (Figure 5.2C). All splenic CD8 CD4 subpopulations were decreased in number (Figure 5.2D), in terms of CD8 CD4 proportions, $CD8^{+}$ $CD4^{-}$ cells were significantly decreased and presumably due to the loss of total cellularity $CD8^{-}CD4^{+}$ cells were increased in proportion. Total liver cellularity was statistically normal between TGRKO and WT mice (Figure 5.3C). However all T-cell CD8 CD4 subpopulations were decreased in number in TGRKO compared to WT (Figure 5.3D) and each subset was proportionally decreased, thus proportions of these cells were normal (Figure 5.3E).

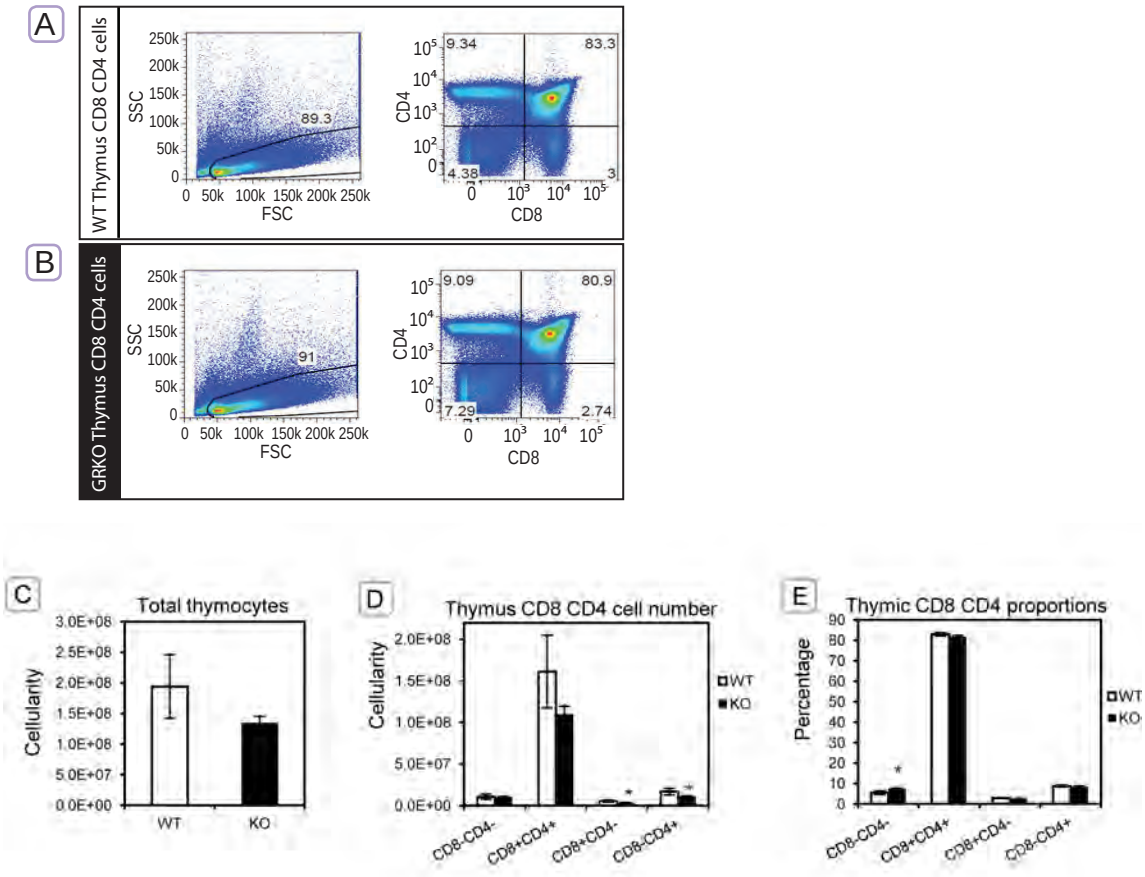


Figure 5.1: Thymic CD8 CD4 subpopulations are mostly normal in GRKO mice

Thymocytes from WT and TGRKO mice were stained for CD8 and CD4 expression and analysed by flow cytometry. A: Representative dot plots for flow cytometry of CD8 CD4 thymic T-cells in WT mice and B: in TGRKO mice. C: Total thymic cellularity in WT and TGRKO mice. D: CD8 CD4 cellularity in thymus. E: CD8 CD4 proportions in thymus. Bar graphs show mean ± SE. Statistical analysis was performed using Mann-Whitney U test. *Significant differences in thymic CD8 CD4 number and proportion between WT and KO mice ($P<0.05$).

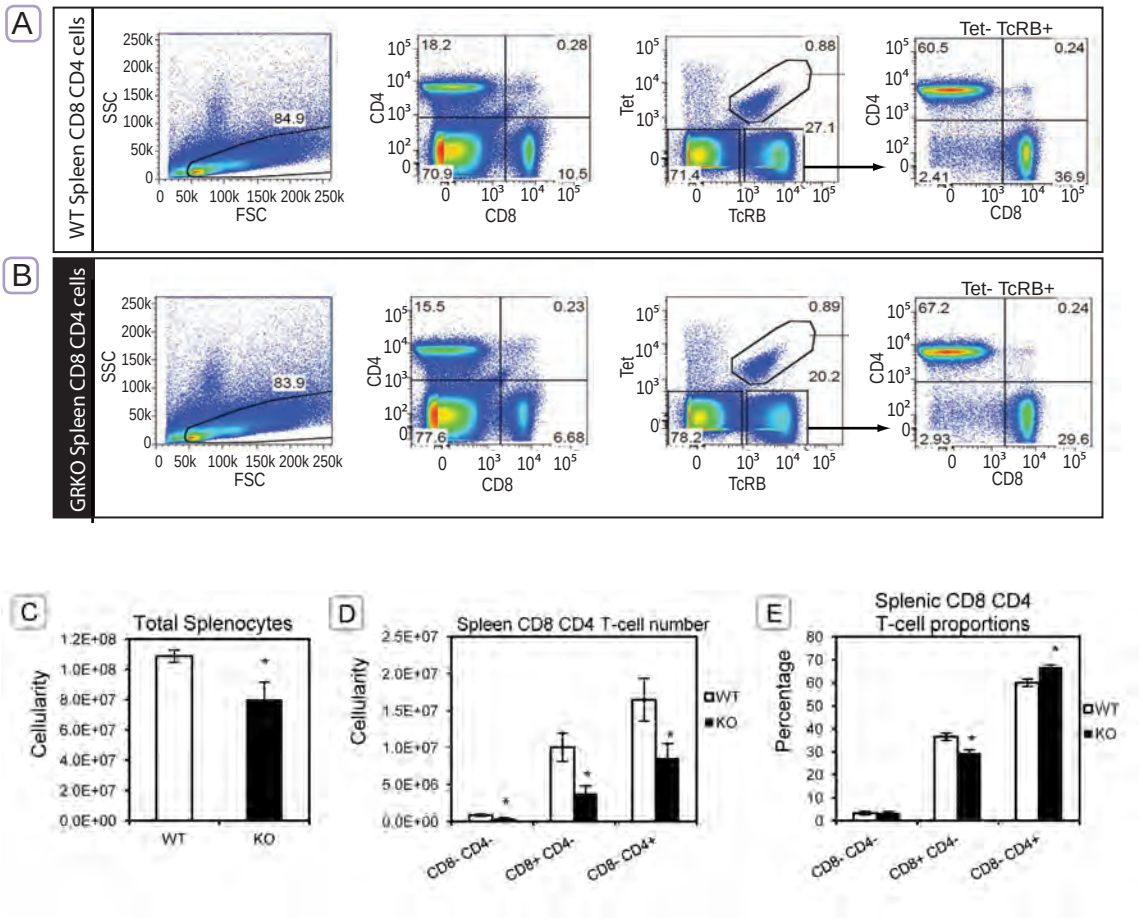


Figure 5.2: Splenic CD8 CD4 subpopulations are reduced in GRKO mice

Splenocytes from WT and TGRKO mice were red cell lysed and stained for CD8, CD4 and TcRβ expression and analysed by flow cytometry. A: Representative dot plots for flow cytometry of CD8 CD4 splenic T-cells in WT mice and B: in TGRKO mice. C: Total splenic cellularity in WT and TGRKO mice. D: CD8 CD4 cellularity in spleen. E: CD8 CD4 proportions in spleen.

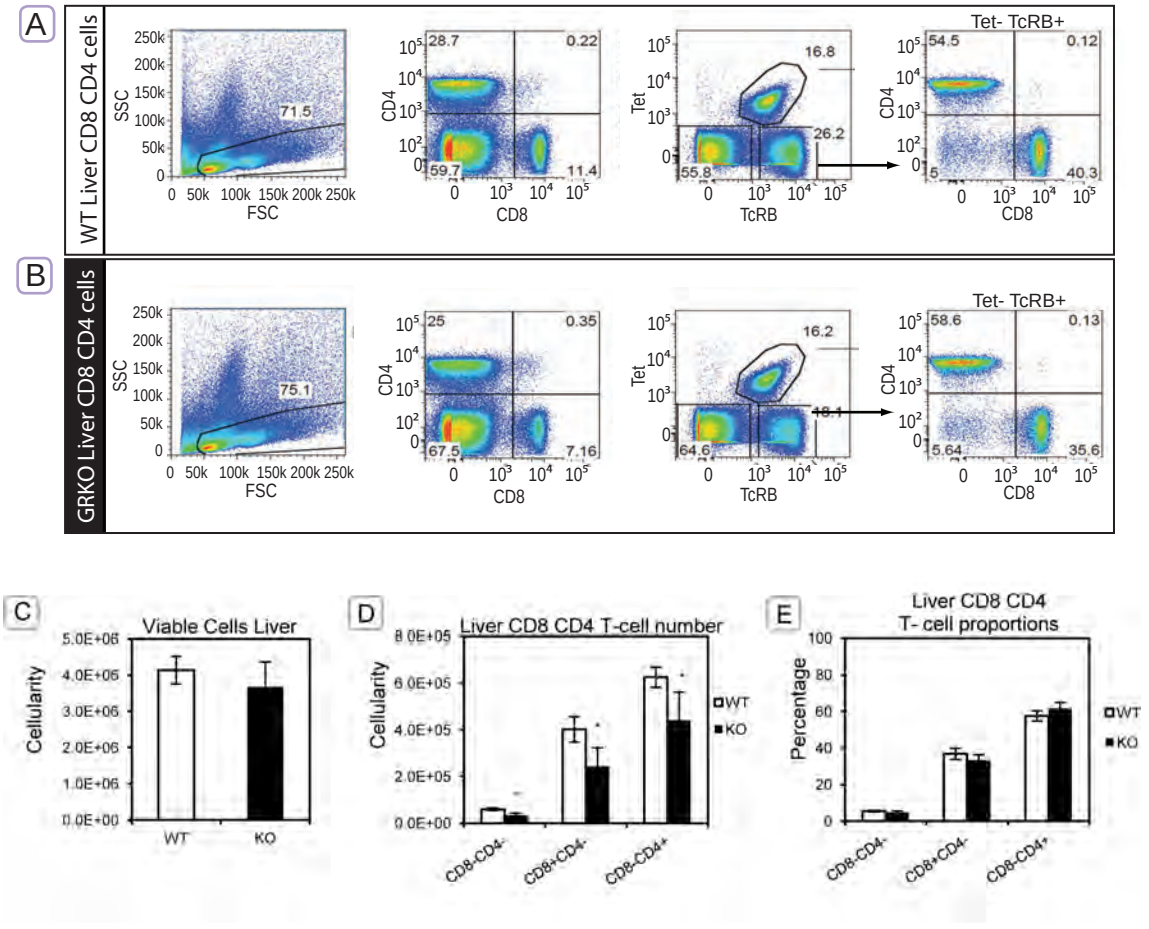


Figure 5.3: Hepatic CD8 CD4 subpopulations are reduced in GRKO mice

Hepatic cells from WT and TGRKO mice were red cell lysed and stained for CD8, CD4 and TcRβ expression and analysed by flow cytometry. A: Representative dot plots for flow cytometry of CD8 CD4 hepatic T-cells in WT mice and B: in TGRKO mice. C: Total hepatic cellularity in WT and TGRKO mice. D: CD8 CD4 cellularity in liver. E: CD8 CD4 proportions in liver. Bar graphs show mean ± SE. Statistical analysis was performed using Mann-Whitney U test. *Significant differences in hepatic CD8 CD4 number and proportion between WT and KO mice ($P<0.05$).

5.4.2: NKT cells develop normally in TGRKO thymus and liver, but not in spleen

NKT cells were sorted from thymus, liver and spleen of WT vs TGRKO mice in order to examine any differences. NKT cells were sorted following staining with α-GalCer loaded CD1d tetramers and TcRβ antibodies, double positive cells were further characterized according to CD4 and NK1.1 status (See section 5.3.6 for antibody information). Representative dot plots for flow cytometry experiments are present in Figure 5.4: (Thymus) 5.5: (Spleen) 5.6: (Liver). Panel A represents cells from wild type animals while Panel B represents samples from TGRKO mice. NKT cell number and proportions were normal in thymus and liver between WT and KO (Figs 5.4C & D and 5.6C & D respectively), however in TGRKO spleen total NKT cell number was decreased in comparison to WT (Figure 5.5C). Despite this decrease in cellularity, the total proportion of NKT cells in the spleen was not effected (Figure 5.5D), presumably due to the decrease in total cellularity in the spleen (Figure 5.2C). Subpopulations were analysed more closely by looking at NK1.1 and CD4 markers. Cellularity and proportions of all NKT cell subpopulations were normal in thymus and liver (Figure 5.4E & F, Figure 5.6E & F respectively). In the spleen however cell numbers of $NK1.1^{-}CD4^{+}$, $NK1.1^{+}CD4^{+}$ and $NK1.1^{+}CD4^{-}$ were significantly reduced (Figure 5.5E, based on Mann-Whitney U test, $p<0.05$) and proportions of $NK1.1^{-}CD4^{+}$, $NK1.1^{-}CD4^{-}$ and $NK1.1^{+}CD4^{+}$ altered (Figure 5.5F). $NK1.1^{-}CD4^{+}$ and $NK1.1^{-}CD4^{-}$ proportions were significantly increased, apparently due to the decrease in cell number of $NK1.1^{+}CD4^{+}$ cells, which causes significant decrease in the proportion of these cells also.

5.4.3: T_{Reg} cells develop normally in TGRKO thymus and liver, with some alterations in spleen

T_{Reg} cells were sorted from thymus, liver and spleen of WT vs TGRKO mice to analyze any differences in T_{Reg} development. T_{Reg} cells were stained with antibodies directed towards FOXP3, CD4, TcRβ and CD25 (see table 5.3.6 for an-

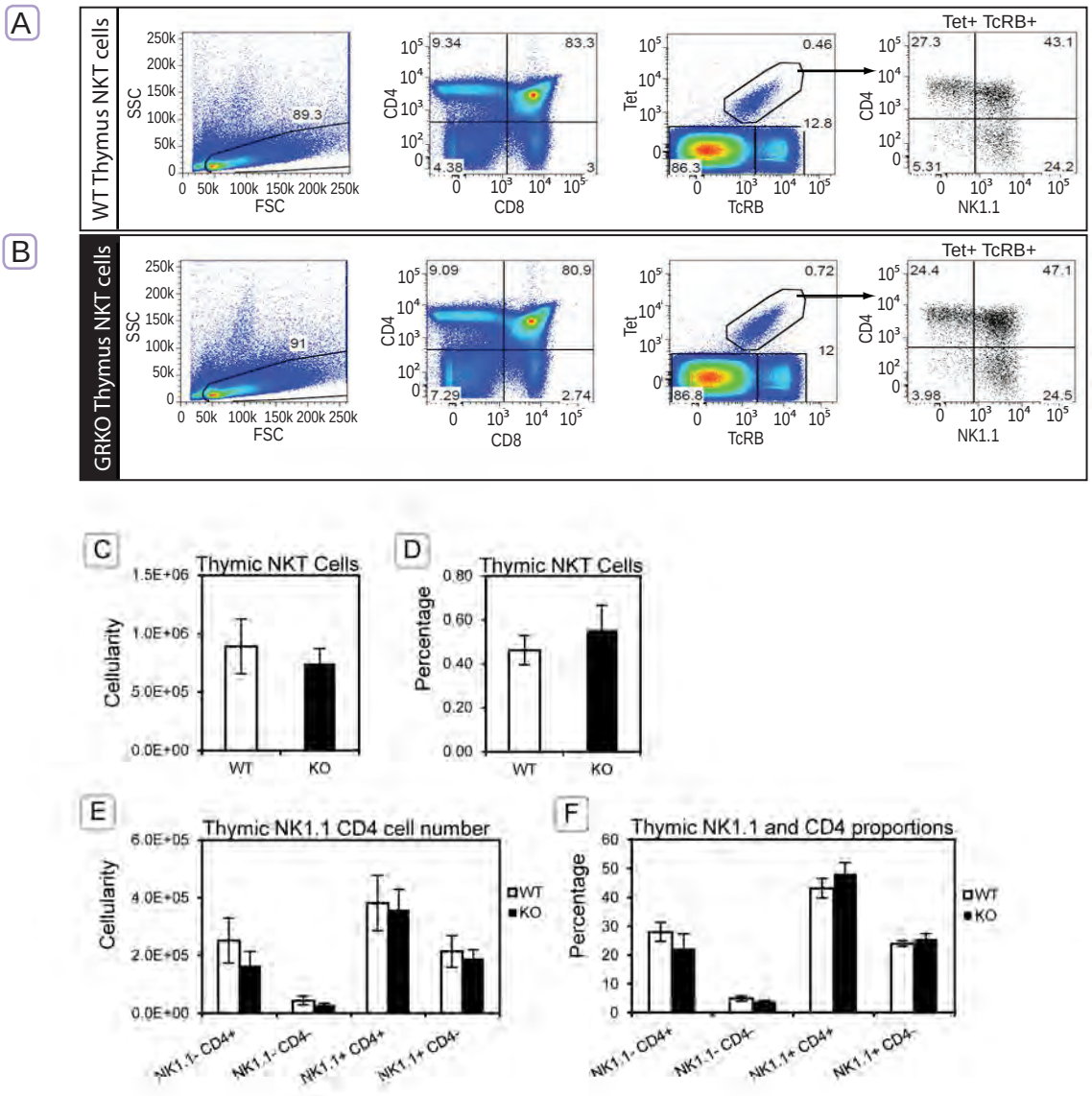


Figure 5.4: Thymic NKT cells are normal in TGRKO mice

Thymocytes from WT and TGRKO mice were isolated and stained for CD8, CD4, α Gal-Cer CD1d tetramer, NK1.1 and TcRβ expression and analysed by flow cytometry. A: Representative dot plots for flow cytometry of thymic NKT cells in WT mice and B: in TGRKO mice. C: Total NKT cellularity in WT and TGRKO mice. D: NKT proportions in thymus of WT and TGRKO mice. E: NKT cell numbers according to NK1.1 and CD4 expression in thymus F: proportions of NKT cells in thymus according to NK1.1 and CD4 expression

