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The Role of TCR and Cytokine Signals in Naïve T cell Homeostasis

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Statement of Declaration

I, **Manoj Saini** confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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It is to them I dedicate this thesis...

Abstract

The peripheral T cell compartment is maintained at a constant size, resulting from a balance of cell development, survival, proliferation and death. Transmission of signals through the TCR and IL-7R on the T cell surface is involved in regulating all these processes, however the precise manner in which these signals together maintain T cell homeostasis is unclear. To investigate the contribution of TCR and IL-7 signals to naive T cell homeostatic responses we established two model systems. To specifically address the role of homeostatic TCR signalling in the development and maintenance of the T cell compartment, we generated transgenic mice that conditionally express the Syk family tyrosine kinase Zap70. The transduction of TCR signals by Zap70 is essential for thymic development and T cell activation. Given the importance of Zap70 expression in T cell antigen receptor signalling, we investigated whether Zap70 was also essential for the transmission of TCR signals, required for the steady state survival of the peripheral naïve T cell compartments. Zap70 deficient mice exhibit a complete block in thymopoiesis at the DP stage in the thymus and as a consequence lack mature peripheral T cells. For this reason we generated mice that express Zap70 in a conditional manner, using the tetracycline responsive gene regulatory system. Thymic selection proceeded normally in these mice, however ablation of Zap70 expression resulted in the disappearance in the peripheral naïve $CD4^+$ and $CD8^+$ T cells, with the naïve $CD8^+$ T cell compartment appearing most affected by the loss of Zap70 expression. This data suggests an important role for Zap70 signalling in the

transmission of homeostatic TCR survival signals. Unexpectedly we also found that TCR signals transmitted by Zap70 had the capacity to influence IL-7R expression and importantly revealed a novel role for positive selection signals in the regulation of peripheral IL-7R expression and therefore the competitive fitness of peripheral T cell clones.

The second model system examined the homeostatic responses of class I MHC restricted $F5^{+/+}$ TCR transgenic *Rag1*$^{-/-}$ $CD8^{+}$ T cells following transfer into MHC class I or IL-7 deficient hosts, to specifically quantify the contribution of TCR and IL-7 signals in naïve T cell homeostasis. Our data reveals that IL-7 signals are more essential than homeostatic TCR signals for the survival of the naïve T cell compartment in conditions of lymphopenia. Interestingly we also demonstrate that IL-7 may exert part of its homeostatic effects by modulating TCR engagement with MHC ligands by regulating T cell – APC interactions *in vivo*.

In conclusion the data presented in this thesis confirms that TCR and IL-7 signals are essential for naïve T cell homeostasis, but also reveals extensive cross-talk between homeostatic TCR and IL7 signals in the control of peripheral T cell homeostasis.

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Abbreviations

+/-	Heterzygous gene knockout
-/-	Homozygous gene knockout
µl	Microlitre
°C	Degrees Celsius
%	Percentage
min	Minute
h	Hour
ADAP	Adhesion and Degranulation-Promoting Adaptor Protein
Ag	Antigen
AIF	Apoptosis Inducing Factor
AIDS	Acquired Immuno Deficiency Syndrome
AKT/PKB	Protein Kinase B
Apaf-1	Apoptosis Protease Activating Factor – 1
APC antibody	Allophycocyanin
APC(s)	Antigen Presenting Cell(s)
Bad	Bcl-2 Antagonist of Cell Death
Bax	Bcl-2-Associated X Protein
Bak	Bcl-2 Antagonist/Killer
B cell	B Lymphocyte
Bcl-2	B Cell Lymphoma-2
BCR	B Lymphocyte Receptor

BH domain	Bcl-2 Homology Domains
Bid	BH3 Interacting Domain Death Agonist
Biotin	Biotinylated
BLAST	Basic Local Alignment Search Tool
BM	Bone Marrow
BMDC	Bone Marrow Derived Dendritic Cell
BrdU	5-bromo-2-deoxyuridine
C	Constant Segment
Ca^{2+}	Calcium
Caspase	Cysteine-Dependent Aspartate-Directed Proteases
CD	Clusters of Differentiation
Cdk	Cyclin-Dependent Kinase
CFSE	Carboxy-Fluorescein Diacetate Succinimidyl Ester
CKI	Cyclin-Dependent Kinase Inhibitors
CLIP	Class II Associated Invariant Chain Peptide
CLP	Common Lymphoid Progenitor
CMV	Cytomegalovirus
CO_2	Carbon Dioxide
cSMAC	Central Supramolecular Activation Clusters
Csk	Carboxy-Terminal Src Kinase
CTL	Cytotoxic T Lymphocyte
D	Diversity Segment

DAG	Diacylglycerol
DC	Dendritic Cell
DD	Death Domain
DETC	Dendritic Epidermal T Cells
DISC	Death Inducing Signalling Complex
DOX	Doxycycline
DN	Double Negative
DNA	Deoxyribonucleic Acid
DP	Double Positive
EF	Expansion Factor
EGFP	Enhanced Green Fluorescent Protein
ER	Endoplasmic Reticulum
ERK	Extra-Cellular Signal Related Kinase
FACS	Fluorescent Activated Cell Sorting
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
Flu	Influenza
Flk-2L	Fetal Liver Kinase 2 Ligand
Foxp3	Forkhead Box p3
FTOC	Fetal Thymic Organ Culture
F5 T cells	$F5^{+/+}$ $CD8^{+}$ TCR transgenic T cells
γ_c	Common Cytokine receptor γ chain

Gata3	Gata-binding protein 3
Gads	Grb2-Related Adaptor Downstream of Shc
GDP	Guanosine Disphosphate
GEF	Guanine Nucleotide Exchange Factor
GEM	Glycolipid Enriched Microdomains
GLUT1	Glucose Transporter Molecule-1
Grb2	Growth Factor Receptor-Bound Protein 2
GTP	Guanosine Trisphosphate
HA	Haemagglutination
Het	Heterozygous
HEK rtTA	Human Embryonic Kidney Cell Line stably expressing the Reverse Tetracyline Transactivator (HEK 293 Tet-On™)
HI	Heat Inactivated
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
Hom	Homozygous
HP	Homeostatic Proliferation
HPK1	Haematopoietic Progenitor Kinase 1
Huβg	Human Beta-globin intron
H5N1	Highly Virulent Influenza A
IDP	Immature Double Positive
IFN	Interferon

Ig	Immunoglobulin
Ii	Invariant Chain
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IP_3	Inositol-1,4,5-triphosphate
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-Linked
IRES	Internal Ribosomal Entry Site
IRF	Interferon-Regulatory Factor
IS	Immunological Synapse
ITAM	Immunoregulatory Tyrosine Based Activation Motif
ITK	Interleukin-2 Inducible T Cell Kinase
i.v.	Intravenous
J	Joining Segment
JAK	Janus Kinase
JNK	c-Jun Amino-Terminal Kinase
kB	Kilobases
kDa	Kilo-Daltons
LAT	Linker for Activated T Cells
Lck	Lymphocyte Specific Kinase
Lck^{ind}	Lck Inducible

LCMV	Lymphocytic Choriomeningitis Virus
LCR	Locus Control Region
LEF-1	Lymphoid Enhancer-Binding Factor 1
LFA	Lymphocyte Function Associated–1
LIP	Lymphopenia Induced Proliferation
LN	Lymph Node
LPS	Lipopolysaccharide
M	Molar
MAPK	Mitogen Activated Kinase
MCL-1	Myeloid Cell Leukemia Sequence 1
MCS	Multicloning Site
MEK	Mitogen-Activated Protein Kinase Kinase
MHC	Major Histocompatibility Complex
MFI	Mean Fluorescence Intensity
MLN	Mesenteric Lymph Nodes
mM	Milli Molar
MOMP	Mitochondrial Outer Membrane Permeablisation
μM	Micro Molar
mRNA	Messenger Ribonucleic Acid
MTOC	Microtubule Organising Centre
Nck	Non-Catalytic Region of Tyrosine Kinase
NCBI	National Center for Biotechnology Information