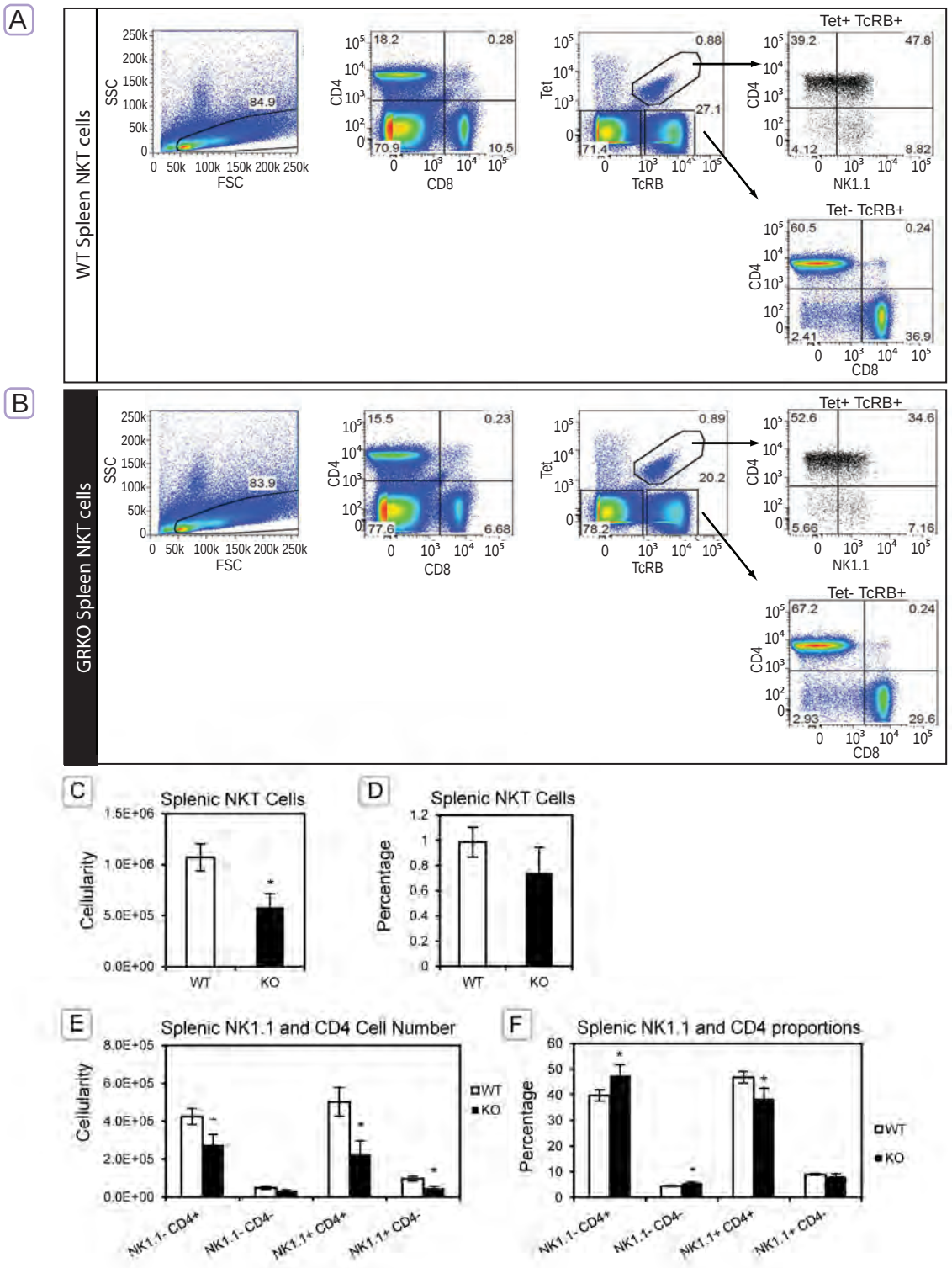


Figure 5.5: Splenic NKT cell numbers are decreased in TGRKO mice

Splenic NKT cells from WT and TGRKO mice were stained for CD8, CD4, α Gal-Cer CD1d tetramer, NK1.1 and TcRβ expression and analysed by flow cytometry. A: Representative dot plots for flow cytometry of splenic NKT cells in WT mice and B: in TGRKO mice. C: Total NKT cellularity in WT and TGRKO mice. D: NKT cell proportions E: NKT cell numbers according to NK1.1 and CD4 expression in spleen F: proportions of NKT cells. Bar graphs show mean ± SE. Statistical analysis was performed using Mann-Whitney U test. *Significant differences in total NKT cellularity, and NKT sub population cell number and proportion between WT and KO mice ($P<0.05$).

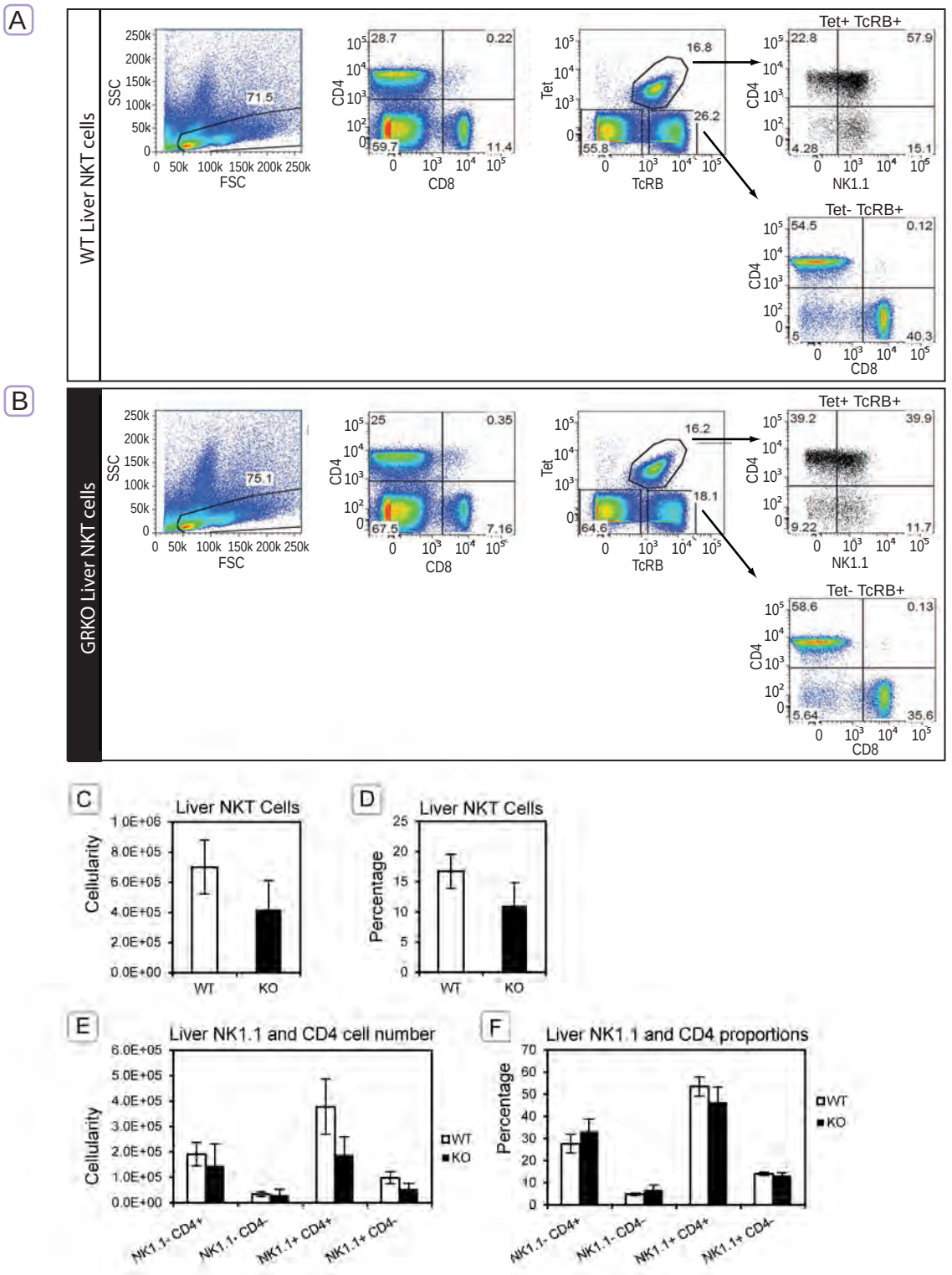


Figure 5.6: Hepatic NKT cell numbers are normal in TGRKO mice

Hepatic NKT cells from WT and TGRKO mice were stained for CD8, CD4, α Gal-Cer CD1d tetramer, NK1.1 and TcRβ expression and analysed by flow cytometry. A: Representative dot plots for flow cytometry of hepatic NKT cells in WT mice and B: in TGRKO mice. C: Total NKT cellularity in WT and TGRKO mice. D: NKT cell proportions E: NKT cell numbers according to NK1.1 and CD4 expression in liver F: proportions of NKT cells

tibody information). Cells were analyzed by flow cytometry. Cells positive for both FOXP3 and CD4 markers were further sorted according to TcRβ and CD25 phenotype. Representative dot plots for flow cytometry experiments are present in Figure 5.7: (Thymus) 5.8: (Spleen) 5.9: (Liver). Panel A shows a representative dot plot from wild type animals while Panel B represents samples from TGRKO mice. Cellularity and percentage of total T_{Reg} cells within thymus, spleen and liver (Figure 5.7C & D, Figure 5.8C & D and Figure 5.9C& D) were all normal in GRKO mice compared to WT counterparts. In thymus and liver, numbers and proportions of $CD25^+$ or $CD25^-$ T_{Reg} cells were consistent between WT and GRKO mice (Figure 5.7E & F and Figure 5.9E & F). In spleen, numbers of $CD25^+$ vs $CD25^-$ were normal between WT and GRKO mice (Figure 5.8E), however the proportion of $CD25^+$ T_{Reg} cells decreased in GRKO mice and the $CD25^-$ cells increased in proportion in GRKO mice compared to WT mice (Figure 5.8F).

5.5: Discussion

5.5.1: General Cellularity of thymus, spleen and liver

Proportions of CD8 CD4 subpopulations were not completely normal between TGRKO and WT mice, in contrast to previous findings (Brewer et al 2002, 2003, Purton et al 2002). The total number of thymocytes did appear decreased although this result was not significant; however there were significant decreases in numbers of both $CD8^+$ $CD4^-$ and $CD8^-$ $CD4^+$ populations. It is known that the variable numbers of CD8 and CD4 cells seen between different mouse strains are attributable to genetic variations in the process of thymic lineage commitment, although there is no literature regarding variation in numbers between individual mice within the C57B6 mouse thus there is the possibility that GR may regulate genes involved in this process, which have not been previously determined (van Meerwijk et al. 1998). Interestingly proportions of most CD8 CD4 subsets were largely normal in thymus, the exception being DN cells which were increased by 2% to a statistically significant degree, if GR was involved in death by neglect as

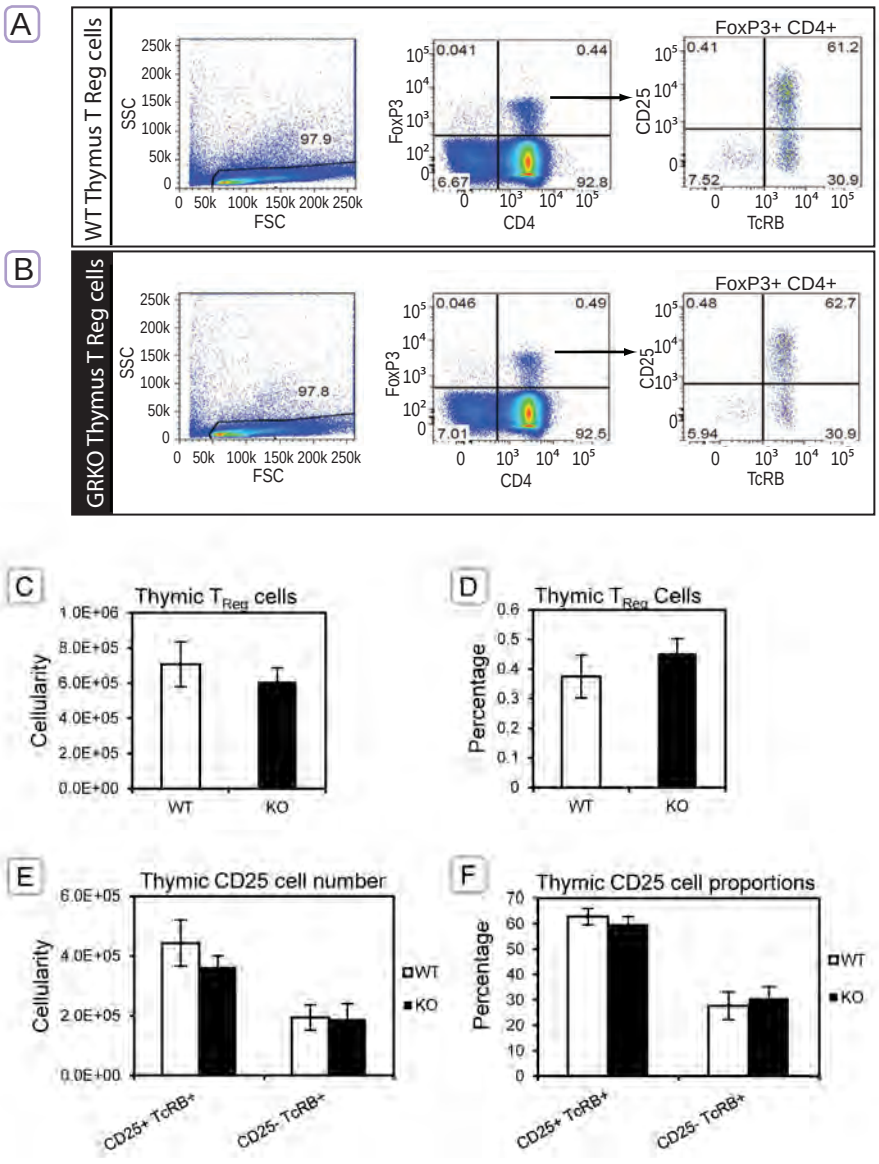


Figure 5.7: Thymic T_{Reg} cell numbers are normal in TGRKO mice

Thymus T_{Reg} cells from WT and TGRKO mice were stained for CD4, FoxP3, CD25 and TcRβ expression and analysed by flow cytometry. A: Representative dot plots for flow cytometry of thymic T_{Reg} cells in WT mice and B: in TGRKO mice. C: Total Thymic T_{Reg} cellularity in WT and TGRKO mice. D: T_{Reg} cell proportions in WT and TGRKO mice E: T_{Reg} cell numbers according to CD25 expression in thymus F: proportions of T_{Reg} cells according to CD25 expression in thymus.

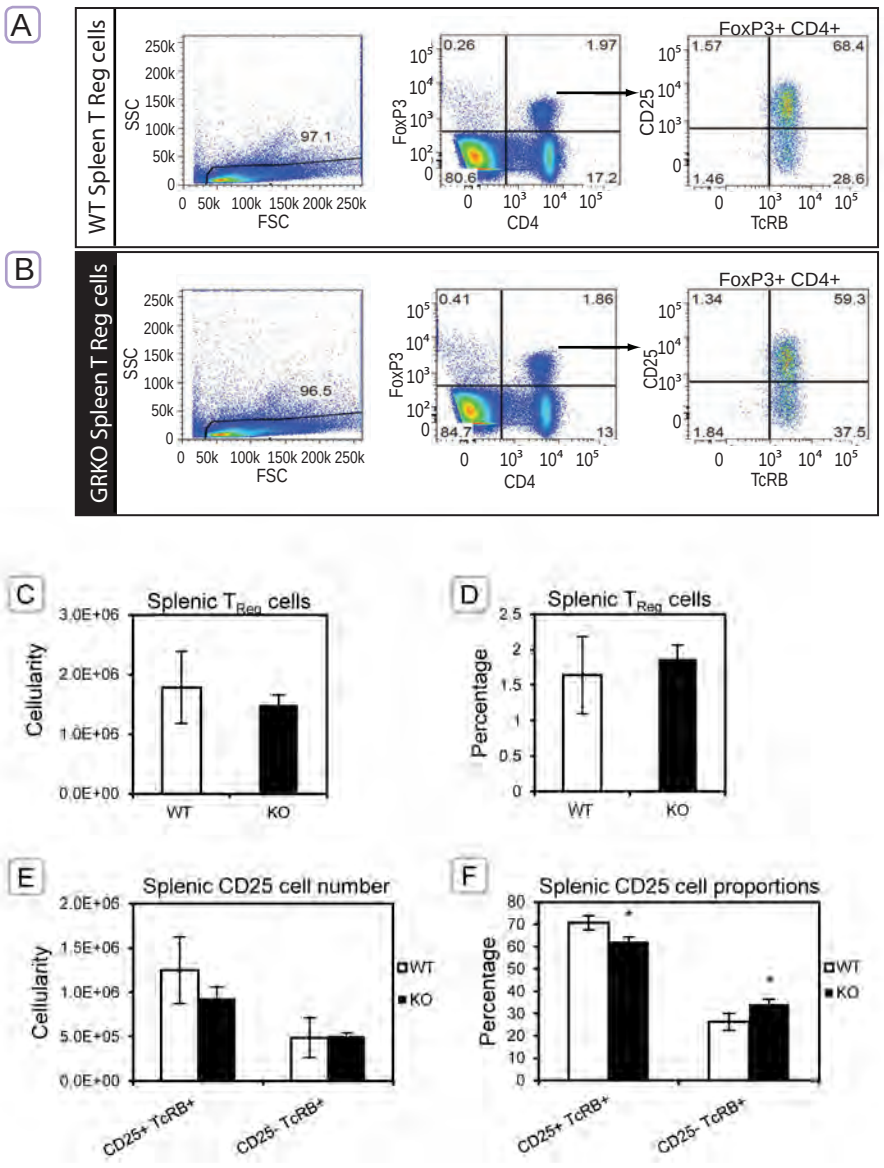


Figure 5.8: CD25⁺ T_{Reg} cell proportions are altered in Splenic TGRKO mice

Splenic T_{Reg} cells from WT and TGRKO mice were stained for CD4, FoxP3, CD25 and TcRβ expression and analysed by flow cytometry. A: Representative dot plots for flow cytometry of splenic T_{Reg} cells in WT mice and B: in TGRKO mice. C: Total splenic T_{Reg} cellularity in WT and TGRKO mice. D: T_{Reg} cell proportions in WT and TGRKO mice E: T_{Reg} cell numbers according to CD25 expression in spleen F: proportions of T_{Reg} cells according to CD25 expression in spleen. Bar graphs show mean ± SE. Statistical analysis was performed using Mann-Whitney U test. *Significant differences in proportions of splenic T_{Reg} cell sub populations between WT and KO mice ($P<0.05$).

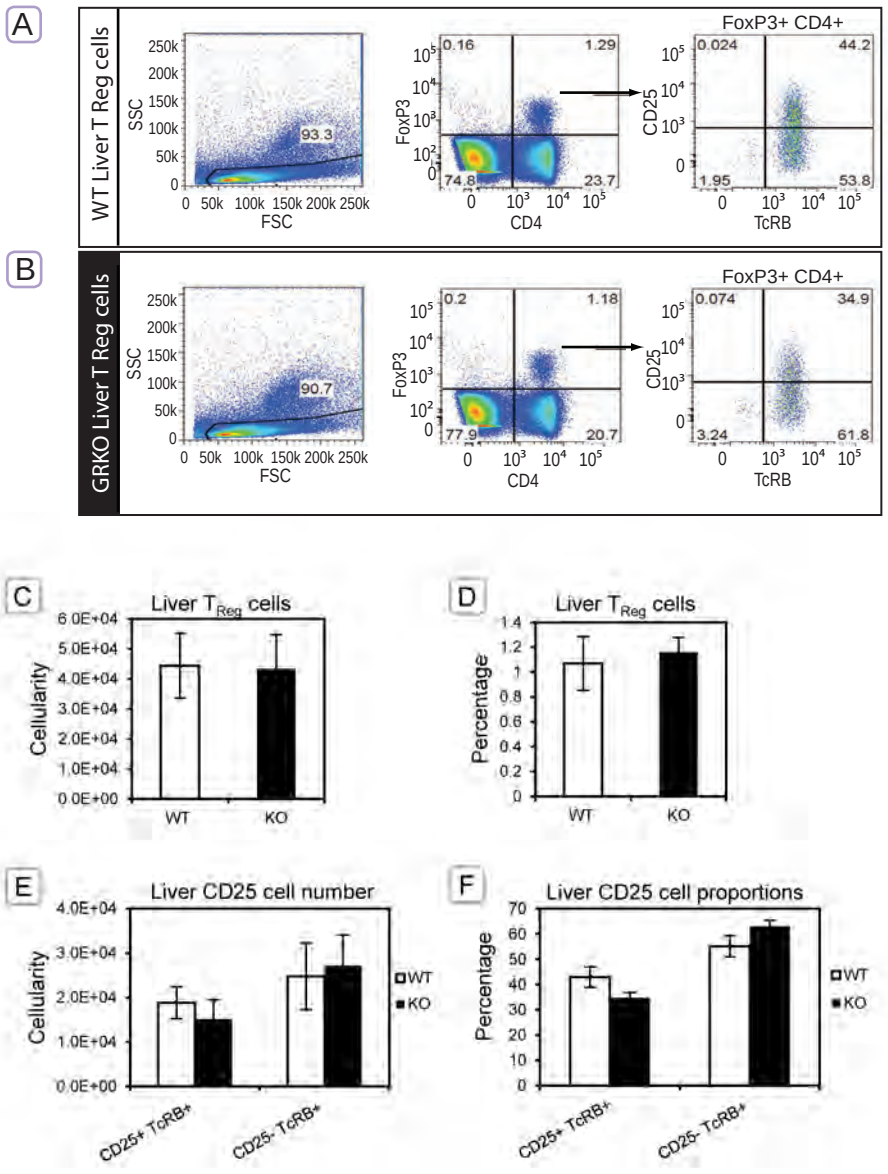


Figure 5.9: T_{Reg} cells are normal in livers of TGRKO mice

Hepatic T_{Reg} cells from WT and TGRKO mice were stained for CD4, FoxP3, CD25 and TcRβ expression and analysed by flow cytometry. A: Representative dot plots for flow cytometry of hepatic T_{Reg} cells in WT mice and B: in TGRKO mice. C: Total hepatic T_{Reg} cellularity in WT and TGRKO mice. D: T_{Reg} cell proportions in WT and TGRKO mice E: T_{Reg} cell numbers according to CD25 expression in liver F: proportions of T_{Reg} cells according to CD25 expression in liver.

described by the mutual antagonism model we might expect to see an increased proportion of DP cells in the absence of GR, which was not observed. Total splenocyte numbers were decreased in TGRKO vs WT mice, with a corresponding decrease in all CD8 CD4 subpopulations, which was not described in a previous study of T-cell specific GRKO mice (Brewer et al. 2003) or in mice irradiated and reconstituted with fetal liver precursors, where total splenocyte numbers were normal at 6 weeks of age (Brewer et al. 2002; Purton et al. 2002). Total numbers of hepatocytes were normal between TGRKO vs WT mice in our study, consistent with previous findings (Brewer et al. 2002; Purton et al. 2002), however CD8 CD4 T-cells were decreased in liver. This observation of decreased CD8 CD4 T-cells in spleen and liver is consistent with the finding that these populations are decreased in the thymus, as this is where they arise. Interestingly the decrease in $CD8^-$ $CD4^+$ cell number seen in the spleen is greater than that observed in the thymus, since T-lymphocytes can expand to maintain population sizes even in the absence of cellular input from the thymus this may indicate their “self renewal” capacity has been disrupted (Freitas, et al. 1986). The known GR target gene *Gata-3* is involved in early stages of T-cell development promoting development of $CD4^+$ cells. Fusion with a LacZ reporter gene showed *Gata-3* expression at the onset of positive selection events, i.e., TcRαβ up-regulation and CD69 expression, and expression remains during maturation of CD4 single-positive (SP) cells in the thymus, but is almost absent in CD8 SP cells (Nawijn, et al. 2001). GCs have been shown to repress *Gata-3* expression and protein synthesis (Liberman, et al. 2009), thus a model of *Gata-3* overexpression may give more insight into whether the affect observed in our GRKO mice is due to a Gata-3-GR interaction. *Gata-3* overexpression decreased $CD8^+$ population numbers in spleen and lymph node, and although numbers were normal in thymus, maturation was affected. $CD4^+$ T-cells were normal in the periphery (Nawijn et al. 2001). Decreased $CD8^+$ cells were observed in our experiment,although in general there was a concomitant decrease in $CD4^+$ cells, with the exceptions of the spleen where $CD8^+$ cells were

significantly decreased in number and proportion, which caused an elevation in the proportion of $CD4^+$ cells, this may be attributed in part to increased *Gata-3* expression, however it is clear that alteration of its expression alone cannot explain our results, given the wide range of targets regulated directly and indirectly by GR and *Gata-3,* there is clearly a large network of genes involved, furthermore the precise role of GR regulation of *Gata-3* in this process requires further investigation.

5.5.2: NKT cells

Our study examined Type I invariant NKT cells, which is the most well characterized subset of NKT cells. NKT cells were sorted for CD4, α-GalCer loaded CD1d tetramer, TcRβ and NK1.1. In our study NKT cells developed normally in the thymus and were present in normal numbers in liver, however NKT cells were altered in spleens of TGRKO mice compared to WT controls. Total NKT cellularity was decreased in the spleens of TGRKO mice, while the percentage of NKT cells was not significantly decreased, most likely due to the total decrease in splenocyte number observed in TGRKO mice compared to WT controls. In keeping with the finding of reduced NKT cell number in the spleen, most subpopulations based on NK1.1 and CD4 status were correspondingly decreased in number, with the exception of $NK1.1^-CD4^-$ of which there are very few cells. The $NK1.1^+CD4^+$ cells were the most affected with cellularity decreasing by more than half, the proportion of these cells was not so severely altered, again presumably because of the total decrease in splenic NKT cellularity, the percentage of $NK1.1^+CD4^+$ cells decreased by 8% in TGRKO mice. NKT cells were shown to develop normally in the thymus and (in contrast to our data) peripheral tissues of irradiated mice reconstituted with WT vs GRKO fetal liver precursors (Purton et al. 2002). There are many factors regulating development of NKT cells, disruption of most factors involved in NKT cell development discovered to date result in abnormalities or deficiencies in thymic populations and/or all NKT cells, however two factors have

been shown to impact NKT cell numbers only in the periphery when their expression is disturbed; these are *Bcl10* and *Gata-3*.

BCL10 is an adaptor signal transduction molecule required for activation NF-κB, well known for its role in T-cell development. Deletion of *Bcl10* leads to a >10 fold reduction of NKT cells in the spleen, liver and bone marrow (Schmidt-Supprian, et al. 2004). This finding is not completely consistent with our results, additionally there is no evidence of *Bcl10* regulation by GR in the current literature.

Gata-3 deficiency results in defects in thymic maturation and peripheral maintenance of murine iNKT cell numbers, thought to be due to disrupted maturation and increased apoptosis. Thymic NKT cell numbers from Gata-3KO mice were normal however surface TcR levels were decreased, in the liver and spleen, NKT cell numbers were markedly reduced in Gata-3KO mice, and overall CD4+ NKT cells were decreased (Kim et al 2006). Thus *Gata-3* overexpression does result in NKT abnormalities similar to that observed in TGRKO mice and may in part explain the differences seen here.

5.5.3: T_{Reg} cells

As described, multiple TReg markers have been shown to be regulated by GCs, for example *Gitr* and *Ctla4* (Chen, et al. 2004), but more significantly *FoxP3* which is crucial for T_{Reg} development (Chen et al. 2004; Chen, et al. 2006; Prado, et al. 2011). Thus it was of interest to study T_{Reg} cells in the context of TGRKO mice, something which has not previously been studied. We examined "nT_{Reg}" cells positive for CD4, FOXP3 and CD25. In our study the only significant difference observed between TGRKO and WT mouse T_{Reg} cells was the proportion of $CD25^{+}FOXP3^{+}CD4^{+}$ cells which were decreased in the TGRKO spleen, with a corresponding increase in $CD25^{-}FoxP3^{+}CD4^{+}$ cells in TGRKO spleen compared to WT. Although CD25 is crucial for the T_{Reg} phenotype, as evidenced by $CD25^{-}$ deficient mice which show autoimmunity (Malek, et al. 2002), in a rat model of diabetes, TxX, $CD25^{-}CD45RC^{low}CD4^{+}$ cells (equivalent to $CD45RB^{low}$ in mice)

from the peripheral thoracic duct lymphocytes were protective against the onset of diabetes, although not to the same degree as $CD25^+$ cells, while $CD25^-$ thymocytes were not able to suppress autoimmune disease and actually accelerated the onset of diabetes (Stephens and Mason 2000). Thus we would not necessarily expect T-cell specific GRKO mice to show severe autoimmune disease of the spleen, particularly as TGRKO mice retained a large number of $CD25^+$ cells, however it cannot be ruled out without further analysis. There is evidence for many subtypes of T_{Reg} cells expressing different markers and in other tissues of the mouse (reviewed in Campbell and Koch 2011; Dimeloe, et al. 2010). Our study focuses on nT_{Reg}s which develop in the thymus and also exist in the periphery. In this study we sorted only for FOXP3, CD4 and CD25 positive cells, thus our analysis is certainly not exhaustive. In addition to nT_{Reg}s there are populations of induced – "i T_{Reg}" cells which develop outside of the thymus in response to TCR stimulation and the cytokines IL-2 and TGF-β, not all of which express *FoxP3*. Additionally there is evidence that T_{Reg} cells developing in the thymus can change phenotypically, for example some begin to express CD44 as they migrate into the periphery thus it would be interesting to study total GR null mice more closely in this context for evidence of autoimmunity in order to further define the role of GCs in development of these sub populations which arise in the periphery.

Functional studies were not performed on the lymphocyte subsets examined, and these will be required to determine whether there are more functional effects from the total loss of GR in T-cells other than decreased cell numbers and modified proportions, such as immune challenge, cytokine assays, cytotoxic capacity of and NKT cells and with reference to T_{Reg}s, protection against autoimmunity. However, this close examination of a few lymphocyte subsets has revealed subtle differences in populations not observed previously (Brewer et al. 2002; Brewer et al. 2003; Purton et al. 2002) and more consistent with the loss of the ubiquitously expressed transcription factor GR.

GR has been shown to effect a multitude of transcription factors involved in lymphocyte development as discussed here, which in turn have their own targets, thus the network of genes that is potentially disrupted is likely to be extensive. It would be interesting to study further the requirement of GR regulation of genes discussed here such as *Gata 3*. Another interesting finding of this study was that generally lymphocyte populations in the spleen were the most affected thus it would be interesting to examine cell emigration, maintenance and self renewal capacity of peripheral lymphocytes in TGRKO mice. In summary this study has revealed the loss of GR in mouse T-lymphocytes does have subtle effects on thymic and peripheral subpopulations of CD8 CD4 cells, NKT and T_{Reg} cells.

CHAPTER 6:

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Glucocorticoids, via their receptors, regulate the transcription of many target genes involved in metabolism, the stress response, immune function, development, reproduction, and apoptosis. Many of these putative targets are as yet unidentified or their mechanism of action is not fully defined. Expression of the GR itself is quite ubiquitous; however there are multiple layers of regulation and mechanisms in place to ensure cell specificity. GR is able to regulate gene transcription in number of ways dependent on the presence of other activating or repressing transcription factors, the presence of translational isoforms provides regulation of GR activity and additionally the multiple untranslated exon 1s confer an extra level of cell specificity to GR expression, with transcripts originating from the 1A promoter, for example, localised to a particular subset of tissues and cell types. In these cells, transcripts originating from the 1A promoter are believed to confer greater GC sensitivity to the cells, serving as a fine tuning mechanism thus ensuring that these cells can rapidly respond to rising levels of GC and aid in the return to the normal state. The cell specificity of the GR transcripts is believed to be enhanced by the presence of multiple individual promoter regions for each of the exon 1s allowing unique combinations of cell specific transcription factors to interact and regulate these promoter regions, thus establishing these complex expression patterns. The identities and interactions of specific factors regulating the individual GR gene promoters are yet to be further resolved, as is the functional role of these transcript isoforms in different cells. The role of elevated levels of GR1A promoter activity in particular has been associated with increasing lymphocyte sensitivity to GICD (Purton, et al. 2004), while direct GR targets in these cells are still to be elucidated. Early studies pointed to the role of GICD in T-cell development, as a way of eliminating cells via negative selection.

Thus there are many aspects of GR regulation, its affects on subsequent downstream targets and in turn on stress-response systems, and T-cell development and function which remain unexplored.