ND	Not Detectable
NFAT	Nuclear Factor of Activated T Cells
NFκB	Nuclear Factor kappa B
NK	Natural Killer
NKT	Natural Killer T
NP68	Nuclear Protein 68
ORF	Open Reading Frame
PAK1	p21-Activated Kinase
PAMPS	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PBL	Peripheral Blood Lymphocytes
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PIP_2	Phosphatidyl Inositol-4,5 Bisphosphate
PI3K	Phosphatidylinositol 3-Kinase
PKC	Protein Kinase C
PLCγ	Phospholipase C Gamma
pMHC	Peptide Loaded Major Histocompatibility Complex
PRR	Pattern Recognition Receptor
pSMAC	Peripheral Supramolecular Activation Clusters

PTEN	Phosphatase and Tensin Homologue Deleted on Chromosome 10
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
Puma	p53-Upregulated Modulator of Apoptosis
Rag	Recombination-Activating Genes
RasGRP	Ras Guanyl-Releasing Protein
RTE	Recent Thymic Emigrants
RORγ	Retinoid-Related Orphan Nuclear Receptor Gamma
rtTA	Reverse Tetracycline Transactivator
RT	Room Temperature
Runx3	Runt-Related Transcription Factor 3
SCID	Severe Combined Immunodeficiency Syndrome
SCF	Stem Cell Factor
SH2	Src Homology Domain 2
SLP-76	SH-2 Domain Containing Leukocyte Protein of 76kDa
SMAC	Supramolecular Activation Cluster
SOCS-1	Suppressor of Cytokine Signalling
SOS	Son of Sevenless
SPF	Specific Pathogen Free
SP	Single Positive
sp	Self Peptide

spMHC	Self Peptide MHC complex
Src	SH2 region Containing Kinase
STAT	Signal Transducer and Activator of Transcription
Syk	Spleen Tyrosine Kinase
T-ALL	T Cell Acute Lymphoblastic Leukemic
TAP	Transporters Associated with Antigen Processing
T cell	T Lymphocyte
TCF-1	Transcription Factor 1
TCR	T Lymphocyte Receptor
Tdt	Terminal Deoxynucleotide Transferase
Tet-Inducible System	Tetracycline Inducible Gene Expression System
Tre-Zap70 Construct 1	Tet Inducible Construct Lacking Intronic Sequence
Tre-huβg-Zap70 Construct 2	Tet Inducible Construct Containing Intronic Sequence
Tre-Zap70	Zap70 Transgene
Tet-Zap70	Mice generated from Tre-Zap70 Construct
Tet-huβg-Zap70	Mice generated from Tre- huβg-Zap70 Construct
T_{CM}	Central Memory T Cell
T_{EM}	Effector Memory T Cell
T_H	T Helper Cell
Th-POK	T helper-inducing POZ/Krüppel factor
TGF-β	Transforming Growth Factor Beta
TLR	Toll Like Receptor

TNFα	Tumour Necrosis Factor alpha
TRADD	TNF-Receptor Associated Death Domain
TRAF	TNF-Receptor Associated Factors
Tre	Tetracyline Responsive Element
T_{Reg}	Regulatory T Lymphocyte
TSLP	Thymic Stromal Lymphopoietin
V	Variable Segment
VSV	Vesicular Stomatitis Virus
WASP	Wiskott-Aldrich Syndrome Protein
WT	Wild Type
Zap70	Zeta Chain Associated Protein Kinase of 70 kDa

Chapter 1

Introduction

1.1 Introduction to The Immune System

The immune system is a collection of overlapping cell-mediated and humoral responses, which protect a host organism from infection by pathogens or host cells, which may have developed into tumors. Given the diverse array of potential pathogens existing in our environment, the ability to resist and eliminate infections is essential for the survival of the host organism. The jawed vertebrate immune system has evolved to provide distinct forms of protection from bacterial, viral and parasitic infection, and is divided into two overlapping branches: the innate immune system (in non-vertebrates and vertebrates) and the adaptive immune system (specific to vertebrates).

The innate immune system is considered the first line of defense involved in the most immediate host response to common bacterial and parasitic infections. It includes the use of mechanical barriers such as external (skin) and internal epithelia (mucosal surfaces), and humoral mechanisms such as the secretion of antimicrobial peptides known as Definsins and Cathelicidins by neutrophils and epithelial cells (Gallo et al., 2002), in addition to cell mediated responses. The cells involved in innate immunity are typically derived from the myeloid lineage and include monocytes, macrophages, dendritic cells (DCs), mast cells, granulocytes (polymorphonuclear leukocytes), neutrophils, basophils and

eosinophils but also include natural killer, (NK) and natural killer T cells (NKT) and γδ T cells of the lymphoid lineage. These cells utilize a range of invariant receptors to maintain the hosts defences against pathogens and infectious agents. These receptors include the complement receptors expressed by phagocytes, which recognise and uptake pathogens opsinised by complement proteins (reviewed in (Frank and Fries, 1991)). Alternatively macrophages and DCs also express germ-line encoded invariant receptors known as pattern-recognition receptors (PRRs). These recognise conserved structures common to microbial pathogens known as pathogen-associated molecular patterns (PAMPs) that are not produced by host cells, allowing the rapid clearance of large groups of microorganisms, which share this common motif. These include receptors, which can directly stimulate phagocytosis of the pathogen such as the macrophage mannose receptor (reviewed in (Apostolopoulos and McKenzie, 2001; Medzhitov and Janeway, 2000)) or receptors that stimulate innate and adaptive immune responses. The best known example of this, are the PRRs known as Toll-like receptors (TLRs). Many PAMPs have been studied and their respective TLRs identified; these include peptidoglycan, which is a TLR2 ligand, lipopolysaccharide (LPS), which activates TLR4, and bacterial unmethylated CpG dinucleotides, which are recognised by TLR9 (reviewed in (Takeda et al., 2003)). TLR signaling plays an important role in innate immunity by activating the nuclear factor-kappa B (NFκB) and interferon-regulatory factor (IRF) signalling pathways leading to the transcription of pro-inflammatory cytokine genes such as tumour necrosis factor α (TNFα), interleukin 1β (IL-1β), interleukin 6 (IL-6) and interleukin 12 p40 subunit (IL-12p40), interferon genes

(IFNs) and costimulatory molecules which induce the inflammatory responses that assist in the clearance of the microbial pathogens (reviewed in (Kopp and Medzhitov, 2003)(Moynagh, 2005)(Kawai and Akira, 2007)). Importantly the PRRs are endowed with a broad specificity, which allows the innate immune system to detect and eliminate broad groups of pathogens. However, if the innate immune system is unable to contain the infection, the adaptive or acquired immune system is engaged to target the specific invading pathogen with a set of more specialized antigen-specific cell-mediated and humoral immune responses.

The cells of the adaptive immune system are of lymphoid origin. B and T lymphocytes both originate from lymphoid progenitors in the bone marrow (BM), however T cells mature in the thymus whereas B cells mature in the BM. The T cells are further subdivided into lineages based on the expression of the cell surface glycoproteins CD4 ($CD4^+$ helper T cells) or CD8 ($CD8^+$ T cells). $CD8^+$ T cells directly kill infected target cells whereas $CD4^+$ T cells commonly referred to as "helper" T cells typically combat infection by activating other immune cells and are further subdivided into many functional classes discussed later. The specificity of the receptors expressed on the surface of the B and T lymphocytes, define the nature of adaptive immune response. Unlike the receptors of the innate immune system, T cell antigen receptors (TCR) and immunoglobulin B cell receptors (Ig) (BCR) are not germ-line encoded invariant receptors but are generated through a series of random TCR and BCR gene rearrangements, producing a diverse array of receptors, which recognise

specific antigens. Following T or B cell activation, the cell undergoes clonal expansion to generate a population of daughter effector cells, which all express the same antigen receptor, and thus can direct an effective immune response against the specific antigen. B cells and T cells both play essential roles in this adaptive immune response, but do so in a variety of different ways. Unlike T cells, B cells are predominantly involved in the humoral responses of the adaptive immune system. Following activation by antigen, naïve B cells differentiate into antibody secreting plasma cells. The antibodies in turn neutralize free pathogens thus preventing the adherence of the pathogen to host cells, or they can opsonise pathogens and enhance the clearance of the infectious agent either through phagocytosis by macrophages or through the activation of complement (reviewed in (Ollila and Vihinen, 2005; Viau and Zouali, 2005)). In contrast, T cells function primarily in cell-mediated adaptive immune responses. Following activation by cognate antigen in peripheral lymphoid organs $CD8^+$ T lymphocytes differentiate into armed effector T cells also known as cytotoxic T lymphocytes (CTL), which directly kill infected host cells. $CD4^+$ T cells differentiate into a number of different types of effector cells, the most common of which are the T helper type 1 (T_H1) $CD4^+$ T cells that secrete IFNγ and lymphotoxin which increases the phagocytic activity of macrophages at the site of infection (Stout and Bottomly, 1989), as well as T helper type 2 (T_H2) $CD4^+$ T cell activity that is essential for B cell differentiation and proliferation (Parker, 1993). A consequence of clonal expansion and survival of effector cells is the generation of memory B and T cells which elicit stronger and faster responses against re-infection by the same pathogen.