In studies presented here I confirmed the presence of GR1A promoter-driven tran-

scripts in the mouse brain cortex and in the pituitary, in agreement with the recent observation of GR1A transcripts in the pituitary and also previous observations of GR1A in the cortex (Bockmuhl, et al. 2011; Purton et al. 2004). In the pituitary the GR1A promoter was subject to auto-regulation. In response to GC treatment GR1A transcript levels were maintained, while total GR mRNA levels were decreased. Thus as a percentage of total GR levels an increase in GR1A transcript levels was observed. Purton et al (Purton et al. 2004) have suggested that expression of the GR gene 1A promoter in the cortex of the brain may serve a similar role to that in thymocytes, i.e. to make cells more sensitive to GC levels, thus ensuring that cells of the cerebral cortex can respond to rising stress-induced levels of GC (Purton et al. 2004). This may be the case in pituitary cells also, especially due to the observation of GR1A transcripts in close proximity to or within Folliculostellate (FS) cells, which play an important role in intra-pituitary signalling by attenuating stress and immune responses. Whether the mechanisms occurring in the brain are the same as those in thymocytes, for example driving increased levels of GR mRNA, increased expression of GRα-B and the maintenance of stable GR protein, remains to be determined.

It is important to confirm that GR1A transcripts are in fact present in FS cells; this could be done by examining GR1A transcript levels in an FS cell line (for example TtT/GF cells) by qRT-PCR. Following this, treatment of FS cells with dexamethasone (dex) and subsequent analysis of GR1A transcript levels and western blotting of GR-B protein would determine whether the mechanisms occurring here are similar to those in the thymus i.e. with elevated GR1A transcripts there is an increased GRα-B:A ratio. Additionally, to determine whether this interaction occurs *in vivo*, *in-situ* hybridization to GR1A and immunodetection of GR-B protein, to co-localise the two in FS cells could be performed. A GR1A knockout mouse is currently under construction which would allow the importance of this promoter to be examined even more closely. Mice could be subjected to various stressors and their ability to cope and return to the normal state could be monitored. GRα-B:A

protein ratios could be analysed in various tissues, differences in gene regulation patterns between WT mice and total GR null or T-cell specific mice would also be interesting to examine.

While the implication of having elevated GR1A promoter activity and thus increased GC responsiveness in T-lymphocytes is that they are more sensitive to GICD, the network of target genes affected by GR has not fully been established. Particularly in terms of a model of the “normal” situation, there is little data. The majority of experiments have been performed on human and cancer cell lines. Our study (presented in Chapter 3) was the first to examine gene regulation in the thymus following acute *in vivo* glucocorticoid treatment. Whole genome expression microarray analysis revealed a large subset of acutely glucocorticoid-regulated genes. Functional cluster analysis showed many of the up-regulated genes were linked to cell proliferation or apoptosis, giving many possible candidates for driving GICD in mouse thymocytes and also demonstrates the intracellular complexity of this process. Some of the genes found to be up-regulated in our study had previously been reported in other microarray analyses of mouse primary thymocytes, these included *Bim*, *Ddit4*, *Fkbp5*, *Nfkbia* and *Txnip* (Bianchini, et al. 2006; Miller, et al. 2007; Woodward, et al. 2010). Interestingly all these targets, excluding Bim, are thought to be anti-apoptotic, thus their early induction may be an effort to protect cells from apoptosis.

In this study we have focussed on the potential role of a strongly dex-induced gene called *Nfil3* in GICD. *Nfil3* was previously thought to be a direct GR target and elevated levels of *Nfil3* shown to precede GICD (Priceman, et al. 2006; Wallace, et al. 1997). Using ChiP we demonstrated for the first time that GR does in fact bind to the *Nfil3* promoter in primary mouse thymocytes and that this most likely accounts for rapid induction of *Nfil3* mRNA and protein levels following dex treatment. We co-localised NFIL3 to apoptotic cells and macrophages in the thymus demonstrating its association with apoptotic thymocytes in vivo. We

characterised NFIL3 induction in the cytoxic T-lymphocyte cell line, Ctll-2, and demonstrated that the response is comparable to thymocytes *in vivo*. By decreasing expression of *Nfil3* in Ctll-2 T cells using siRNA technology our study is the first to definitively show that *Nfil3* for normal GC-induced apoptosis in Ctll-2.

Although in normal Ctll-2 cells 3h of dex treatment was when apoptosis began to occur and after 6 h Annexin V was no longer able to detect early apoptotis, it would be of interest to extend the study and do a longer treatment time for the *Nfil3* knockdown samples to determine whether knockdown of *Nfil3* only delays GICD. However if other mechanisms do take over this may not represent the true case of acute GICD in these cells, and may simply be the affect of GR on the multiple other genes that can contribute to GICD.

Nfil3 has been characterised as an active transcriptional repressor, binding to response elements in the promoters of target genes and inhibiting their transcription directly, not by inhibiting binding of an activator by competitive binding to DNA or by protein-protein interactions with an activator (Cowell and Hurst 1994; Cowell et al. 1992). From our microarray data we have identified genes which are down-regulated in mouse thymus following 3 h of dex treatment, that have been previously associated with apoptosis and which have putative NFIL3 response elements within their promoters. Three genes of particular interest are *c-Myc*, *Ccnd3* and *Traf1*.

Ccnd3 and *c-Myc* have previously been studied in the context of GICD, and seem to work together: both are down regulated by GCs prior to cell cycle arrest, (Forsthoefel and Thompson 1987; Ma, et al. 1992; Rhee, et al. 1995) and over-expression of both *c-myc* and *ccnd3* protected P1798 cells from GC-induced cell cycle arrest or apoptosis, although individual overexpression of these genes was not protective (Rhee et al. 1995; Thompson, et al. 1992).

In silico promoter analysis identified two putative NFIL3 binding elements in the promoter of the *Ccnd3* gene and also the *c-Myc* promoter, however GC regulation of these genes has not been confirmed as occurring directly. GC regu-

lation of *Ccnd3* has been shown to occur largely via RNA-protein interactions occurring in the 3'UTR of the *Ccnd3* gene (Garcia-Gras, et al. 2000), although one of the proteins identified as binding to the mRNA in this region is in fact a 52kda protein, there is no evidence of NFIL3 acting as an RNA binding protein. It remains to be determined whether NFIL3 influences regulation of this gene from the 5'UTR. While the rapid decrease in *c-Myc* in response to GCs (Forsthoefel and Thompson 1987) does suggest direct GR action upon the *c-Myc* promoter, conflicting promoter analysis using luciferase reporters leaves this in question (Ashwell et al. 2000; Zhou et al. 2000).

Tumour necrosis factor Receptor Associated Factor 1 (*Traf1*) is a protein that interacts with the TNF receptor protein via TRAF2 (Rothe, et al. 1994) and has an anti-apoptotic affect on antigen induced $CD8^+$ cells, additionally over expression of TRAF1 has been observed in cases of CLL and non-Hodgkin's lymphomas, indicating its role in promoting cell survival. There are no previous studies demonstrating GC regulation of this gene. We have identified one consensus NFIL3 binding element in the upstream region of this gene.

Preliminary experiments examining the regulation of *c-Myc* by dexamethatsone in *Nfil3* deficient cells revealed an increase in *c-Myc* expression, indicating NFIL3 may normally repress *c-Myc* in the presence of GCs, and thus providing a possible mechanism for the protective effect of *Nfil3* knockdown against GICD. It will be interesting to determine whether any of these potential binding elements found in the *c-Myc* promoter and other gene promoters discussed here are in fact bound by NFIL3 following Dex treatment and how NFIL3 regulation of these gene targets is important in GICD. This could be performed using ChIP experiments and further knockdown experiments targeted to these specific genes. Additionally, these NFIL3 targets were chosen via functional analysis looking for genes involved in cellular growth or pro survival genes, thus the list may be incomplete, not recognising novel targets which have not been studied in this context. With the increasing use of sequencing technologies, ChIP-Seq could extend these ex-

periments further, identifying direct GR and NFIL3 target genes to provide further insight into the complex gene and signalling networks occurring and perhaps identify novel targets which may help to complete the puzzle. These target genes have been studied in the context of GICD in different cell lines than used in this study thus it would be interesting to nullify *Nfil3* activity in additional cell lines to compare results or to study these genes in *Nfil3* KO mice and primary cells.

Interestingly the loss of *Nfil3* in mice eliminates NK cell development. Although NK cell development occurs largely in the bone marrow and it is unknown whether GR up-regulates *Nfil3* in the bone marrow as it does in thymocytes, it would be interesting to examine a role for GC signalling during NK cell development. There is little evidence of GC regulation of the other major transcription factors involved in NK cell development, *TOX*, *PU.1*, *Ets1* and *Ikaros* although the latter have all been implicated in cooperative or competitive actions with GR to regulate various target genes (Wargnier et al. 1998; Aittomaki et al. 2000; Geng and Vedeckis 2011). Dexamethasone inhibited expression of ID2 in adipocyte cells and erythroid cells (Zilberfarb et al. 2001; Gabbianelli et al. 2003).

In the context of GR and lymphocyte development, we were able to study this more closely with the availability of “TGRKO” mice, where GR expression has been ablated in vivo in T-cell derived lineages only (Brewer, et al. 2003). The involvement of GR during T-cell development was proposed in the 90s following studies in culture using specific antigens to simulate AICD of T-cells, where application of either Dex or antigen alone induced apoptotis whereas co-application of GCs and antigen did not induce death (Zacharchuk, et al. 1990). The “Mutual antagonism model” suggested endogenous glucocorticoids can cause thymocyte programmed cell death and only those thymocytes that were activated by self antigen would be permitted to survive (positive selection). Too much avidity for self molecules, and thus too strong an activation signal, would overcome the antagonism provided by the GCs, resulting in activation-induced cell death (negative

selection) (Zacharchuk et al. 1990).

Studies of total GRKO mice under control of various promoters gave conflicting results (King et al. 1995; Morale et al. 1995; Reichardt et al. 1998), however irradiated mice reconstituted with exon 1C-2 or exon 2 GRKO murine fetal liver precursor cells showed normal numbers of total T-lymphocytes in the thymus, spleen, lymph node and liver between both GR null models and their wild type counterparts (Brewer, et al. 2002; Purton, et al. 2002). Additionally, subpopulations of DN, DP or SP, and NKT, memory and activated T-cells were also not significantly different in either model or genotype indicating normal T-cell development and maintenance (Brewer et al. 2002; Purton et al. 2002). These models are more reliable as GR is completely absent from thymocytes and circulating GCs are normal, unlike some adrenalectomized models; models where GC release is inhibited or models where GR activity is only partially reduced, although these studies are still continuing and have shown differences in CD8 CD4 subsets (Pazirandeh et al. 2005; Reichardt et al. 1998; Sato et al. 2010; Stojic-Vukanic et al. 2009). Potentially the best model is the T-cell specific “TGRKO” mouse model generated by crossing GR exon 1B/C/2 floxed alleles with Lck-cre mice. Analysis of this model revealed normal thymic cellularity and normal subset distribution in thymus, spleen and lymph nodes, although the analysis of T-cell subset distribution was not shown, and specialized T-cells subsets were not examined (Brewer et al. 2003). Thus there are aspects of the involvement GR and GCs in T-cell development and selection that remain controversial, particularly some subsets which have not been studied in detail. Following T-cell selection and differentiation into CD8 or CD4 cells, T-cells receive a multitude of signals specifying them toward their function. With GCs known to regulate so many target genes the potential for GCs to impact this development is enormous thus we studied two particular T-cell subtypes further, NKT and T_{Reg} cells, to analyse the requirement of GR for development.

Proportions of CD8 CD4 subpopulations were not completely normal be-

tween adult TGRKO and WT mice, in contrast to previous findings (Brewer et al. 2002; Brewer et al. 2003; Purton et al. 2002). Although not significant, total thymocyte numbers did appear decreased in TGRKO mice. The large variation observed in WT thymocyte numbers may account for this figure being non-significant. There is no literature describing the extent of natural variation in differences in total thymocyte number between C57B6 mice, additionally CD8 CD4 subpopulations were disproportionally decreased in number, thus cell collection is most likely not the issue, and the variation seen is most likely a result of random variation. The expected observation if GR was involved in T-cell selection as described by the mutual antagonism model would be an increased proportion of DP cells in the absence of GR, which is not observed. In fact in TGRKO mice DP numbers seemed decreased, although this was not statistically significant. Total splenocyte numbers were decreased in TGRKO vs WT mice, with a corresponding decrease in all CD8 CD4 subpopulations, and a decrease in the proportions of SP sub-populations which was not described in previous studies (Brewer et al. 2002; Brewer et al. 2003; Purton et al. 2002). Total numbers of hepatocytes were normal between GRKO vs WT mice in our study, consistent with previous findings (Brewer et al. 2002; Purton et al. 2002), however all CD8 CD4 T-cell subpopulation numbers were decreased in the liver.

As previously described, the GR repressed transcription factor GATA-3 is involved in early stages of T-cell development, and is important for promoting maturation of CD4 single-positive (SP) cells in the thymus and is almost absent in mature CD8 SP cells (Nawijn, et al. 2001). Thus *Gata-3* could be a GR regulated targeted candidate whose disregulation may be responsible for the alterations in T-cell populations observed in this study in the absence of GR. In a model of *Gata-3* over-expression, decreased numbers of $CD8^+$ were observed in the spleen and lymph node, and although numbers were normal in thymus, maturation was affected. $CD4^+$ T-cells were normal in the periphery (Nawijn et al. 2001). In our study decreased $CD8^+$ cell numbers were observed, although in general there

was a concomitant decrease in $CD4^+$ cells, with the exception of the spleen where $CD8^+$ cells were significantly decreased in number and proportion, which caused an elevation in the proportion of $CD4^+$ cells. Thus, the data presented here is different to that expected if *Gata-3* alone was the major target disrupted by loss of GR signalling. Gene expression analysis in a mouse model of *Gata-3* overexpression occurring in CD2 cells, revealed 276 genes were found to be differentially regulated with a fold change ±1.4 and p-value <0.0001. Additionally ChIP-Seq has revealed the extent of GATA-3 binding in a number of T-cell subsets (Wei, et al. 2011). It is clear that removal of GR alone affects many targets, 444 target GR genes were differentially regulated in our own microarray studies of total thymus, and with the secondary aspect of the role of GATA-3 regulation itself in T-cell development and the large subset of genes affected it is likely that removal of GR would affect many GATA-3 target genes indirectly. The precise role of GR regulation of *Gata-3* in this process requires further investigation.

In general T-lymphocytes can expand to maintain population sizes even in the absence of cellular input from the thymus, thus the decrease in peripheral CD8 CD4 cells in the periphery may indicate their “self renewal” capacity has been disrupted (Freitas, et al. 1986). It is difficult to comment further on factors which influence maintenance of CD8 CD4 population numbers in the periphery, as these represent a wide range of differentiated cells, factors affecting individual subtypes studied here will be discussed.

In our examination into development of type I invariant NKT cells in the absence of GR we observed normal NKT cell development in the thymus and normal numbers in liver. Total NKT cellularity was decreased in the spleens of TGRKO mice, and there was a concomitant decrease in most subpopulations based on NK1.1 and CD4 markers. The percentage of total splenic NKT cells was not significantly decreased; however the proportions of the individual subpopulations were altered. This is in contrast to previous studies where NKT cells were shown to develop normally in the thymus and peripheral tissues (Brewer et al. 2002; Purton

et al. 2002). As described there are two factors that have been shown to impact NKT cell numbers exclusively in the periphery when their expression is disturbed; these are *Bcl10* and *Gata-3*. *Gata-3* is the more interesting candidate, due to the effect seen on peripheral NKT cells following *Gata-3* gene ablation, however the likely affect of loss of GR would be an increase of *Gata-3* expression and NKT populations have not been studied in a model of *Gata-3* overexpression.

Some genes known to be involved in NKT development have been identified as differentially regulated by *Gata-3* overexpression and include *Sap* and *c-Myc* (van Hamburg et al. 2009). Additionally, some genes involved in NKT cell development and maturation have not been described as being regulated by GCs in T-cells in current literature, however they were observed to be significantly altered in our microarray analysis in 3h Dex-treated GRKO vs WT mice, these were: *Fos12*, *Egr2*, and *Runx*. *Fos12* deficient mice have increased numbers of iNKT cells and NKT cells in the periphery are hyper-responsive to antigen, showing increased expansion and produce increased amounts of IL-2 and IL-4 compared with their wild-type counterparts (Lawson et al. 2009). *Egr2* is important in the selection, survival and maturation of NKT cells (Lazarevic et al. 2009). *Runx1* seems to be involved in selection or subsequent expansion of iNKT precursor cells (Egawa et al. 2005) and thus is an interesting candidate gene for GR regulation during NKT development and maintenance in the periphery and its dis-regulation may account for the decreased splenic NKT cells observed here. Further investigation of the role these GR target genes would be of interest.

Our study is the first to examine the development of regulatory T-cells in the absence of GR. Given the known regulation of markers including the crucial lineage specifying transcription factor FoxP3, it is an important study (Chen et al. 2004; Chen et al. 2006; Prado et al. 2011). In our study of nT_{Reg} cells the only significant difference observed between TGRKO and WT mouse T_{Reg} cells was the proportion of CD25+FoxP3+CD4+ cells which were decreased in the TGRKO spleen, with a corresponding increase in CD25-FoxP3+CD4+ cells in TGRKO

spleen compared to WT.

As explained, although CD25 is crucial for the T_{Reg} phenotype and immunosuppression, there is evidence that the altered proportion of $CD25^+$ cells observed in the spleens of TGRKO may not necessarily mean they show autoimmune disease, particularly since there are normal cell numbers which presumably can compensate functionally (Malek et al. 2002; Stephens and Mason 2000). From studies of Scurfy mice lacking FOXP3 and the observation that high-levels of FOXP3 is sufficient to confer suppressive activity to normal non-T_{Reg} cells it is evident that the transcription factor FOXP3 confers suppressive activity to T_{Reg} cells presumably via regulation of an as yet undefined network of genes, which so far is known to include *Nfat* (nuclear factor of activated T cells), *Aml1* (acute myeloid leukemia-1)/*Runx1* (runt-related transcription factor 1), the histone acetyl transferase (*Hat*)/histone deacetyl transferase (*Hdac*) complex, and possibly *Nf-κB*. Thus although in this study T_{Reg} cells were able to develop relatively normally, there are many aspects which may affect normal T_{Reg} function. Therefore it is important to study these mice in terms of regulatory functionality as it is possible that GR regulation of *FoxP3* is important in the suppressive process, if not in development, and that these mice could develop adult autoimmune disease.

An interesting outcome of this study was that all T-cell subsets examined were most significantly affected in the spleen. The reason for this is unknown, and while some genes have been shown to impact peripheral populations only, none quite fit this observed pattern. In fact this close examination of a few lymphocyte subsets has revealed subtle differences in populations not observed previously (Brewer et al. 2002; Brewer et al. 2003; Purton et al. 2002) and is more consistent with the loss of the ubiquitously expressed GR transcription factor. In our microarray analysis, 444 genes were found to be differentially regulated in thymus from Dex treated WT or GRKO mice, with a fold change of ±1.5 and p value ≤0.05, additionally GR has been shown to affect a multitude of transcription factors involved in lymphocyte development as discussed here, which in turn have their

own targets, thus the network of genes that is potentially disrupted is likely to be extensive. With the emerging technology of ChIP-Seq these complex interactions may begin to be investigated and unravelled. In particular it would be interesting to study further the requirement of GR regulation of genes discussed here such as *Gata-3* and *Runx1*.

Conclusions

It is clear that there are many factors involved in the pathways of stress, T-cell development and GICD, and the timing and fine regulation of GR target genes in these processes is complex. This study has shed light on some of these mechanisms.

We have identified rapidly induced GR target genes in thymocytes by whole genome expression microarray analysis.

The transcriptional repressor, *Nfil3,* was characterized as direct GR target gene in normal mouse thymocytes. In Ctll2-T cells we have shown that *Nfil3* is necessary for GICD.

We have shown that the regulation of GR expression in the pituitary may occur via similar mechanism to thymocytes, via auto-upregulation of the GR1A promoter, an action which likely increases sensitivity to elevated GC levels and may account for the rapid return to a normal state following stress.

We have demonstrated T-cell development is subtly affected by loss of GR which has not been described in a reliable model as has been used here.

As previously mentioned, ChIP-Seq is likely to provide valuable insights into mechanisms of GR actions in T-cell development, GICD and stress responses in the future which will provide an important contribution to furthering knowledge into the complex transcriptional networks occurring in these systems. An improved understanding of these systems may translate to better management of a multitude of conditions and complications such as hematopoietic malignancies, autoimmune diseases and chronic stress.

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APPENDIX 1:

CONSTRUCTION OF GR EXON 1A-GFP REPORTER MICE

A1.1 Introduction

Analysis of the promoter region of the mouse Glucocorticoid Receptor gene has indicated that five promoters direct expression of the mouse GR (mGR) gene with each promoter differing in its tissue and cell specificity (Chen et al., 1999a; Strahle et al., 1992). The five promoters; 1A, 1B, 1C, 1D and 1E each drive expression of a full GR transcript containing one of the corresponding exon 1s, with the majority of basal GR expression occuring from promoters 1B and 1C. An inframe splice site within exon 2 just prior to the translation initiation site means that exons 1A through 1E are noncoding (Strahle et al., 1992; Chen et al., 1999a). It is believed the function of the various promoters is to provide increased levels of GR in response to the appropriate signal in a tissue specific manner adding a higher level of regulation to the ubiquitous GR. It is thought that the difference in tissue and cell specific patterns for each of the exon 1 transcript isoforms is due to the ability of unique combinations of transcription factors to interact with individual promoter regions, thus establishing these complex expression patterns, such as C-Myb in the case of the GR1A promoter (Chen et al., 1999b, Geng and Vedeckis, 2005). Exon 1A transcripts in particular show a highly tissue specific expression pattern and have previously been observed in T-lymphocytes, spleen and the brain of humans and mice, with this being narrowed down to the cortex in mice (Breslin et al., 2001; Strahle et al., 1992; Purton et al., 2004). Techniques to identify tissues or cell lines positive for GR1A transcripts so far have included RNAse protection assay and qRT-PCR. There are many limitations to this; analysis of an entire tissue does not give an indication of which cell types specifically are expressing the GR1A transcripts, and analysis of various immortalised cell lines has not been exhaustive. There is difficulty in further narrowing down the location of GR1A transcripts to more specific regions and cell types within the brain and also to T-cell subsets. Low transcript levels mean GR1A transcripts are difficult to detect by in situ hybridisation and co-immunohistochemistry with markers for T-cell subsets would most likely be confounding. In an effort to overcome some of

these issues it was decided to generate a transgenic mouse with the Enhanced Green Fluorescent Protein (EGFP) reporter gene under control of the GR1A promoter to allow definitive determination of the precise location of GR1A-promoter positive cells within the brain (See Figure A1.1). GFP transgenic reporter gene mice have been used successfully to monitor endogenous promoter activity since 1997 (Zhuo et al., 1997) where it was used to visualize astrocytes. They have since been used to monitor other neuronal specific promoters (Matsushita et al., 2002; Metzger et al., 2002; Soda et al., 2003), to selectively examine development in heart (Fleishmann et al., 1998) and kidney (Srinivas et al., 1999; Shao et al., 2002) in studies of the immune system (Manjunath et al., 1999; Buckland et al., 2000; Shimizu et al., 2001) and osteoblast differentiation (Dacic et al., 2001; Kalajzic et al., 2002) to name a few. The benefits of analysis of promoter activity via GFP reporter are numerous. Positive T-cells can be examined directly via FACS with co staining for various markers to allow identification of positive cells into subtypes, GR1A transcripts can localized in fresh frozen tissue sections from brain by simple fluorescence microscopy, and live animal imaging is also a possibility, especially in examining the changes in promoter activity in response to various stressors. A GFP transgenic mouse has benefits over a lacZ reporter in that it will fluoresce without the need for an exogenous substrate or cofactors. GFP transgenic mice are generated by microinjection of the appropriate construct into pronuclei of fertilized oocytes. The DNA integrates randomly into a chromosome and is expressed alongside the endogenous gene (Gama Sosa et al., 2010).

A1.2 Methods

A1.2.1 Animals and housing

Mice used in this study were bred and housed under standard conditions at the animal house and ARL of the Biochemistry Department, Monash University, and Mouseworks, Monash University. All animal experimentation was approved by the School of Biomedical Sciences Animal Ethics Committee, Monash Univer-

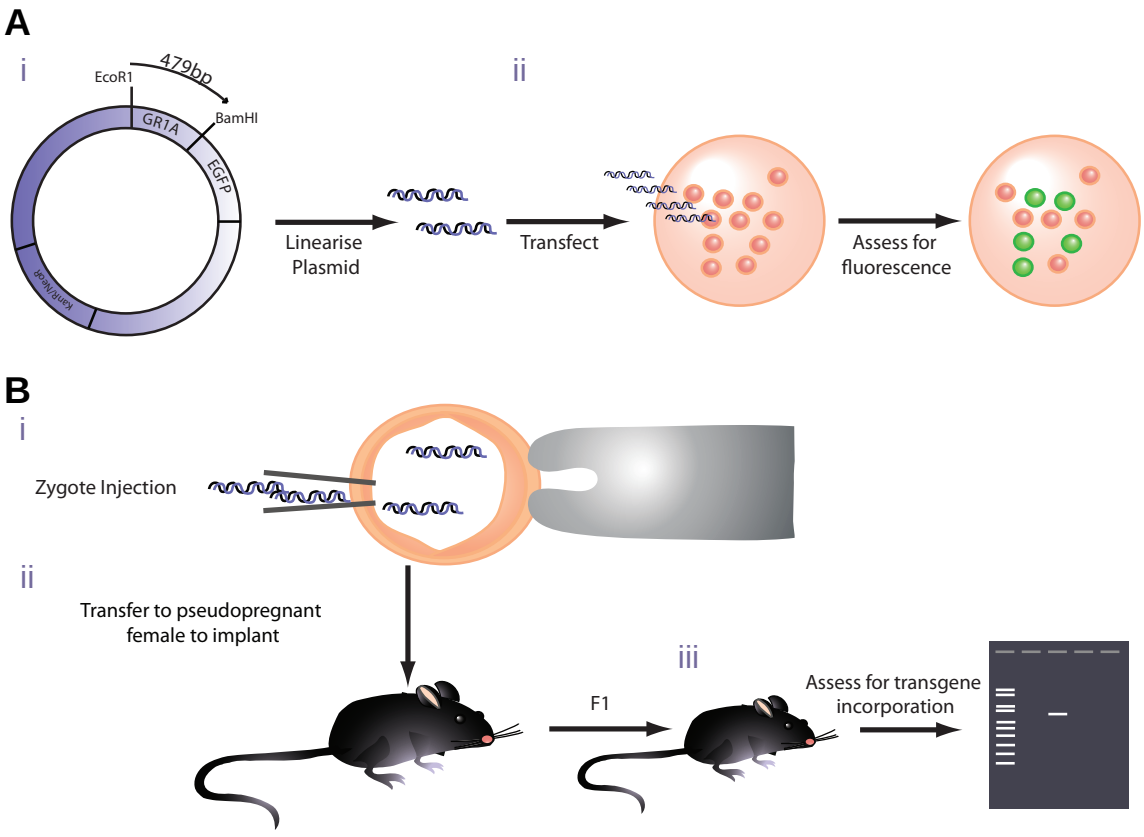


Figure A.1.1 Generation of GR1A/GFP Reporter gene transgenic mouse

Schematic diagram demonstrating the process undertaken to generate transgenic mice. **Ai)** Constructs were produced with the GFP gene under the control of the GR1A promoter. **Aii)** Reporter gene constructs were transfected into Jurkat T-cells to test the ability of each promoter fragment to induce fluorescence. **B)** Suitable contruct was chosen for injection. Construct was linearised and dissolved into microinjection buffer at a concentration of 2ng/ul **Bi)** DNA was microinjected into mouse blastocysts. **Bii)** Blastocysts were transferred into pseudopregnant female mice. **Biii)** F1 offspring were assesed for transgene incorporation by DNA PCR.

sity (Ethics no. 2009/63), and was carried out according to the National Health and Medical Research Council of Australia guidelines for the breeding, care and use of genetically modified and cloned animals for scientific purposes 2007. Transgenic and wildtype mice were sacrificed and thymi, spleens, livers, brains and kidneys removed for further analysis. Tissues were stored as appropriate for each application. For flow cytometry, tissues were placed immediately in FACS buffer and stored on ice until cell isolation was performed as described in A1.2.7. If RNA was to be extracted tissues were snap frozen in liquid nitrogen and stored at -80˚C or RNA was isolated immediately as described in A1.2.8. For Immunohistochemistry tissues were fixed by immersing in 4% PFA (in PBS) immediately after dissection for 2 h and gradually dehydrated as described in A1.2.11.

A1.2.2 Genotyping

To determine genotypes with respect to target genes in transgenic mice, genotyping PCRs were performed. Genomic DNA was prepared from tail or toe clips using REDExtract (Sigma-Aldrich, Castle Hill, NSW - according to manufacturers instructions) or Tail Digestion Buffer. Approximately 4mm of tail was digested with 0.5 mL Tail Digestion Buffer and 10 µL Proteinase K overnight at 55°C. Samples were centrifuged for 1 min at 15,000 rpm in an Eppendorf 5415R. Supernatant was transferred to fresh 1.7 mL tube eliminating as much fur as possible. DNA was precipitated with 1.0mL 95% ethanol containing 1/20th volume 3M NaOAc pH 7.0 at RT for 1.5 h. Sample was centrifuged 10 min at max speed, ethanol removed and pellet air dried and resuspended in sterile water. PCR was performed using primers listed in sections A1.2.13.

Genotyping of GFP transgenic mice was performed using 0.5 µg of tail DNA, 1X Taq Buffer, 200µM each dNTPs (NEB, Ipswich, MA), 200µM sense and antisense primers (Sigma-Aldrich, Castle Hill, NSW) (GFP primers in A1.2.13) 0.05 u/µl Taq DNA polymerase (NEB, Ipswich, MA) in a total volume of 20 µl with nucelase free water. 32 cycles of PCR were performed at the following temperatures:

Initial Denaturation: 1 minute at 95˚C

Denaturation: 1 minute at 95˚C

Annealing: 1 minute at 63˚C

Extension: 1 minute at 72˚C

Final extension: 5 minutes at 72˚C

Resulting amplified DNA was run on 1% Agarose gel in 0.5X TBE against a 1 Kb Plus DNA Ladder (Life Technologies, Mulgrave, Victoria) with Ethidium bromide or GelRed (Life Technologies) detection and visualised using the Gel Dock UV transilluminator.

A1.2.3 Construction of plasmids containing a mouse GR1A promoter fragment

In order to study the transcriptional activity from the GR1A promoter three GR1A-GFP plasmid constructs were generated for the purpose of microinjection into mouse oocytes to generate GR1A-GFP transgenic mice. Three cDNA fragments were generated by PCR of a BAC clone that contained the *Mus musculus* glucocorticoid receptor gene, promoter, exon1A and 5'UTR (Accession Number: AY429467). PCR was performed using primers specific for these regions of the mouse GR gene. Primer sequences were generated using Primer 3 software, (http://frodo.wi.mit.edu/primer3/) GC content was between 40 - 60%, with minimal secondary structure formation favoured. Primer sequences are given in section A1.2.13. Amplification of the appropriate region of the BAC clone was performed using 0.5 µg of DNA, 1X Thermo Pol Taq Buffer (NEB, Ipswich, MA), 200µM each dNTPs (NEB, Ipswich, MA), 200µM sense and antisense primers (Sigma-Aldrich, Castle Hill, NSW) (GR1A primers in 2.2.13) 0.05u /µl Taq DNA polymerase (NEB, Ipswich, MA) in a total volume of 50µl with nuclease free water. 30 cycles of PCR were performed at the following temperatures:

Initial Denaturation: 1 minute at 95˚C

Denaturation: 1 minute at 95˚C

Annealing: 1 minute at 62˚C

Extension: 1 minute at 72˚C

Final extension: 5 minutes at 72˚C.

The resulting three PCR fragments of each were separated by gel electrophoresis. Targeted fragments were 479bp, 2kb and 3kb in size. The band corresponding to the molecular weight of the specific amplicon, determined by comparison to 1 Kb Plus DNA Ladder (Life Technologies) was excised from the gel. DNA was purified from the gel using the Perfect Prep Gel Cleanup kit (Eppendorf) following the manufacturers specifications, with the DNA eluted in ddH_2O. Sanger sequencing of the DNA was performed at the Gandel Charitable trust sequencing facility (MIMR, Clayton) to confirm the band was in fact the specific DNA fragment required. The respective DNA fragments were digested with restriction enzymes and ligated into pEGFP-N1 (clontech) vector, which had the Cytomegalovirus (CMV) promoter of pEGFP-N1 (Clonetech, distributed by Scientifix, Clayton, Vic) removed, using a DNA ligation reaction kit (Promega, Alexandria, NSW). Detail on restiction enzymes used for subcloning is provided in Appendix 1, including plasmid maps. Each of the resulting plasmids was transformed into Escherichia coli JM 109 cells (Promega) which were then grown in Luria Broth (LB) medium for 2 h at 37˚C with shaking and grown on selective Kanamycin plates at 37˚C overnight. LB medium supplemented with 50µg/mL Kanamycin was innoculated with an individual colony picked from the selective plate and was grown shaking at 37˚C overnight. The plasmid DNA was isolated from E.Coli cells using the Gen Elute Plasmid Miniprep kit (Sigma-Aldrich). The concentration of plasmid DNA recovered was measured at OD260 using a Biophotometer (Eppendorf, North Ryde, NSW). For plasmids where ligation of the DNA fragment into the cloning site of the plasmid was not directed, the DNA was digested with restriction enzymes to check the insert was in fact there and also in the correct orientation and subsequently sequenced. The ability of each promoter fragments to drive GFP expression was tested in Jurkat T cells.

In preparation for injection into mouse oocytes, 10µg of pmGR1A-GFP2kbp plasmid DNA was digested with 8µl HindIII (NEB, distributed by Genesearch, Arundel,

QLD) in a 200µl for 1 h at 37˚C, after which time 8µl of SfiI (NEB) was added and the incubation temperature increased to 55˚C for a further hour. Digestion yielded the linearised promoter fragment and GFP gene. The digested DNA was separated by electrophoresis on a 0.9% agarose Gel at 100V for 1.5 h. The gel was stained with 0.5µg/mL ethidium bromide (Biorad, Gladesville, NSW) for 15 minutes. The DNA was excised from the gel using a scalpel blade and purified from the gel using the Perfect Prep Gel Cleanup kit (Eppendorf) following the manufacturers specifications, with the DNA eluted in sterile injection buffer. The concentration of DNA recovered was measured at OD260nm and the DNA diluted to 2ng/µL for microinjection.