Importantly, in order for the immune system to function efficiently the innate and adaptive branches must converge to successfully contain and resist infection. This occurs at many stages in the immune response to pathogens. Innate mechanisms of immunity have been shown to be important in priming adaptive immune responses. It has been shown that TLR signaling enhances the activation of DCs which upregulate co-stimulatory proteins involved in $CD4^+$ T cell activation (Medzhitov et al., 1997; Schnare et al., 2001). Other examples include the important role DCs play in the degradation of soluble antigens into peptides that must be presented to T cells for their activation and the generation of effector functions. Other examples of overlapping adaptive and innate immune responses include the manner in which B cells and DCs also cooperate in order to phagocytose antibody opsinised pathogens. Similarly IL-12 secreted by macrophages and DCs of innate immune system, influence adaptive immune responses by polarizing $CD4^+$ T cells to adopt a T_H1 phenotype (Heufler et al., 1996; Macatonia et al., 1995) releasing cytokines which in turn assist the activation of macrophages.

Clearly there is much cross-talk between the innate and adaptive immune systems at the level of humoral and cellular mechanisms of immunity. Given the complexity of the immune responses to different pathogens, and the number of distinct cell types and receptors, which orchestrate innate and adaptive immune responses, immune homeostasis is essential to maintain the most efficient balance of the different cellular constituents. The maintenance of homeostasis is particularly important within the T cell mediated adaptive

immune responses, given that the various T cell subsets have a critical role in the activation of humoral and cell-mediated immunity, and have the capacity if dysregulated to recognize and destroy self, resulting in autoimmunity. Therefore immune homeostasis throughout the lifespan of a T cell is essential for the maintenance of a T cell repertoire that can respond efficiently to, and maintain long-term memory to infection.

1.2 T cell Development

Efficient T cell homeostasis is evident in the thymus where the mature T population is enriched with antigen-specific clones. This occurs though a set of ordered steps that, positively select T cells, which possess useful TCRs and delete potentially autoreactive T cells.

1.2.1 The Clonotypic αβ T cell receptor and CD3 Complex

The specialised and antigen-specific responses that are critical for adaptive immunity are dependent on the clonal expression of antigen receptors on the surface of mature $CD4^+$ and $CD8^+$ T lymphocytes. This occurs through a set of ordered cell fate decisions marked by the successive rearrangement of TCR β and α loci and timed surface expression of the co-receptors CD4 and CD8.

The TCR is a heterodimer of α and β polypeptides, and functions to specifically recognize peptide fragments complexed with major histocompatability complex molecules (MHC). For $CD8^+$ T cells, peptide antigen is presented by MHC class I molecules whereas MHC class II molecules present antigen to $CD4^+$ T

cells. The αβ TCR possesses short transmembrane and cytoplasmic domains and as a consequence has limited signalling capabilities. The interaction of the TCR with MHC ligands is tranduced into an appropriate intracellular response, through its association with accessory invariant δ, γ, ζ and ε (Ashwell and Klusner, 1990) polypeptide chains of the CD3 complex and the coreceptors CD4 and CD8. The accessory polypeptide chains of the CD3 complex are able to propagate TCR signals by the presence of conserved tyrosine residues in their extended cytoplasmic domains (Reth, 1989) (Samelson et al., 1985; Weiss, 1993). These motifs are termed immunoreceptor tyrosine based activation motifs (ITAMs) and are defined by the presence of two tyrosine residues separated by 6-8 amino acids and surrounded by number of semi-conserved residues (Reth, 1989; Weiss, 1993). Phosphorylated ITAMs act as adaptor sites, facilitating the recruitment of proximal signalling molecules and adaptor proteins to the TCR complex (reviewed in (Pitcher and van Oers, 2003)). The ITAMs present within the TCR/CD3 complex are notable for their sheer number. Ten ITAM motifs reside within the CD3 complex, with the disulphide-linked CD3ζ chains containing six of the total ten (3 in each chain) and the remaining chains each contributing one ITAM. The presence of multiple ITAMs has been suggested to serve many functions (reviewed in (Wange and Samelson, 1996)). Firstly it has been proposed that the presence of a large number of ITAMs within one receptor complex may amplify the initial signal received by the each TCR, promoting a more rapid and efficient cellular response. This is supported by data reporting that the trimerisation of the ITAMs of the CD3ζ chains yield enhanced intracellular signals than single

ITAMs (Irving et al., 1993), implying that ITAMs signalling occurs in a cumulative manner. Secondly, it has also been suggested that the individual ITAMs may have distinct effector preferences, examples of this include phosphatidylinositol 3-kinase (PI3K) whose preferred interaction is with the most TCR proximal CD3ζ chain ITAMs (Exley et al., 1994), other adaptors exhibiting similar ITAM binding preferences include the Zeta-chain associated protein of 70kDa (Zap70) (Isakov et al., 1995).

A minor population of T cells express an alternative highly restricted TCR composed of γ and δ polypeptides. In contrast to T cells, which bear the αβ TCR, mature γδ T cells are not MHC restricted and are predominantly found in the epithelial layers of tissues, including the intestinal epithelium, reproductive tracts, lung, skin and the tongue (reviewed in (Carding and Egan, 2002)). γδ T cells, in contrast to conventional αβ T cells, express TCRs generated from a less diverse set of variable (V), diversity (D) and joining (J) TCR genes, that appear to differ depending on the anatomical location of the particular γδ T cell subset. The reason for this is unclear but it has been suggested that the expression of a highly restricted TCR by different γδ T cells, in these specific locations allows them to recognise specific ligands common to that particular anatomical area (Hayday, 2000).

1.2.2 DN Stages of T cell Development

During thymopoiesis, the differentiating thymocytes begin to develop characteristics essential for their function and concurrently lose the properties

associated with immature thymocytes. T cell development begins in the primary lymphoid organs, where committed lymphoid progenitors arising in the adult BM (or fetal liver in the fetus) migrate to the thymus via the bloodstream. These thymic lymphoid progenitor cells lose the potential to differentiate into B cells (Pui et al., 1999)(Wilson et al., 2001) and NK cells (Michie et al., 2000) and lack the expression of the CD4 and CD8 coreceptors and hence are referred to as double negative thymocytes (DN).

The DN population can be subdivided further into four phenotypically discrete populations, identified through the surface expression of CD25 (interleukin-2 Rα chain/IL-2Rα) and CD44 (phagocyte glycoprotein 1) (Godfrey et al., 1993)(Pearse et al., 1989) as follows: DN1 $CD44^+$ $CD25^-$, DN2 $CD44^+$ $CD25^+$, DN3 $CD44^-$ $CD25^+$ and DN4 $CD44^-$ $CD25^-$. DN1 thymocytes upregulate CD25 expression and differentiate into DN2 thymocytes **(Fig 1.1)**. These DN2 thymocytes mature further and down regulate expression of CD44 and initiate rearrangement of the TCR β, γ and δ loci (Godfrey et al., 1994; Livak et al., 1999; Petrie et al., 1995).

The TCR β gene is made up of four gene segments that encode V, D, J and constant (C) regions. In order to generate a productive β chain, the DNA segments must rearrange by a process known as somatic recombination. This is mediated by recombination-activating genes (Rag1 and Rag2) which are the lymphocyte specific components of the enzyme complex referred to as the V (D) J recombinase (Yancopoulos et al., 1986). Mice lacking expression of the Rag1 or Rag2 genes exhibit a complete block in T and B cell development and

this correlates with an inability to perform V (D) J recombination (Mombaerts et al., 1992) and consequently express a mature antigen receptor. Thymocytes unable to form a successfully rearranged TCRβ chain die by apoptosis (Falk et al., 2001). In contrast, in thymocytes that have successfully rearranged the TCRβ locus, the TCRβ chain is assembled with a pre-Tα chain (pTα) (Groettrup et al., 1993) and CD3 signal transduction machinery (Malissen et al., 1995) which together form the pre-TCR complex on the surface of the DN3 thymocytes (Saint-Ruf et al., 1994; von Boehmer and Fehling, 1997). Thymocytes that fail to pair with a pTα exhibit a profound block in thymopoiesis and fail to generate into $CD4^+$ $CD8^+$ DP thymocytes (DP) (Fehling et al., 1995).