A1.2.4 Cell lines and cell culture

Human T-ALL Jurkat cell line clone E1-6 (kind gift of Alfons Lawen, Monash University, Australia) were used to test the ability of the various GR1A promoter regions in the GR1A-GFP contrsucts to drive GFP expression. Jurkat cells were grown in RPMI media (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 1% penicillin and streptomycin (JRH biosciences, Australia) and 2mM L-Glutamine (Sigma-Aldrich) in a humidified 5% CO2 atmosphere at 37°C. Cells were split every second day at a 1:5 dilution.

A1.2.5 Plasmid transfection of the Jurkat T cell line

GR1A-GFP constructs were transfected into Jurkat cells to test their fluorescence. Viable Jurkat cells were transfected with the various GFP constructs with the aid of DMRIE-C transfection reagent (Life Technologies) according to the manufacturer's instructions, briefly: 6µL of transfection reagent was first complexed with 6µg of plasmid DNA in 1 mL of serum free OPTI-MEM media (Life Technologies) for 45 min in a 6 well plate. In the meantime a single cell suspension of Jurkat cells was prepared from stock cells growing in log phase, viable Jurkat cells were separated from non-viable cells by centrifugation with Ficoll-Paque

Plus (GE Healthcare, Rydalmere, NSW) according to the manufacturers instructions. Separated cells were pelleted and rinsed in serum free OPTI-MEM. Cells were again pelleted and resuspended in fresh OPTI-MEM media so that the final concentration of cells was 2.5 $X10^6$ /mL. 200µL of this cell suspension was added to each well so that the total number of cells transfected in each well was 5.0 $X10^6$. Cells were incubated in a 5% CO_2 atmosphere for 5 h at 37˚C. After 5 h 2mL of Opti-MEM media supplemented with 1µg/mL Phorbol 12-myristate 13-acetate (PMA) (Life Technologies) and 50ng/mL Lectin from Phaseolus vulgaris (PHA) (Life Technologies) was added to each well. Cells were tested for expression of the transgene 36 hours post-transfection.

A1.2.6 Fluorescence Microscopy

Jurkat T-cells transfected with EGFP plasmids were visualised using the Olympus IX71 microinjection/fluorescence microscope at the relevant wavelength and magnification. Images were captured using the camera attachment and Spot software and modified using image J software. Live cells were kept at 37˚C during microscopy.

A1.2.7 Cell preparation from organs

For flow cytometry experiments lymphocytes were isolated from thymus and spleen by first removing any residual fat or connective tissue using fine forceps. Organs were then scored over a petri dish containing FACS Buffer (see Appendix 2) using the frosted ends of 2 glass slides and then gently ground between rough surfaces till cells spilled into the FACS Buffer. Cells were resuspended and transfered to a tube through a 100 µm mesh.

A1.2.8 Isolation of total RNA

Total RNA was isolated from mouse tissues, primary cells or cell lines by homogenisation in TRIzol reagent (Life Technologies). After chloroform extrac-

tion, RNA was precipitated from the aqueous phase with isopropanol, washed in 70% ethanol and redissolved in nuclease-free sterile water. The concentration and purity of the isolated RNA were measured at OD260 using a spectrophotometer (Eppendorf). A260/280 ratios >1.6 were considered of satisfactory quality. The integrity of total RNA was evaluated by electrophoresis in a 1% agarose gel containing Morpholinopropanesulfonic acid (MOPS) and ethidium bromide. 1µg of RNA was added to: 2µL of 10X MOPS, 1.75µL of Formaldehyde, 5µL of Formamide, 1.25µL of ddH_20. These reagents were incubated at 55˚C for 5 min at which time 1µL of RNA loading dye was added and the total solution was loaded onto the gel. RNA was separated by electrophoresis at 70V for 1 h.

A1.2.9 Synthesis of cDNA

cDNA was generated using the random-primed Promega Two-Step Reverse Transcriptase Polymerase Chain reaction (RT-PCR) kit (Promega), 1µg of total RNA was used to synthesise cDNA according to the manufacturers instructions. cDNA yields were increased by changing the duration of the transcription step from 50 minutes to 90 minutes at 42˚C. The concentration of cDNA was measured at OD260 using a spectrophotometer (Eppendorf)

A1.2.10 Reverse transcriptase PCR

To detect GFP mRNA in potential GR1A-GFP transgenic mice, non-quantitative reverse transcriptase PCR was performed on cDNA prepared as above. PCR was performed using the same conditions described for genotyping GFP transgenic mice (section A1.2.2) with GFP primers (Section A1.2.13). Resulting amplified cDNA was run on 1% Agarose gel in 0.5X TBE with Ethidium bromide or GelRed (Life Technologies, Mulgrave, Victoria) detection and visualized using the Gel Dock UV transilluminator.

A1.2.11 Immunohistochemistry

Tissues were fixed by immersing in 4% PFA (in PBS) immediately after

dissection for 2 h. Tissue was gradually dehydrated into 70% ethanol and processed into paraffin. 5 µm sections were collected onto superfrost plus slides and incubated at 40°C overnight. Prior to experiment, slides were placed at 60°C for 30 min and then dewaxed through 3 changes of xylene, 5 min each. Slides were rehydrated by taking through decreasing ethanol concentrations, finally bringing them to water and placed in 1X PBS for 15 min. Antigen retrievel was performed by boiling slides in 0.01M Sodium Citrate for 20 min and allowed to cool to room temp. Sections were washed in Wash Buffer (see Appendix 2) 3 X 5 min. For Horseradish Peroxidase detection, endogenous peroxidases were blocked by incubating slides in 3% H_2O_2 (in water) for 5-10 min. Slides were washed 3 X 5 min in Wash Buffer before being placed in Block Buffer (see Appendix 2) for at least 30 min at room temperature (RT). Sections were incubated with primary GFP antibody (see table A1.2.14) diluted to 400 ng/mL in Block Buffer for 1 hr at RT or 4°C overnight. Primary antibody was washed off with Wash Buffer 3 X 5 min before the addition of the secondary antibody diluted 1:5000 in Block Buffer for 30 - 60 min. Secondary antibody was washed off with Wash Buffer 3 X 5 min, and if necessary a streptavidin conjugated tertiary antibody was applied, diluted in Block Buffer, for 30 – 60 min. Tertiary antibody was washed off with Wash Buffer 3 X 5 min, for HRP detection, DAB solution (1 drop DAB Chromogen /1mL DAB substrate Buffer) was applied to sections until brown staining appeared, after which time slides were washed 3 X 5 min in 1 X PBS. Slides were then counterstained with Hemotoxylin and mounted using DPX mounting medium.

A1.2.12 Flow Cytometry

Cells were isolated as described in section A1.2.7.Samples were analyzed using a 488-nm Argon laser on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey). Data analysis was performed using Cell Quest software.

A1.2.13 DNA Primer Sequences

Gene	Forward (5'-3')	Reverse (5'-3')
GR1A 3kb 5'UTR	GCGAATTCTTGACAG-GAGTGTGGGCATCC	GCGAATTCCCAGCTG-CAGAATGGGAATCA
GR1A 2kb 5'UTR	GCGAATTCCATGGAGTC-CGTTCTGCCTTC	GCGAATTC-GAGGGAAAGGGAGGAGA-
GR1A 471bp 5'UTR	GCGAATTCCATGGAGTC-CGTTCTGCCTTC	GCGAATTC-GAGGGAAAGGGAGGAGA-
GFP (for DNA and cDNA)	CAAGGGCGAGGAGCT-GTT	CTTGTACAGCTCGTCCATGC

A1.2.14 Antibody Information

Specificity	Conjugate	Company
Immunohistochemistry		
Primary		
GFP	N/A	Chemicon International
Seconday		
Chicken IgG	Biotin	Chemicon International
Tertiary		
Streptavidin	HRP	Invitrogen

A1.3 Results

A1.3 .1 Preparation of a GFP construct under the control of a GR1A promoter fragment

As an initial step to generate GR1A-GFP transgenic mice, to allow analysis of Glucocorticoid Receptor 1A (GR1A) promoter driven GFP gene expression, three plasmids with the EGFP reporter gene under the control of a GR1A promoter fragment were generated and the activity of each GR1A promoter EGFP constructs was tested after transfection into the Jurkat T cell line.

Three cDNA fragments were generated by PCR of a BAC clone that contained the genomic region of the GR gene 1A promoter. One of the fragments was 479bp in size and contained 352bp of mouse genomic sequence upstream of start of transcription and included 119 bp of GR exon 1A (Figure A1.3.1a). This fragment was digested with ApaI and BamHI and ligated into the corresponding sites in the MCS of pEGFP-N1. The second fragment was 2kb in size and consisted mostly of genomic sequence upstream of start site of transcription and included 124 bp of the GR exon 1A (Figure A1.3.1a). The third fragment was 3kb in size and contained 1919bp of mouse genomic sequence upstream of start of transcription and included the entire exon GR1A (1024bp) plus 32bp of downstream intron (Figure A1.3.1a). The 2- and 3kb fragments were both digested with EcoRI and ligated into the corresponding site within the MCS of pEGFP-N1. Following subcloning, purified plasmid miniprep DNA was digested with restriction enzymes to confirm the presence of the insert, the correct orientation, and the integrity of other cleavage sites essential for further use of the plasmid (Figure A1.3.1b). The plasmid pmGR1A-GFP 479bp was digested with ApaI and BamHI, (Figure A1.3.1b, Lane 10) to check for the correct insert. The low molecular weight band corresponds to the insert of approximately 480bp in size. The plasmid pmGR1A-GFP 2kb was digested with EcoRI (Figure A1.3.1b, Lane 15) to check for the correct insert. The low molecular weight band corresponded to an insert of 2kb in size. It was also digested with ApaI (Figure A1.3.1b, Lane 16) to check the insert is in the correct orientation. The bands produced correspond to DNA of molecular weight

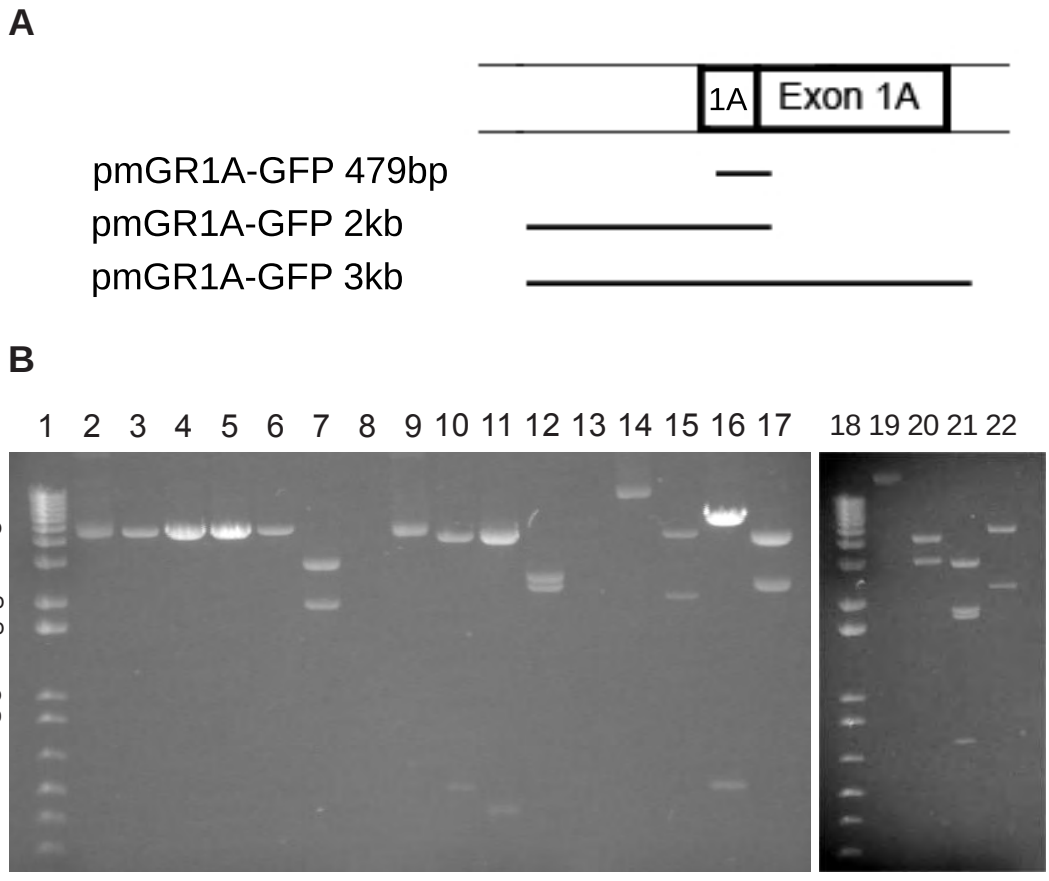


Figure A1.3.1 Assessment of GR1A/GFP constructs by restriction digest mapping

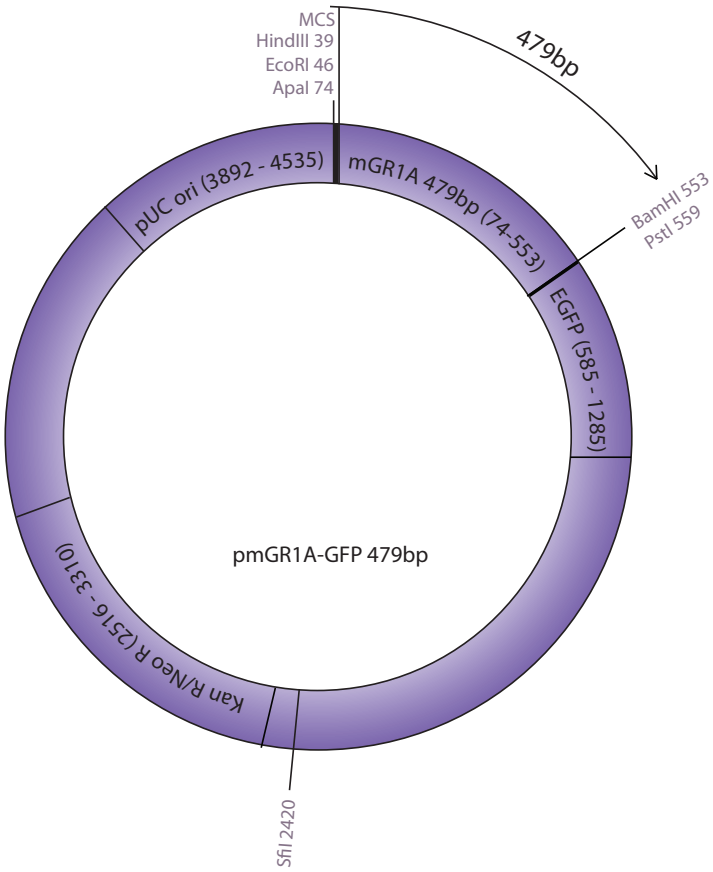
A: Schematic diagram showing the region that each promoter fragment spans relative to the gene. **B:** 0.9% Agarose gel electrophoresis of plasmid DNA digested with restriction enzymes. Lane 1: 1kb plus standard DNA ladder. Lane 2: pEGFP-N1. Lane 3: pEGFP-N1 digested with EcoRI. Lane 4: pEGFP-N1 ApaI. Lane 5: pEGFP-N1 PstI. Lane 6: pEGFP-N1 ApaI/BamHI. Lane 7: pEGFP-N1 HindIII/SfiI. Lane 8: Empty. Lane 9: pmGR1A-GFP 479bp. Lane 10: pmGR1A-GFP 479bp ApaI/BamHI. Lane 11: pmGR1A-GFP 479bp PstI. Lane 12: pmGR1A-GFP 479bp HindIII/SfiI. Lane 13: Empty. Lane 14: pmGR1A-GFP 2kbp. Lane 15: pmGR1A-GFP 2kbp EcoRI. Lane 16: pmGR1A-GFP 2kbp ApaI. Lane 17: pmGR1A-GFP 2kbp HindIII/SfiI. Lane 18: 1kb plus standard DNA ladder. Lane 19: pmGR1A-GFP 3kbp. Lane 20: pmGR1A-GFP 3kbp EcoRI. Lane 21: pmGR1A-GFP 3kbp NcoI. Lane 22: pmGR1A-GFP 3kbp SfiI/HindIII

505bp and 5651 bp, indicating the insert is in the correct orientation. The plasmid pmGR1A-GFP 3kb was digested with EcoRI to check for the presence of the correct insert (Figure A1.3.1b, Lane 20). The lower molecular weight band corresponds to the insert 3kb in size. pmGR1A-GFP 3kb was also digested with NcoI to check the insert was in the correct orientation. The bands corresponding to DNA 1916bp and 2379bp in molecular weight present in Lane 21 (Figure A1.3.1b) indicate that the 3kb fragment is in the correct orientation within the plasmid. All plasmids were digested with SfiI and HindIII, which is necessary to remove vector sequences and isolate the promoter fragment and GFP gene together in a linear form, to check the sites were in tact. The bands corresponding to DNA of molecular weight 2420bp, 3956bp, and 4924bp (Figure A.1.3.1b, Lanes 12, 17 and 22, respectively), the high molecular weight bands in each case, correspond to the linear portion of the of plasmid comprising the GR1A promoter fragment and the EGFP gene. Following sequencing and restriction mapping the constructs were ready for testing (See Figure A1.3.2 for plasmid maps).

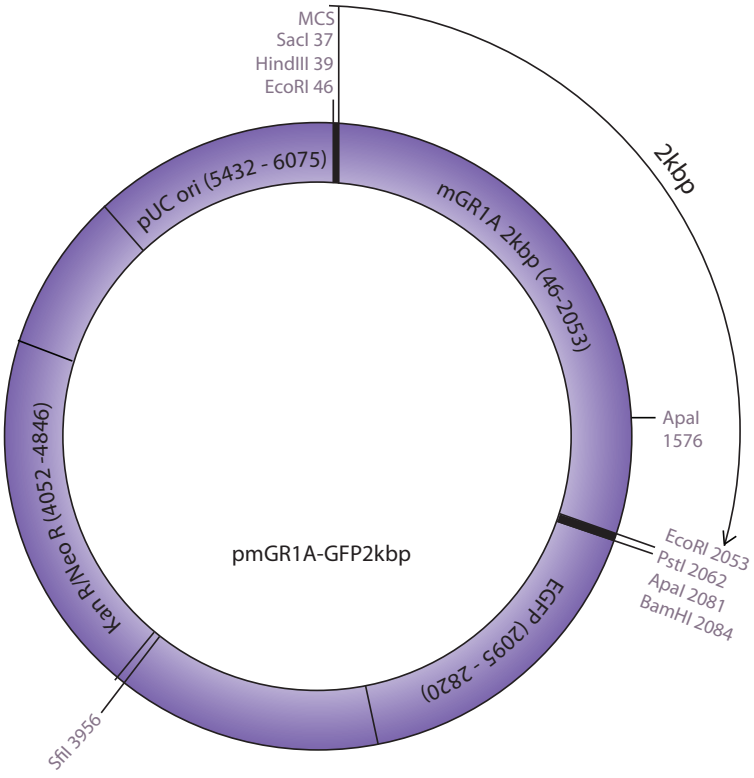
A1.3.2 Analysis of the GR1A promoter driven GFP expression in Jurkat T-cells

To test the ability of each of the promoter fragments to drive expression of the GFP gene, they were each transfected into Jurkat T-cells. Jurkat T-cells have previously been shown to have elevated 1A promoter activity and would therefore be expected to express transcription factors compatible with activation of the GR1A promoter. Jurkat T cells were successfully transfected with a GFP construct, and were able to express the green fluorescent protein as exemplified in cells transfected with pEGFP-N1, where fluorescence was observed, compared to those cells where DNA was not applied, which showed no fluorescence (Figures A1.3.3a and A1.3.3f respectively). A lower proportion of cells transfected with GFP constructs pmGR1A-GFP 479bp or pmGR1A-GFP 2kbp showed fluorescence (Figure A1.3.3b and A1.3.3c respectively), indicating that the transfection process may have been less efficient in comparison to cells transfected with

A


MCS
HindIII 39
EcoRI 46
ApaI 74
479bp
BamHI 553
PstI 559
mGR1A 479bp (74-553)
EGFP (585 - 1285)
pUC ori (3892 - 4535)
Kan R/Neo R (2516 - 3310)
SfiI 2420
pmGR1A-GFP 479bp

B


MCS
SacI 37
HindIII 39
EcoRI 46
2kbp
mGR1A 2kbp (46-2053)
ApaI
1576
EcoRI 2053
PstI 2062
ApaI 2081
BamHI 2084
EGFP (2095 - 2820)
Kan R/Neo R (4052 -4846)
SfiI 3956
pUC ori (5432 - 6075)
pmGR1A-GFP2kbp

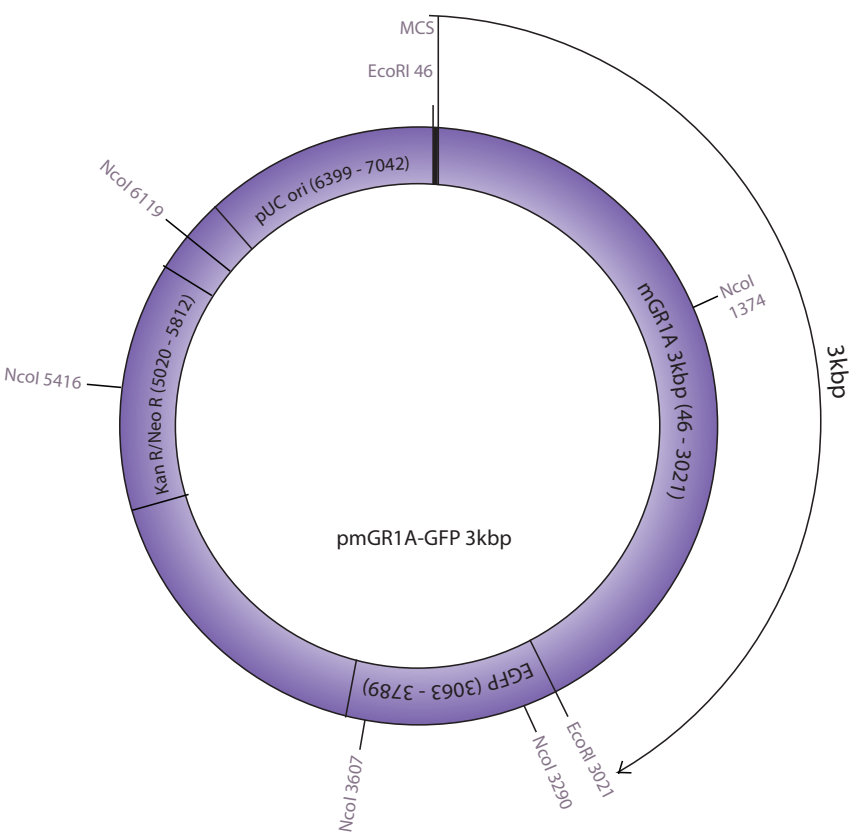


Figure A1.3.2 Plasmid Maps of pmGR1A-GFP contructs

A: Plasmid map of *pmGR1A-GFP 479bp*. The original CMV promoter (from 8-592) of the pEGFP-N1 plasmid was removed by digestion with restriction endonucleases AseI and NheI. The vector was cut with ApaI and BamHI, which lie within the Multi Cloning Site (MCS), and a 479bp fragment of the mGR1A promoter was ligated into this site. The fragment contains 352bp of mouse genomic sequence upstream of start of transcription and includes 119 bp of Glucocorticoid exon 1A (see Fig A1.3.1a) **B:** Plasmid map of *pmGR1A-GFP 2kbp*. A 2007bp fragment of the GR1A promoter was inserted into the EcoRI site within the MCS. The fragment contains approximately 2kb of mouse genomic sequence upstream of start of transcription and includes 124 bp of Glucocorticoid exon 1A (see Fig A1.3.1a). **C:** Plasmid map of *pmGR1A-GFP 3kbp.* A 2975 base pair fragment of the GR1A promoter was inserted into the EcoRI site within the MCS. The fragment contains 1919bp of mouse genomic sequence upstream of start of transcription and includes the whole exon GR1A (1024bp) plus 32bp of downstream intron.

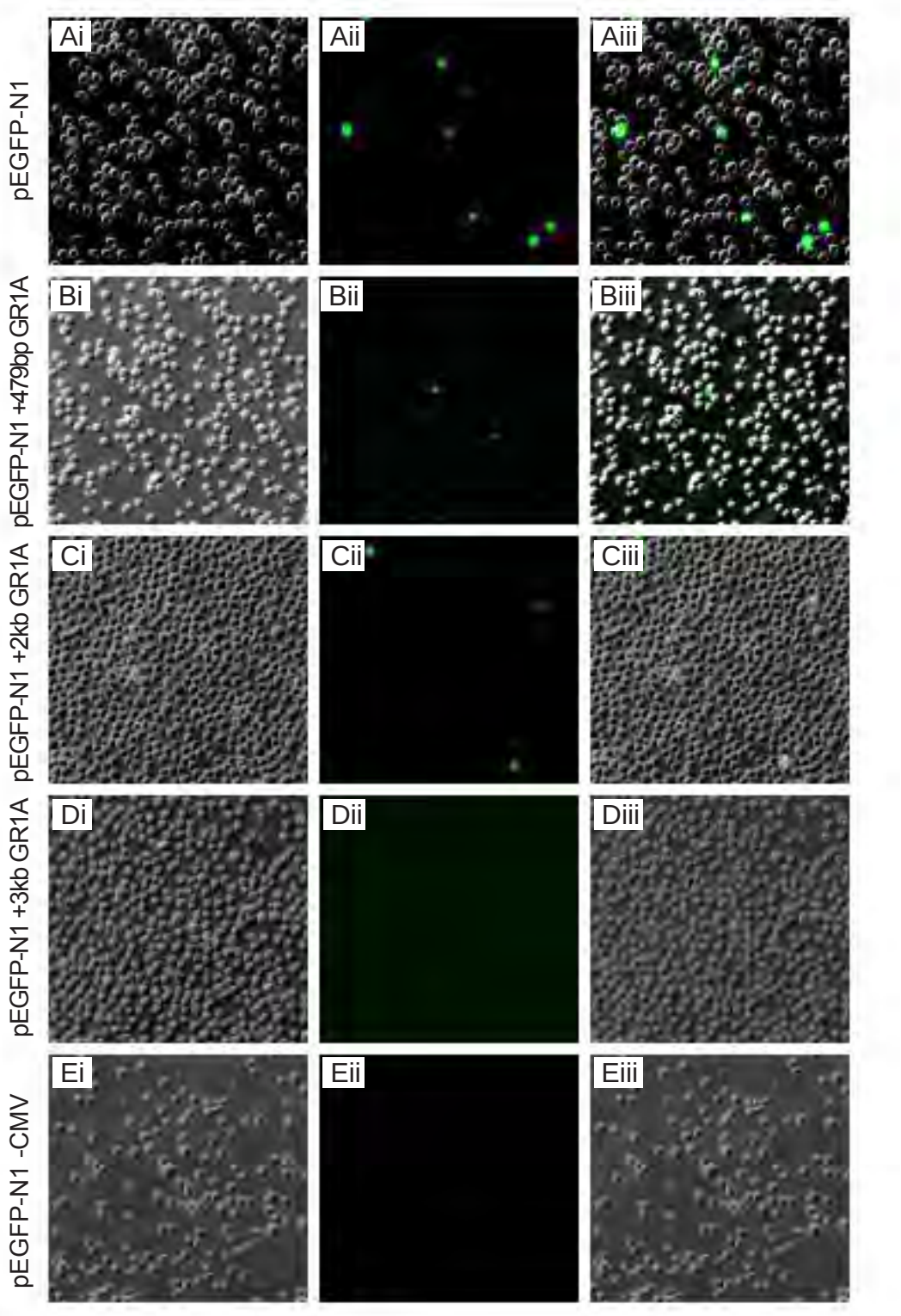


Figure A1.3.3 Jurkat T-cells transfected with GFP constructs.

A i-iii) Jurkat T cells transfected with pEGFP-N1, 20X magnification. **i)** Brightfield. **ii)** Fluorescent. **iii)** Overlay. **B i-iii)** pmGR1A-GFP 479bp. **C i-iii)** pmGR1A-GFP 2kbp. **D i-iii)** pmGR1A-GFP 3kbp. **E i-iii)** pEGFP-N1 –CMV promoter. **Fi-iii)** No DNA

pEGFP-N1, or may reflect a reduced strength of the GR1A promoter in these cells (Figure A1.3.3a). The transfection process appeared even less efficient when pmGR1A-GFP 3kbp was applied. Few fluorescent cells were observed, and many of the ones which did in fact show fluorescence were non-viable (Figure A1.3.3d). GFP expression was found to be entirely under the control of the GR1A promoter in cells transfected with pmGR1A-GFP 479bp, pmGR1A-GFP 2kbp or pmGR1A-GFP 3kb, by comparison to a negative control, where Jurkat T-cells were transfected with pEGFP-N1 in which the CMV promoter had been removed by digestion with restriction endonucleases AseI and NheI. In these cells, no fluorescence was observed (Figure A1.3.3d) suggesting that in cells transfected with GR1A promoter containing constructs which showed fluorescence, the GFP gene was not induced by other factors within the cell or the construct itself, but by the GR1A promoter. Interestingly, it appears that only a small portion of the GR1A promoter is required to induce gene expression as exemplified by the 479bp promoter fragment, which contains only a small portion of the promoter. It comes as no surprise that glucocorticoid response elements (GREs) were identified in the GR1A promoter region of the 2kb fragment. It would be interesting to observe the effects of GC regulation on these promoter fragments and subsequent effects on GFP expression.

A.1.3.3 Preparation of pmGR1A-GFP 2kbp for microinjection

The plasmids pmGR1A-GFP 479bp and pmGR1A-GFP 2kbp were chosen for further analysis in mice as they were best able to induce fluorescence in Jurkat T-cells. Plasmids were digested with SfiI and HindIII to isolate the GR1A promoter/GFP gene (Figure A1.3.4a). Digestion of pmGR1A-GFP 2kbp with SfiI/HindIII (represented in Figure A1.3.4a, Lane 3) yielded products of size 2239bp and 3917bp. The 3917bp fragment contained the GR1A promoter and EGFP gene. Digestion of pmGR1A-GFP 479bp with SfiI/HindIII (represented in Figure A1.3.4a, Lane 6) yielded products of size 2239bp and 2389bp. The 2389bp fragment con-

tained the GR1A promoter and EGFP gene. In each case, the higher molecular weight band containing the GR1A promoter and EGFP gene promoter was excised from the gel and purified using the eppendorf Perfect Prep Gel Cleanup kit. The resulting fragment was analysed by gel electrophoresis to check its size and purity (Figure A1.3.4a, Lane 4 (pmGR1A-GFP 2kbp) and Lane 7 (pmGR1A-GFP 479bp)). In both lanes, a single band is present, which indicates the product is pure. In addition the size of each of the bands corresponds to that of the desired fragment. These purification fragments were injected into mouse zygotes to generate GR1A/EGFP transgenic mice.

A.1.4 Identification of mice positive for the GR1A/GFP transgene

Genotyping was performed using primers specific for the GFP portion of the transgene. The first round of injections yielded no transgenic mice. A second round was performed yielding 4 mice which appeared positive for the transgene, (see Figure A1.3.4b) 1 female and 3 males. The 3 males were selected for further breeding. The F1 contained a number of mice postive for the transgene (Fig A1.3.4c). These mice were bred and checked for GFP expression.

A.1.5 Transgenic GR1A/GFP mice do not express GFP mRNA or produce GFP protein

Reverse transcriptase PCR was performed on cDNA synthesised from RNA isolated from thymuses of transgenic F1 mice to detect GFP transcripts. There did appear that there may be GFP expressed in the liver thymus and brain of mouse T446 A3 (Figure A1.3.5a) so this was further investigated by immunohostochemistal staining for GFP protein. GFP staining was performed on 8um frozen thymus sections. It appears that both mice were negative for GFP (Figure A1.3.5b). As a final check FACS analysis of purified thymocytes was performed to assess GFP content. Cells appeared negative for GFP (Figure A1.3.5c).

Offspring from microinjected mice do not express GFP protein, although they do contain the transgene. The transgene may have incorporated into a tran-

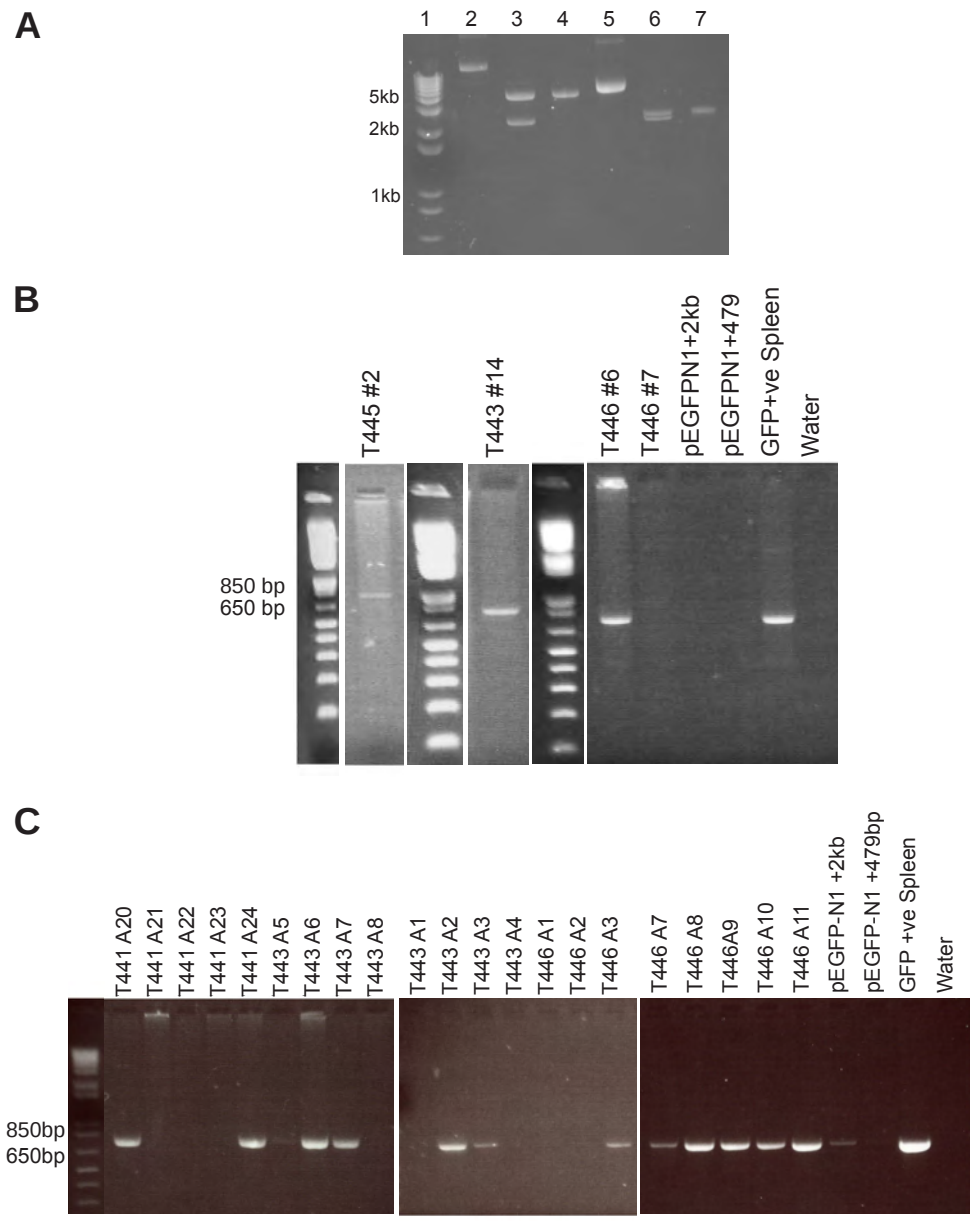


Figure A1.3.4 Transgene incorporation into the mouse genome

A: Plasmid DNA digested with restriction enzymes in preparation for microinjection into mouse zygotes. 2: pmGR1A-GFP 2kbp. 3: pmGR1A-GFP 2kbp SfiI/HindIII. 4: Gel purification product of 2389bp product generated by digestion with Sfi/HindIII containing 2kbpGR1A fragment and GFP gene. 5: pmGR1A-GFP 479bp. 6: pmGR1A-GFP 479bp SfiI/HindIII. 7: Gel purification product containing 479bpGR1A fragment and GFP gene. **B:** Four mice were postive for the transgene after the second round of microinjection as indicated by a 709bp GFP PCR product. **C:** Many F1 generation mice were also positive for the transgene.

scriptionally silent portion of the genome. It was decided this part of the project, to identify specific cell types within mouse tissues postive for GR1A transcripts, will be performed using in situ hybridisation and immunohistochemistry for cell specific markers.