Signals through the pre-TCR complex in DN3 thymocytes that have achieved productive rearrangement of the TCR β locus (also known as β-selection) rescue the cells from programmed cell death (Penit et al., 1995) and leads to the downregulation of CD25 expression by the thymocytes and progression to the DN4 stage. The pre-TCR signals also upregulate the expression of the CD4 and CD8 cell surface glycoproteins and induce cell cycle progression and clonal expansion of thymocytes, to generate a large population of immature thymocytes expressing both $CD4^+$ and $CD8^+$ coreceptors (DP thymocytes) (Hoffman et al., 1996)(Vasseur et al., 2001). Importantly, the β-selection signals transmitted by the pre-TCR complex also prevent further unnecessary rearrangment of the TCR β locus (allelic exclusion) and initiate the rearrangement of the TCRα gene within the immature DP population.

This early β-selection checkpoint, requires the transmission of pre-TCR signals by a cascade of TCR proximal signalling proteins, beginning with the SH2 region containing (Src) family kinase members lymphocyte-specific kinase ($p56^{Lck}$ hereon abbreviated to Lck) (Molina et al., 1992) or $p59^{Fyn}$ (Fyn) (Appleby et al., 1992), however the activity of these kinases in pre-TCR signalling is partially redundant, given that the transmission of pre-TCR signals is only fully abrogated in the absence of both kinases (Groves et al., 1996; van Oers et al., 1996b). This pre-TCR signal is further propagated by the non-receptor cytoplasmic tyrosine kinase Zap70 and related family member spleen tyrosine kinase (Syk) (Cheng et al., 1997). Similarly, there also appears to be redundancy between the requirement of Zap70 and Syk in pre-TCR signalling (Cheng et al., 1997) since neither Zap70 deficient (Negishi et al., 1995) nor Syk deficient mice (Cheng et al., 1995; Turner et al., 1995) show major abnormalities in early thymocyte maturation. Further downstream adaptors proteins including the Zap70 substrates linker for activation of T cells (LAT) and SH2-domain containing leukocyte specific phosphoprotein of 76kDa (SLP-76) also appear to be critical for effective pre-TCR signalling, since DN T cell development is completely arrested at the DN3-DN4 transition following ablation of these signalling intermediaries (Clements et al., 1998; Pivniouk et al., 1998; Zhang et al., 1999).

1.2.3 Positive and Negative Selection

After the pre-TCR checkpoint the DP thymocytes begin to rearrange the TCRα locus to form a functional TCRα chain. However, the assembly of the TCR itself is insufficient for further thymocyte maturation. DP thymocytes that express an

αβ TCR proceed to a further checkpoint in which the strength TCR ligation/signalling is tested, based on its continuous interaction with self peptide (sp) in the context of appropriate MHC complexes (spMHC) (Kisielow et al., 1988b; Teh et al., 1988). This is performed to purge the repertoire of potentially autoreactive and useless T cells. Thymocytes expressing TCRs unable to interact with spMHC fail to mature and die by neglect. In contrast thymocytes expressing overtly self-reactive TCRs, which bind very well to spMHC and are potentially autoreactive are rapidly deleted from the repertoire through a process of apoptotic cell death referred to as negative selection (Ashton-Rickardt et al., 1994; Kappler et al., 1987; Kisielow et al., 1988a; Sebzda et al., 1994; Sha et al., 1988). Only DP thymocytes whose MHC Class I or Class II restricted TCRs recognise and interact with spMHC complexes with a avidity between those that induce death by neglect or negative selection, produce signals that terminate Rag gene expression, mediating allelic exclusion of the TCRα locus and rescue the thymocytes from programmed cell death (PCD) leading to their long-term survival and maturation, a process collectively known as positive selection (reviewed in (Sebzda et al., 1999)).

1.2.4 Proximal Signalling Events in Thymic selection

Ultimately the goal of TCR ligation by antigen, is to instigate a pattern of gene expression that is necessary for the function of the cell. Given that the TCR lacks intrinsic kinase activity, the transmission of TCR signals for positive and negative selection, as well as peripheral T cell activation, is performed by the recruitment and the sequential activation of intracellular kinases, phosphatases

and adaptor proteins, which culminate in new gene transcription. Signalling via this complex yields different functional outcomes depending on the cell type, influence of various costimulator molecules and nature of the TCR stimulus.

1.2.4.1 Src Kinases

The transmission of positive and negative selection signals utilising these signalling intermediaries, begins with the sequential activity of Src and Syk family tyrosine kinases (Straus and Weiss, 1992; van Oers et al., 1996a). One of the earliest signalling events following interaction of the TCR with spMHC in the thymus, is the activation of the Src kinase Lck. The role of Lck in T cell selection was initially studied by the overexpression of a catalytically inactive form of Lck in murine thymocytes, which abrogated positive selection (Hashimoto et al., 1996). Although, negative selection appeared to proceed normally in these mice, superantigen mediated deletion was reduced in the thymocytes expressing the HY transgenic TCR (specific for the male HY antigen (Kisielow et al., 1988a)), suggesting a modest role for Lck in negative selection. Similarly, a role been suggested for Fyn in negative selection, however, the reduction in clonal deletion in the absence of Fyn expression is less dramatic than with a loss of Lck expression (Stein et al., 1992; Utting et al., 1998) suggesting, that Lck is more essential for thymic selection.

1.2.4.2 CD45 and Csk

Essential roles in thymic selection have also been reported for CD45 and carboxy terminal Src kinase (Csk) which positively and negatively regulate Lck and Fyn activity respectively (reviewed in (Mustelin and Tasken, 2003)). CD45

null mice exhibit reduced numbers of DP and single positive thymocytes (SP) as a result of blocks in β-selection and positive selection (iByth et al., 1996). Furthermore, in the absence of CD45 expression, thymocytes are significantly impaired in their ability to induce apoptotic responses to cross linking of the TCR in fetal thymic organ cultures (FTOCs) (Kishihara et al., 1993). In addition these mice permit the selection of self-reactive cells when crossed onto TCR transgenic backgrounds (Mee et al., 1999) suggesting that CD45 is essential for both positive and negative selection events. In contrast, the ablation of Csk, results in the positive selection of DP thymocytes to the CD4 lineage, even in the absence of TCRβ or MHC expression (Schmedt and Tarakhovsky, 2001), suggesting that Csk works to oppose positive selection signals during thymopoiesis.

1.2.4.3 Zap70

One of the most important TCR proximal adaptors immediately downstream of Lck, and involved in antigen receptor signalling is the tyrosine kinase Zap70. Zap70 is a 70kDa protein and a member of the Syk family of tyrosine kinases. Zap70 like its related family member Syk, is composed of two tandemly arranged amino terminal SH2 domains, followed by a central linker region called interdomain B, which connects the SH2 domains with the carboxyl terminal kinase domain (Chan et al., 1992; Folmer et al., 2002) **(Fig 1.2)**. It is an essential component of the signalling cascade that propagates pre-TCR signals influencing β-selection, positive and negative selection signals as well as the transmission of antigenic stimulation in peripheral T cells. Stimulated TCRs recruit Zap70 to phosphorylated ITAM motifs within the CD3 complex,

thereby coupling TCR engagement to the activation of downstream signalling cascades including the phospholipase C γ (PLCγ) (Williams et al., 1999) and mitogen-activated protein kinase (MAPK) pathways (Shan et al., 2001).

Zap70 is expressed in T cells, NK and B cells (Chan et al., 1992; Schweighoffer et al., 2003). In resting thymocytes and lymph node (LN) T cells, Zap70 is primarily found in the cytoplasm, however a small fraction of total enzyme has been reported to translocate to the cell membrane and is found associated with the constitutively tyrosine phosphorylated form of CD3ζ homodimer (21kDa) (van Oers et al., 1994). Surprisingly, (Sloan-Lancaster et al., 1997) also report that a large pool of Zap70 resides within the nucleus in both resting and activated Jurkat T cells, however it is not clear whether this fraction of Zap70 is recruited to the TCR or has a separate nuclear function.

The critical role of Zap70 in thymic selection has been addressed in both mice and humans. In humans, a rare autosomal recessive form of severe combined immunodeficiency, results from mutations in Zap70 that lead to the translation of truncated or inactive forms of Zap70 protein (Arpaia et al., 1994; Chan et al., 1994). CD8 single positive (CD8 SP) thymocyte generation is completely arrested in these patients. Although they do generate mature peripheral $CD4^+$ T cells, the loss of Zap70 expression renders these cells non-functional as they are unable to properly transduce TCR mediated signals. The ablation of Zap70 expression in murine thymocytes completely blocks thymocyte development at the DP stage of thymopoiesis, preventing the generation of mature CD4 single positive (CD4 SP) and CD8 SP thymocytes (Negishi et al., 1995; Wiest et al.,

1997), inferring that Zap70 is essential for positive selection. This is supported by (Sakaguchi et al., 2003) who report that a specific point mutation in the gene encoding the SH2 domain of Zap70, causes chronic autoimmune arthritis in mice, by permitting the selection of otherwise negatively selected self reactive T cells. A role for Zap70 in negative selection has also been suggested by (Negishi et al., 1995). since Zap70 deficient DO10 TCR transgenic thymocytes are resistant to deletion by chicken ovalbumin *in-vitro*

It was initially reported that only αβ T cell development was affected in Zap70 deficient mice, given that CD3 positive thymocytes primarily of the γδ lineage could be detected in the LN of these mice. However, more extensive analysis performed by (Kadlecek et al., 1998) has revealed that the dendritic epidermal γδ T cell subset (DETC) exhibit an abnormal morphology and are fewer in numbers as are γδ intestinal intraepithelial lymphocytes, suggesting that Zap70 expression is also required for their optimal development.

Initiation of Zap70 Signalling

Following TCR engagement, Zap70 translocates to the TCR. Until recently, the molecular manner in which Zap70 is recruited to the activated TCR/CD3 complex in thymocytes or peripheral T cells was largely unknown. However, a study by (Gu et al., 2006) has reported an interesting interaction between Zap70 and RhoH, a haematopoietic-specific GTPase-deficient member of the Rho GTPase family. This study showed that RhoH could interact with Zap70, through the association of the tandem SH2 domains of Zap70 with ITAM-like

motifs within the RhoH protein. Importantly, ablation of RhoH expression in mice resulted in the complete arrest of thymopoiesis at the DP stage mimicking the phenotype of *Zap70*$^{-/-}$ mice. Furthermore, following TCR stimulation RhoH was found to be localised with Zap70 at the immunological synapse, in contrast translocation of Zap70 to the cell membrane was abrogated in the absence of RhoH expression. These surprising findings suggest that RhoH may shuttle Zap70 to the plasma membrane thus permitting the activation of signalling events essential for normal thymic development.

Following recruitment to the activated TCR, the tandem SH2 domains permit the association of Zap70 with phosphorylated ITAMs within the cytoplasmic domains of the CD3 subunits in a highly specific and cooperative manner (Hatada et al., 1995). Zap70 is then activated by phosphorylation of tyrosine residues 493 and 492 located in the activation loop of the kinase domain (Chan et al., 1995; Mege et al., 1996) as determined in human Jurkat cell lines. These residues have positive and negative regulatory functions on Zap70's enzymatic activity respectively and once phosphorylated appear to stabilise Zap70 in an active conformation (Jin et al., 2004). The manner in which these tyrosine residues become phosphorylated, is still unclear, but it is reported that this occurs by interaction with Lck and Fyn (Chan et al., 1995; Fusaki et al., 1996; Mege et al., 1996; Wange et al., 1995) and/or auto-phosphorylation by Zap70 to achieve its full activation (Brdicka et al., 2005; Neumeister et al., 1995).

Regulation of Zap70 Activity by Key Tyrosine Residues

Several other tyrosine residues present within interdomain B also have important roles in the regulation of Zap70 activity. Initial studies, using transformed human Jurkat cell lines, identified three tyrosine residues Y292 (Kong et al., 1996; Zhao and Weiss, 1996), Y315 (Wu et al., 1997) and Y319 (Di Bartolo et al., 1999; Williams et al., 1999) which following recruitment of Zap70 to the TCR become phosphorylated by Lck (Williams et al., 1999) or by Zap70 itself (Di Bartolo et al., 1999). These tyrosine residues do not influence the enzymatic activity of Zap70 but act in trans by coupling Zap70 to downstream SH2 containing effectors proteins or by permitting optimal recruitment of Zap70 to phosphorylated ITAM motifs.

Further analysis of the role these tyrosines play in TCR signal transduction has been addressed either by generating knockin mice, that express Zap70 molecules with point mutations within the specific tyrosine residues (Magnan et al., 2001), or by generating endogenous Zap70 deficient mice, reconstituted with transgenes encoding mutant Zap70 molecules. There are significant defects in thymic selection when these tyrosine residues are mutated (Gong et al., 2001b). In both studies mice expressing the transgenic HY TCR, showed that mutation of the Y315 impaired both the positive and negative selection of mature T cells, suggesting that this residue enhances Zap70 function. This was initially attributed to the association of Y315 with the SH2 domain of Vav1 the guanine nucleotide exchange factor for Rho family GTPases Rac1 and Cdc42 (Tybulewicz, 2005). However mutation of Y315 in different studies has

produced conflicting data, with reports suggesting this association is essential for TCR signal transduction (Katzav et al., 1994; Wu et al., 1997) and others refuting the requirement (Di Bartolo et al., 1999; Magnan et al., 2001). More recent studies have suggested an alternative role for Y315. (Di Bartolo et al., 2002) have reported that mutation of Y315 reduced the binding affinity of Zap70 to the phosphorylated ITAM motifs within the CD3 subunits suggesting Y315 may have a critical role in stabilising SH2 domain-ITAM contacts.

Similarly, substitution of Y319 to phenylalanine also resulted in reduced positive and negative selection possibly as a result of reduced contact with Lck (Pelosi et al., 1999). In contrast mutation of Y292 failed to influence positive or negative selection events, however *in-vitro* stimulation of the mature cells expressing the Y292 mutation showed a greater number of cells producing interleukin 2 (IL-2) and IFNγ. This mutation did not enhance cytokine production but instead appeared to increase the number of responding T cells. Analysis of the phosphorylation states of CD3ζ and Zap70 revealed that these proteins appeared more tyrosine phosphorylated in Y292 mutated cells compared to wildtype controls, suggesting that the Y292 residue has a negative regulatory role, and functions to attenuate TCR signalling possibly through its ability to bind a novel phosphotyrosine binding domain in the N-terminal of Cbl the product of the *c-Cbl* protooncogene and putative negative regulator of TCR signal transduction (Lupher et al., 1997; Naramura et al., 1998; Rao et al., 2000).

Divergence of Positive and Negative Selection Signals

Once phosphorylated on the key tyrosine residues, activated Zap70 in turn phosphorylates downstream adaptor proteins LAT (Zhang et al., 1998a) and SLP-76 (Wardenburg et al., 1996) creating phosphorylated docking sites for SH2 domain bearing signalling proteins. While it is appreciated that the activity of intracellular kinases and adaptor proteins are essential for thymic selection, how the intracellular signals discriminate between thymocyte positive and negative selection and at which point TCR signals driving positive or negative selection diverge remain poorly understood. Recent experiments suggest that positive and negative selection signals may diverge at the level of the extracellular-signal related kinase (ERK) and c-Jun amino-terminal kinase (JNK) activation. (Neilson et al., 2004) have shown that deletion of the locus encoding the regulatory B1 subunit of calcineurin in thymocytes *in vivo*, completely abrogated positive selection but was not essential for negative selection. Furthermore the ablation of calcineurin activity resulted in the inefficient activation of ERK suggesting it too is essential for positive but not negative selection. In contrast (Gong et al., 2001a) showed that inhibition of JNK and p38 phosphorylation *in vivo* selectively impaired negative but not positive selection furthermore, the authors also observed that there were differences in the signaling thresholds of JNK, ERK and p38, such that ERK had a lower activation threshold than either JNK or p38, and thus could provide a mechanism by which quantitative differences in signal strength could influence positive or negative selection (reviewed in (Siggs et al., 2006)).