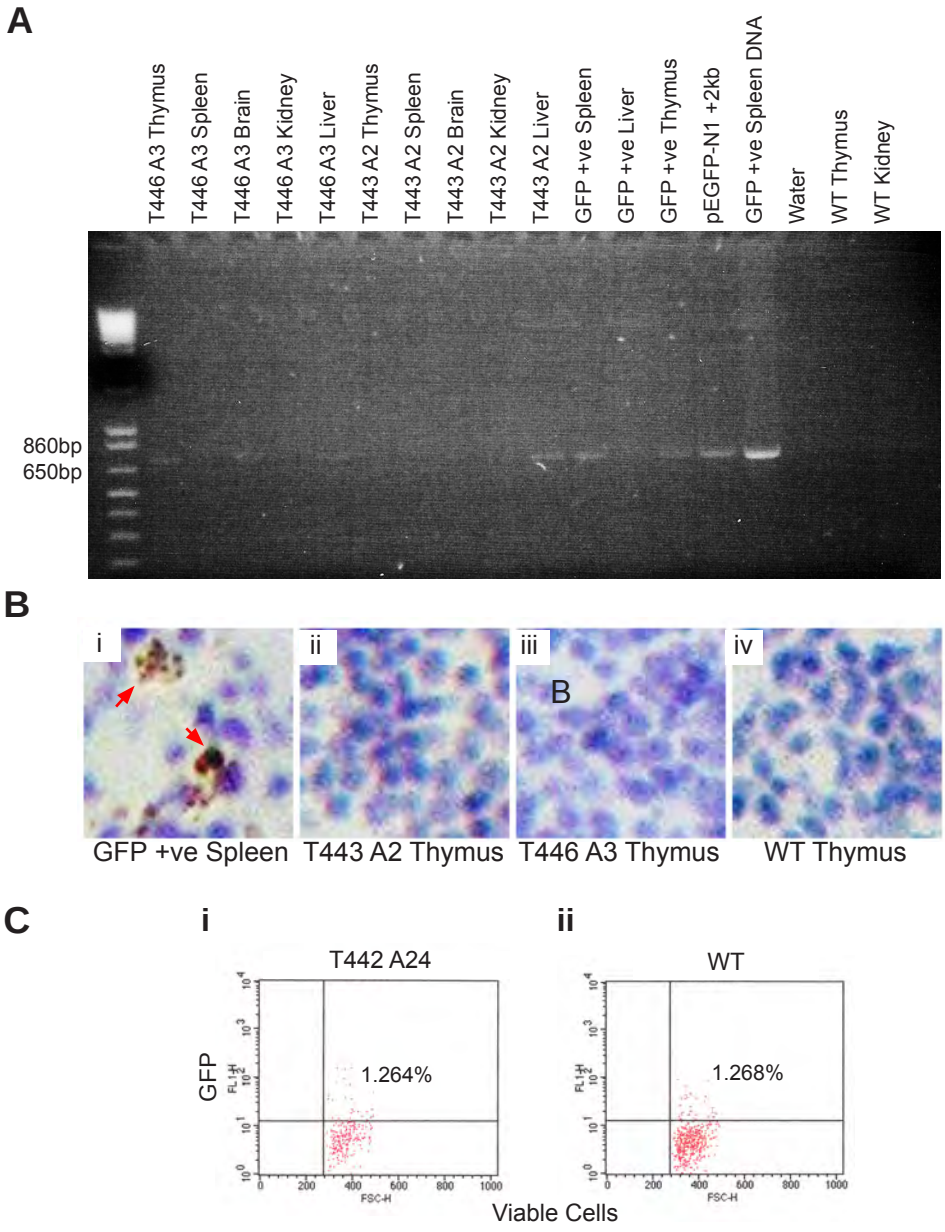


Figure A1.3.5 Transgenic mice do not express GFP mRNA or produce GFP protein

A: Representative RT-PCR performed on cDNA synthesised from tissues expected to express GR1A transcripts taken from transgenic mice T443 A2 and T446 A3 gave mostly negative results. **B:** Immunohistochemical staining for GFP protein in thymus sections from transgenic mice T443 A2 and T446 A3 (Bii and iii) was negative compared to a positive control (Bi, staining indicated by red arrows) **C:** Representative of dot plots for FACS analysis of GFP transgenic mouse T442 A24

APPENDIX 2:

SOLUTIONS

0.5M EDTA

EDTA	146.12 g

pH to 8.0, make up to 1 L with ddH_2O, autoclave

20% Paraformaldehyde (in PBS)

Paraformaldehyde	20 g

Add DEPC ddH_2O up to 80 mL, Heat to 65°C, Add 10M NaOH until PFA dissolves

10 X PBS	10 mL

pH to 7.6, Make up to 100 mL with DEPC dd H_2O, Filter sterilise

20X PBS

NaCl	160 g
KCl	4 g
Na_2HPO_4	28.8 g
KH_2PO_4	4.8 g

pH to 7.4, make up to 1 L with ddH_2O, add 1mL DEPC, autoclave after 30 mins

20X SSC

Sodium Chloride	175.3 g
Tri-Sodium Citrate	88.25 g

Make up to 1 L with ddH_2O, add 1 mL DEPC, autoclave after 30 mins

5M Sodium Chloride

NaCl	292.2 g

make up to 1 L with ddH_2O, autoclave

1M Sodium Citrate, DEPC

Trisodium Citrate dehydrate	23.52 g

add ddH_2O up to 800 mL, add 0.8 ml DEPC, autoclave after 30 mins

Tail Digestion Buffer

1M Tris-HCl pH 8.0	10 mL
0.5M EDTA pH 8.0	20 mL
5M NaCl	5 mL
20% SDS	25 mL

Make up to 1 L ddH_2O

10X Taq Buffer

IM Tris-HCl pH 8.8	1 mL
1M MgCl2	0.2 mL
IM KCl	5 mL
10% Trition X-100	1 mL

Make up to 1 mL ddH_2O, Filter sterilise

10X Tris-Borate EDTA (TBE)

Tris Base	106 g
Boric acid	55 g
0.5M EDTA	40 mL

Make up to 1 L with ddH_2O, autoclave

1M Tris HCl

Tris Base	121 g

Dissolve in 800 mL ddH_2O, Adjust pH with HCl, Make up to 1 L with ddH_2O, autoclave 20 minutes

Wash-buffer (Immunohistochemistry)

1M Tris pH 6.8	40 mL
5M NaCl	24 mL
IGEPAL/NP-40	4 mL

Make up to 800 mL with ddH_2O

Block buffer (immunohistochemistry)

Add 5% serum from host of secondary antibody to Wash Buffer

FACS buffer

Add 5% FCS to 1X PBS

APPENDIX III:

QRT-PCR PRIMER EFFICIENCY

qRT-PCR was used to quantify activity from the GR1A promoter with primers spanning the exon 1A and exon 2 boundaries to detect GR1A transcripts. To determine the level of GR1A promoter activity as a percentage of total GR transcript levels, the relative level of GR1A transcripts was divided by the relative level of total GR transcripts. Thus it was important that the two PCRs amplified at a similar efficiency. To determine the efficiency of cDNA amplification by the specific primer sets qRT-PCR was performed at a number of primer dilutions. The amplification plots are shown in Figure A 3.1 A and B. The Ct values were plotted against the log primer amounts to generate a linear regression graph. The slope of the graph was used to calculate the efficiency of the PCR using the following equation.

E = (10^(-1/slope)-1)*100

Thus for primers targeted towards exon 1A the slope was -3.18 (see Figure A 3.2 A)

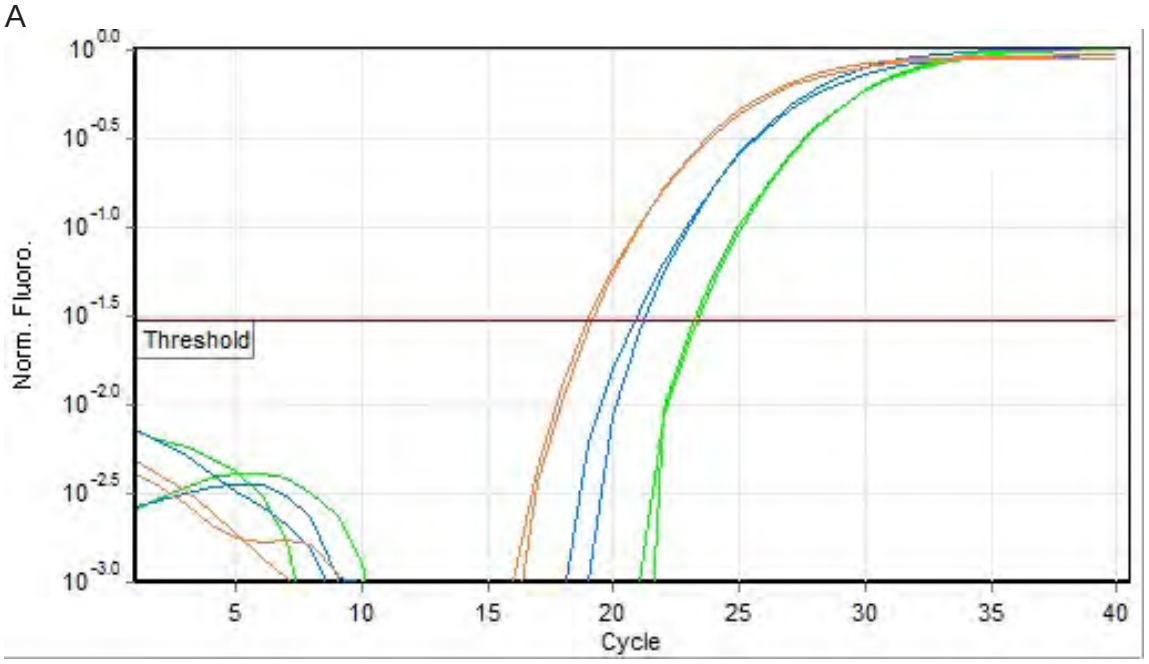
E = (10^(-1/-3.18)-1)*100
= 106%

For primers targeted towards exon 2/3 the slope was -3.15 (see Figure A3.2B)

E = (10^(-1/-3.15)-1)*100
= 107.6%

As the PCRs are of similar efficiency the activity of the GR1A promoter can be calculated as a percentage of total GR.

A



B

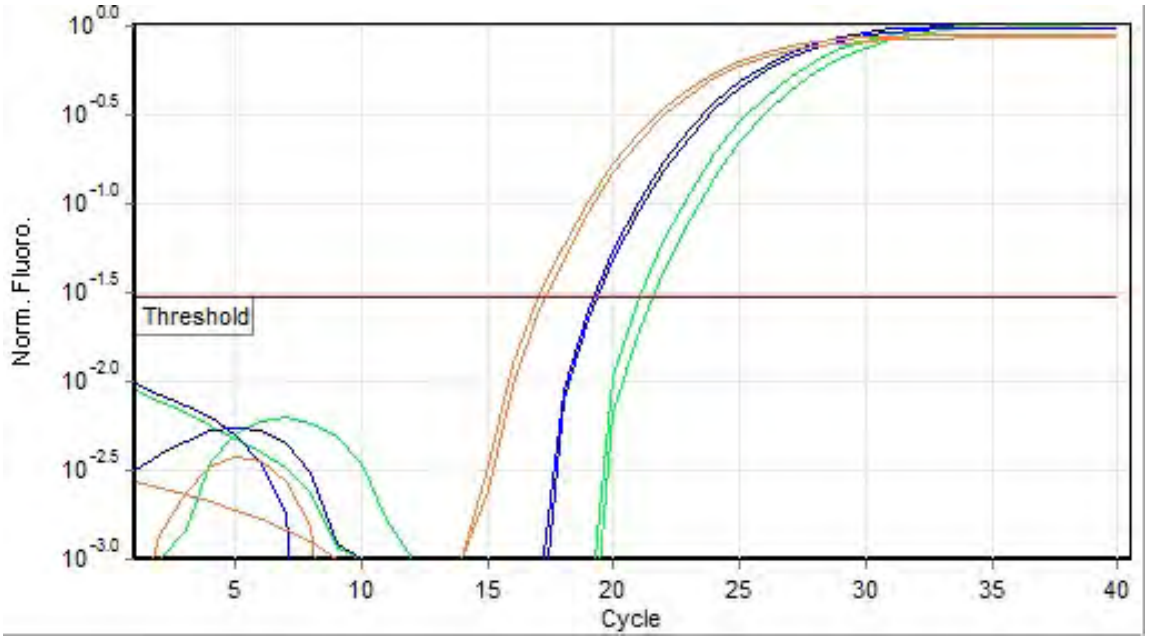


Figure A 3.1: Amplification plots for qRT-PCR

qRT-PCR was performed on cDNA synthesised from thymic mRNA. cDNA was used at a 1/25 dilution. Primer dilutions of 1000ng/µl (indicated by orange curves), 200ng/µl (blue) and 50ng/µl (green) were used. Amplification plots for GR1A (A) PCR and GR2/3 (B) PCRs.

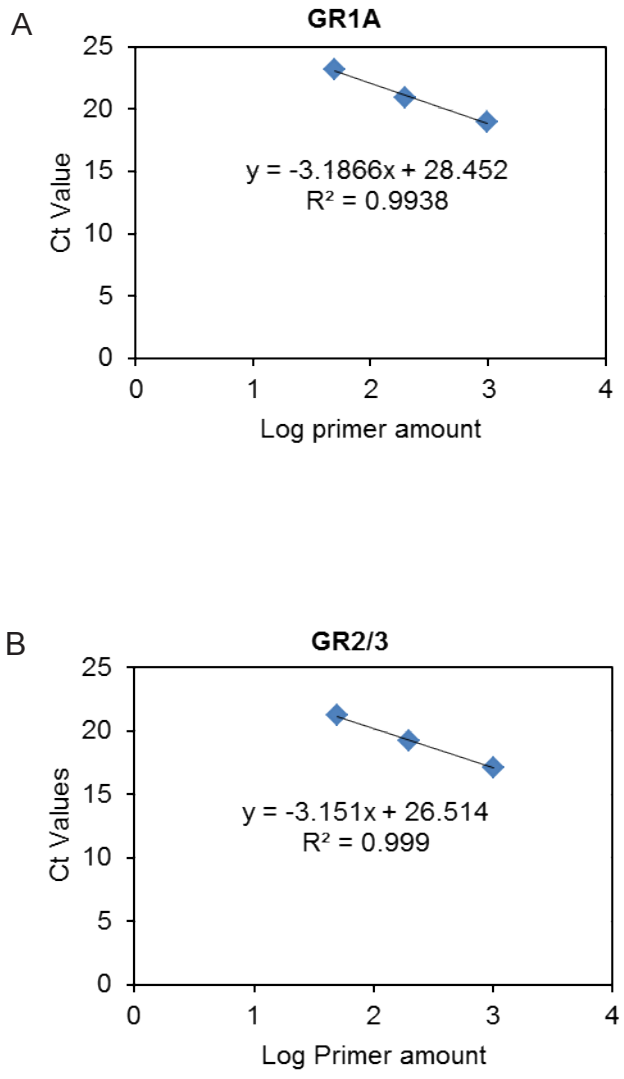


Figure A 3.2: Linear Regression plots for qRT-PCR primer efficiency determination

Ct values obtained following qRT-PCR of thymic cDNA were plotted against the Log values of the primer amounts in nanograms. The equation of the line was obtained and the slope value used to calculate the PCR efficiency for each primer pair. Linear regression plots for GR1A (A) PCR and GR2/3 (B) PCRs.