1.2.5 CD4/CD8 Lineage Decision

The decision to become either a CD4 or a CD8 T cell is of great importance given that these populations play differential effector roles that are critical for the clearance of infection, as well the generation of protective immunity. How cell fate decisions are regulated is still not understood in detail and is a topic of much debate (reviewed in (Kappes et al., 2005)).

The two original hypotheses regarding lineage commitment propose different models for the how a T cell matches its MHC and coreceptor specificity with its TCR (reviewed in (Germain, 2002)). The instructive model proposes that engagement of the TCR and CD4 coreceptor with MHC class II molecules initiates qualitatively different intracellular signals compared to when a TCR and CD8 coreceptor corecognises a MHC class I ligand. These different signals were thought to "instruct" the cell to commit to the CD4 or CD8 lineage (Borgulya et al., 1991; Robey et al., 1990). In contrast the stochastic/selective model suggests that DP thymocytes randomly make lineage decisions that are unrelated to the specificity of their TCR and that a subsequent selective process eliminates thymocytes that have inappropriate coreceptor expression (Chan et al., 1993; Davis et al., 1993).

Initial studies hoping to provide a definitive answer to how lineage commitment occurred have given conflicting results. It has been shown that MHC class II restricted cells can under some circumstances commit to the CD8 lineage (Matechak et al., 1996) suggesting that lineage decisions are not a result of

qualitatively distinct signals that are induced by class I ligands or class II ligands. Similarly, constitutive expression of the CD4 or CD8 coreceptors in TCR transgenic mice have failed to direct substantial numbers of mismatched thymocytes expressing incorrect coreceptor-TCR combinations to the correct lineage, thus contradicting the stochastic model of lineage commitment (Borgulya et al., 1991; Robey et al., 1994). In order to reconcile these results a revised instructive model has been proposed. This suggests that lineage commitment is instructed by quantitative rather than qualitative differences in TCR signals depending upon which coreceptor is engaged, such that CD4 and CD8 commitment results from 'stronger' and 'weaker' TCR signals, respectively (Itano et al., 1996; Matechak et al., 1996). The basis for this "strength of signal" model comes from the finding that the CD4 coreceptor binds Lck with a higher affinity that that of the CD8α chain (Ravichandran and Burakoff, 1994; Veillette et al., 1988), thus TCR engagement by MHC class II ligands is thought to induce stronger signals leading to CD4 commitment, while engagement by MHC class I ligands would induce weaker signals leading to CD8 commitment. Indeed substitution of the CD8α cytoplasmic domain with that of CD4 coreceptor re-directs development of class I-restricted thymocytes to the CD4 lineage (Itano et al., 1996). Furthermore *in vivo* MHC class II–restricted thymocytes develop into functional $CD8^+$ T cells when Lck activity is reduced whereas, class I restricted thymocytes develop into functional $CD4^+$ T cells when Lck activity is increased (Hernandez-Hoyos et al., 2000). Recent studies have reported that the strength of TCR signalling which influences lineage decisions may be modulated by the duration of TCR signalling, such that long

exposure to TCR ligands induces commitment to the CD4 lineage whereas a shorter duration of TCR signalling directs thymocytes to the CD4 or CD8 lineage (Liu and Bosselut, 2004; Yasutomo et al., 2000). How such quantitative differences in TCR signalling are transmitted intracellularly into CD4 or CD8 lineage-determining signals still remains unknown. However, there is evidence that different lineage specific transcription factors are essential for commitment to the CD4 or CD8 lineages such as runt-related transcription factor 3 (Runx3), which silences CD4 expression at the SP CD8 stage and Gata-binding protein 3 (Gata3) which favours differentiation to the CD4 lineage (reviewed in (Kappes et al., 2005)). More recently a "master regulator" of lineage commitment has been identified as the transcription factor T helper-inducing POZ/Krüppel factor (Th-POK also called cKrox) which is essential for CD4 lineage commitment since mutation of the *Thpok* gene in mice, redirects MHC class II expressing thymocytes to the CD8 lineage, resulting in a complete loss of CD4 SP thymocytes (He et al., 2005) (Sun et al., 2005). More work is still required to determine how and which TCR proximal signalling proteins are necessary for the activation of the transcription factors essential for lineage commitment.

1.2.6 Development of Other T cell Subsets

1.2.6.1 γδ T cells

In contrast to αβ T cell development, the generation of γδ T cells is not as well understood in part due to an inability to identify unique surface markers common to γδ T cell precursors (reviewed in (Hayday and Pennington, 2007)).

Currently it is thought that γδ and αβ T cells arise from a common multipotent DN precursor population in the thymus (Dudley et al., 1995). Following maturation to the DN2 stage of development, the immature thymocytes begin to rearrange the β, γ and δ loci (Capone et al., 1998). Lineage commitment to the αβ or γδ lineage is also believed to occur within the bipotent DN2 subset. Recent studies testing the developmental potential of the DN thymocyte subsets *in vitro* using OP9 BM stromal cell monolayers in the presence Notch ligands, have shown that αβ and γδ lineage decisions are completed during the DN2-DN3 transition (Ciofani et al., 2006). Once committed to the γδ lineage, γδ thymocytes mature along a developmental pathway distinct from the αβ lineage. Unlike αβ thymocytes, γδ cells do not require pre-TCR signals for their maturation nor do the γδ T cells mature through a DP stage of development.

Recently, the question of how γδ T cells generated in the thymus localise to distinct anatomical sites and acquire different functions has been investigated. It has been suggested that like αβ T cell precursors in the thymus, at least some γδ T cell subset precursors undergo some form of thymic selection, although this appears to be MHC independent since γδ T cell development is normal in MHC class I or class II deficient mice (Bigby et al., 1993; Correa et al., 1992; Grusby et al., 1993). However, limited knowledge regarding the nature of natural γδ ligands has made the analysis of γδ selection complicated. For this reason many studies have been performed in transgenic adult G8 and KN6 mouse strains which harbour γδ TCRs specific for T10 and T22 non-classical MHC class I molecules (Bonneville et al., 1989; Ito et al., 1990; Schild

et al., 1994), which also constitute a small subset of naturally occurring γδ T cells found in wildtype mice (Crowley et al., 2000). Using these transgenic mice (Dent et al., 1990) reported that γδ T cells underwent negative selection following interaction of the γδ TCR with strong ligands, which was not observed in response to weaker ligands. Whether γδ T cells also undergo positive selection, is less clear. While there is evidence to both support (Haks et al., 2005; Pereira et al., 1992; Wells et al., 1993) and refute (Schweighoffer and Fowlkes, 1996) the requirement of positive selection in the generation of mature γδ T cell subsets in adult mice, it is difficult to come to a definitive answer since the requirement for positive selection appears to differ depending on the transgenic or wildtype γδ subset analysed and the genetic background of the mouse strains used. Recently other groups have begun to address this question in the fetal thymus, given that the first cells that develop in the fetus express γδ TCRs. These develop in progressive waves, generating discrete subsets, which populate different anatomical positions in the adult mouse (Dunon et al., 1997). To date most of the work has studied the cutaneous DETC subset of γδ T cells. Using transgenic mice in which different TCR γ gene segments specific to DETCs were deleted (Cγ1 cluster) revealed that while γδ T cells populating other peripheral sites developed normally there was a complete absence of DETCs, suggesting the generation of this population was dependent on the rearrangement and selection of specific γ chains (Xiong et al., 2004). This was supported by a further study, which showed that selection of DETC γδ T cells is dependent upon the expression of thymic stromal ligand, such than mice deficient for this putative γδ ligand completely lack DETCs

(Lewis et al., 2006) implying that positive selection does occur during γδ T cell development. Whether this occurs for other γδ subsets that populate different anatomical areas, is still unclear.

1.2.6.2 CD4 Regulatory T cells

$CD4^+$ $CD25^+$ $Foxp3^+$ T cells (T_{Reg}) cells are a naturally arising specialised population of regulatory T cells, which suppress conventional immune responses and prevent autoimmune pathology *(discussed in more detail in section 1.4.3.2)*. Natural T_{Reg} cells develop in the thymus and are positively selected on the cortical medullary epithelial cells (Bensinger et al., 2001). This population constitutes ~5% of the CD4 SP thymocyte compartment and exhibits a phenotype and function similar to peripheral T_{Reg} cells in that they have the capacity to suppress T cell proliferation *in vitro* and prevent immunopathology (reviewed in (Hori and Sakaguchi, 2004)).

Similar to conventional $CD4^+$ T cells, the selection of natural T_{Reg} thymocytes appears to require a TCR signal, although the nature of this still remains contentious. Some studies suggest that T_{Reg} cells are positively selected in response to TCR engagement by self peptides of an intermediate affinity (Jordan et al., 2001). However other groups suggest that T_{Reg} cells are selected as a result of defective negative selection, such that this allows the T_{Reg} repertoire to be enriched with autoreactive cells by eliminating high affinity clones that have failed to upregulate the transcription factor forkhead box p3 (Foxp3) (Kawahata et al., 2002; Liston and Rudensky, 2007; Romagnoli et al., 2005; van Santen et al., 2004).

While the nature of the TCR signal that influences the generation of T_{Reg} cells in the thymus is unclear, what is known is that the transcription factor Foxp3 is important for T_{Reg} development. This was shown by (Fontenot et al., 2003), who generated mixed BM chimeras reconstituted with a mixture of allotypically marked Foxp3 negative and Foxp3 positive BM cells. Importantly all the $CD25^+$ $CD4^+$ T cells generated in these mice were of Foxp3 origin, indicating that Foxp3 was necessary for their development. The signals that induce the activation of Foxp3 transcription have yet to be elucidated, however it has been suggested that high-avidity TCR interactions may be responsible for Foxp3 induction (Hori and Sakaguchi, 2004).

More recently a requirement for cytokine and costimulatory signals have been identified for T_{Reg} development (Fontenot and Rudensky, 2005). A key factor known to influence T_{Reg} development is the cytokine IL-2 (Malek and Bayer, 2004). This is most evident in the phenotype of *IL-2*$^{-/-}$ and *IL-2Rα*$^{-/-}$ mice, which display lymphoproliferative autoimmune disorders. However, $CD4^+$ $Foxp3^+$ thymocytes still develop in *IL-2Rα*$^{-/-}$ deficient mice (Fontenot et al., 2005), furthermore adoptive transfer of peripheral $CD4^+$ T cells isolated from IL-2-deficient mice still conveyed protection in a TCR-transgenic model of experimental allergic encephalomyelitis, suggesting that T_{Reg} cells can develop in absence of IL-2 indicating that IL-2 may dispensible in T_{Reg} differentiation (Furtado et al., 2002).

A role in T_{Reg} differentiation has also be identified for the costimulatory molecule CD28. Interaction of CD28 with its ligands CD80 (B7.1) or CD86 (B7.2) is

reported to influence thymic T_{Reg} development, by the observation that the number $CD4^+$ $CD25^+$ $Foxp3^+$ thymocytes is significantly reduced in *CD28*$^{-/-}$ mice and mice treated with antibodies to B7 (Sansom and Walker, 2006; Tang et al., 2003). However, as observed in the absence of IL-2 signalling, functional T_{Reg} cells are generated in the absence of CD28 (Salomon et al., 2000) suggesting that these pathways may be more important for maintenance than generation of T_{Reg} cells.

1.2.7 Role of IL-7 in T cell Development

The generation of a mature lymphocyte repertoire is dependent on the proliferation and survival of early thymocyte progenitors committed to the T or B cell lineage. The cytokines interleukin-7 (IL-7) and stem cell factor (SCF) influence the maturation and survival of the early T cell precursors, whereas IL-7, thymic stromal lymphopoietin (TSLP) and fetal liver kinase 2 ligand (Flk-2L) are essential for the maturation of B cell precursors (reviewed in (Kang and Der, 2004)). For T cells, IL-7 signalling via the interleukin-7 receptor (IL-7R) and SCF via its receptor c-kit (CD117) support the survival of the early DN T cell progenitors whereas at later stages DP cells depend on signals through the $\alpha\beta$ T cell receptor (Cantrell, 2002).

The non-redundant role of IL-7 during ontogeny varies depending on the lineage and the differentiation state and is most evident in IL-7 (von Freeden-Jeffry et al., 1995) and IL-7Rα deficient mice (Maraskovsky et al., 1994; Peschon et al., 1994). These mice exhibit dramatically reduced numbers of $\alpha\beta$ thymocytes and are completely devoid of $\gamma\delta$ T cells (Maki et al., 1996) since IL-

7 is essential for rearrangement of the γ TCR locus (Maki et al., 1996) and to a lesser extent TCR δ and β loci by increasing chromatin accessibility (Candeias et al., 1997; Crompton et al., 1997; Durum et al., 1998; Schlissel et al., 2000). Interestingly the defect in thymic cellularity appears most pronounced in *Il-7Rα*$^{-/-}$ mice than *Il-7*$^{-/-}$. This is due to the additional loss of TSLP signalling which also utilises the IL-7Rα chain (Pandey et al., 2000; Park et al., 2000) and can suboptimally substitute for IL-7 in lymphopoiesis (Al-Shami et al., 2004; Chappaz et al., 2007). IL-7 signalling is also important for human thympopoiesis, since mutations in the IL-7Rα gene result in severe combined immunodeficiency (SCID) characterised by normal B and NK cell development but abrogated T cell development (Puel and Leonard, 2000; Roifman, 1995).

IL-7Rα expression is tightly regulated during ontogeny (reviewed in (Fry and Mackall, 2005) and (Mazzucchelli and Durum, 2007)). Typically IL-7Rα is first expressed on the common lymphoid progenitor (CLP) in the BM, which gives rise to T and B cells (Kondo et al., 1997b). Although CLPs express IL-7Rα it appears that IL-7 signalling is not required for their survival (Miller et al., 2002). Interestingly IL-7Rα is not a prerequisite for T cell lineage development since lymphoid progenitors in which IL-7Rα expression is either low or absent can effectively generate T cells in the thymus (reviewed in (Bhandoola et al., 2003; Montecino-Rodriguez and Dorshkind, 2003)).

In the thymus IL-7Rα is expressed at different developmental checkpoints. IL-7Rα expression appears high on DN thymocytes, is absent from the DP subset of thymocytes and is re-expessed on mature SP thymocytes (Fry and Mackall,

2002; Sudo et al., 1993). In the DN subsets, IL-7Rα expression is essential for their survival, and does so by protecting the cells from apoptosis via the upregulation of the anti-apoptotic protein B cell lymphoma-2 (Bcl-2) (Kim et al., 1998; von Freeden-Jeffry et al., 1997). Over expression of Bcl-2 or deletion of the pro-apoptotic protein Bcl-2-associated X protein (Bax) in *Il-7Rα*$^{-/-}$ mice can partially restore αβ thymocyte development, confirming the importance of the protective role IL-7 plays in thymocyte development (Akashi et al., 1997; Khaled et al., 2002). However, Bcl-2 overexpression and Bax deletion could not rescue γδ T cell development suggesting that IL-7 may regulate several independent processes during thymopoiesis.

IL-7 signalling has also been shown to play a role in DN thymocyte differentiation and proliferation (Conlon et al., 1989; Murray et al., 1989; von Freeden-Jeffry et al., 1997; Watson et al., 1989). More recently (Dadi et al., 1994) have reported that IL-7 can activate PI3K and (Pallard et al., 1999) have shown that the proliferative effects of IL-7 may be mediated by PI3K in human thymocytes. (Hagenbeek et al., 2004) have further reported that the lipid phosphatase known a phosphatase and tensin homologue deleted on chromosome 10 (PTEN) can negatively regulate PI3K mediated thymocyte proliferation, and importantly that deletion of PTEN in the absence of IL-7 (γc deficient mice) can restore αβ T cell development.

At the β-selection checkpoint IL-7Rα expression is maintained by pre-TCR signals and possibly reflects a mechanism whereby the survival of potentially useful T cell clones is facilitated allowing their maturation to the DN4 stage of

development (Trigueros et al., 2003). However, following maturation to immature DP stage (iDP) of thymopoiesis, the thymocytes silence IL-7Rα expression (Van De Wiele et al., 2004) and constitutively express the suppressor of cytokine signalling (SOCS-1) which suppresses the pro-survival effects of γ_c cytokines (Yu et al., 2006). As a consequence, survival of useful thymic clones is maintained by positive selection signals through the αβ TCR only.

The reason for this strict control of IL-7Rα expression during ontogeny has been a matter of debate, but recent reports have shed light on this matter. Forced expression of IL-7Rα during development by (Munitic et al., 2004) resulted in a surprise decrease in thymocyte number with age and was attributed to increased DN thymocyte apoptosis as a result of reduced Bcl-2 expression. The reason for this is unclear, but (Munitic et al., 2004) suggest that competition for endogenous IL-7 by the DP thymocytes, decreased available IL-7 for the DN population and thus IL-7Rα downregulation during thymopoiesis may reflect an altruistic mechanism, of maintaining sufficient levels of IL-7 for DN consumption and thus maintenance of normal numbers of thymocyte subsets. Alternatively, it has also been reported that forced expression of IL-7Rα in the iDP thymocyte compartment (also referred to as immature single positive thymocytes in the literature), inhibits the expression of HMG domain containing transcription factors Transcription factor 1 (TCF-1) (Okamura et al., 1998) and lymphoid enhancer-binding factor 1 (LEF-1) (Verbeek et al., 1995), as well as by the retinoid-related orphan nuclear

receptor γ (RORγ) and its thymus specific isoform RORγt transcription factor (Sun et al., 2000b), which allow iDP thymocytes to differentiate into mature DP cells (Yu et al., 2004), leading to a complete block in thymocyte development, indicating that IL-7R expression must be terminated at this developmental checkpoint in order for T cell development to progress.

Following positive selection, IL-7Rα is re-expressed on murine CD4 SP and CD8 SP thymocytes, and is expressed at high levels on recent thymic emigrants (derived from human umbilical cord blood) (Hassan and Reen, 2002). Interestingly is has also been suggested that lineage commitment to the $CD8^+$ lineage is in part mediated by IL-7 signalling, such that IL-7 can upregulate Bcl-2 expression, silence CD4 coreceptor expression and upregulate the surface expression of the glucose transporter molecule-1 (GLUT1) increasing nutrient uptake and metabolism facilitating the differentiation to the CD8 SP lineage (Yu et al., 2003).

1.3 Peripheral T cell Homeostasis

Following their positive selection, CD4 SP and CD8 SP exit the thymus and are released into the circulation adding to the peripheral T cell compartment. In order for an efficient immune response to occur, this peripheral T cell pool must be homeostatically regulated to control its size and composition. This is achieved in the face of highly variable dynamics of T cell generation and losses. Additions to the T cell pool arise not only from the constant production of new T cells by the thymus but also the expansion of mature T cells during

immune responses in the peripheral lymphoid compartment. Conversely, T cells are lost as a result of naturally occurring events, such as following infections, or clinically as a consequence of medical treatments involving irradiation or administration of cytotoxic drugs.

The peripheral T cell compartment exists to detect and combat potential sources of infection and to do so efficiently requires finely balanced numbers of immune cell subsets at different states of differentiation, for example: naïve versus antigen experienced memory cells. This ensures that the immune system can respond optimally to the potential pathogens it encounters, be it a primary response to a new infection or mounting rapid recall memory responses to pathogens previously encountered. Dysregulation of these processes, however, can also result in immune pathology, such as lymphoproliferative disorders, autoimmunity and malignancy highlighting the necessity of regulatory processes that maintain homeostasis within the peripheral lymphoid organs.

1.3.1 The role of TCR Signals for Naïve αβ T cell Survival

The transduction of signals received by the TCR from foreign antigen leads to the activation and differentiation of naïve T cells. However, less is known about the precise TCR signals required for the survival of peripheral naïve T cells, whether in fact TCR signals exist and the nature of their contribution to the long-term steady state survival of the lymphocytes within the peripheral lymphoid compartment remains controversial.

1.3.1.1 Self-peptide MHC/TCR Interactions

Early studies examining the survival of the peripheral T cell populations, reported that naïve T cells were relatively long-lived (with a half life in the order of months) and had slow turnover rates as determined by the level of 5-bromo-2-deoxyuridine (BrdU) incorporation (Sprent et al., 1991) and slow decay in naïve T cell numbers following thymectomy of adult mice (Di Rosa et al., 1999; Jameson, 2002; Tough and Sprent, 1994). Until relatively recently, it was thought that naïve T cells circulating through the peripheral tissues, were in state of passive quiescence maintained independently of environmental stimuli. However, about 10 years ago several seminal studies suggested that naive T cells require exogenous signals for their survival. It was initially proposed that naive $CD4^+$ and $CD8^+$ T cells were sustained in the periphery by continuous interaction of the TCR/CD3 complex with spMHC ligands (Takeda et al., 1996) similar to those that influence positive selection. Since then, the study of naïve T cell survival has been a topic of great debate and controversy with many studies supporting or refuting the requirement of TCR signals in peripheral naïve T cell maintenance. In the case of naïve $CD4^+$ T cells, the first seminal study addressing whether these cells required TCR spMHC contact for their survival was performed by (Takeda et al., 1996). This group reported that MHC class II expression was not required for short-term survival, but was needed for the longevity of murine polyclonal naïve $CD4^+$ T cells. Later studies performed by (Rooke et al., 1997) employing adenoviruses to transiently express MHC Class II genes in the thymus of T cell deficient *MHC Class II*$^{-/-}$ hosts or by transplanting *Rag1*$^{-/-}$ MHC class II deficient hosts with wildtype thymus grafts

(Kirberg et al., 1997) or mature TCR transgenic $CD4^+$ T cells (Brocker, 1997) added more controversy to the field, by showing that naïve $CD4^+$ T cells were markedly short-lived in the absence of MHC class II expression. Conversely, (Dorfman et al., 2000b) reported that in their system, polyclonal naïve $CD4^+$ T cell survival appeared the same in MHC class II sufficient and deficient hosts, suggesting that TCR spMHC contact was not essential for peripheral T cell survival.

In the case of naïve $CD8^+$ T cell survival, early studies performed by (Tanchot et al., 1997) tested the ability of HY TCR transgenic H-$2D^b$ restricted monoclonal $CD8^+$ T cells to survive following adoptive transfer into irradiated mice expressing different MHC class I alleles. These authors showed that the survival of the naive TCR transgenic $CD8^+$ T cells required interaction with allele specific MHC class I complexes. Further studies involving the transplantation of lymphopenic MHC class I deficient hosts with wildtype thymic grafts, demonstrated that polyclonal $CD8^+$ T cells could not be detected in the periphery of these mice in the absence of MHC class I expression, but importantly could be restored following injection of syngeneic MHC class I expressing cells (Nesic and Vukmanovic, 1998). These early studies also appeared to indicate that naïve $CD8^+$ T cells in the absence of MHC class I expression declined with a kinetic faster than that of naïve $CD4^+$ T cells.

However, analysis of the methodology used to asses the role self-recognition plays in naïve T cell survival revealed, that while it appeared that TCR spMHC contact could maintain the survival of cohorts of naïve T cells transferred to T

cell deficient hosts, it could also under these conditions of lymphopenia stimulate their proliferation a process termed homeostatic proliferation (HP) or lymphopenia induced proliferation (LIP) (Goldrath and Bevan, 1999). Furthermore, it was found that different TCR transgenic T cell clones showed varied profiles of LIP, depending on TCR avidity for self antigen as well as the availability of these TCR ligands (Barthlott et al., 1998; Kassiotis et al., 2003; Kieper et al., 2004). This was further complicated by the finding that the common practice of irradiation used to render hosts T cell deficient (in some of the studies described so far) could also augment the observed LIP (Schluns et al., 2000) by stimulating the release of cytokines and growth factors not present in normal unirradiated hosts (Hong et al., 1999; Limanni et al., 1995; Peterson et al., 1994). The inability to distinguish between T cell expansion and steady state survival led to the assumption that spMHC recognition was inducing proliferation and not maintaining T cell survival (Grandjean et al., 2003), and that the differences in cell numbers obtained from recipients in the presence or absence of spMHC were due to differential expansion. Not only is the use of such a model made difficult by the risk of cotransferring MHC expressing APCs (Dorfman et al., 2000a), but also the LIP serves to mask steady state survival. More sophisticated genetic studies employing gene ablation and inducible gene expression in transgenic mice, have shed further light on this question.

A study conducted by (Polic et al., 2001) involving the generation of TCR deficient naïve T cells by MX-cre induced ablation of the gene encoding the

constant region of the TCRα chain, showed that naïve $CD4^+$ and $CD8^+$ T cells lacking TCR expression exhibited reduced half lives in comparison to TCR expressing cells and with a similar rate of decay observed in T cells lacking MHC contact. This was supported by data generated by (Witherden et al., 2000) using a mouse model which allowed the tetracycline controlled expression of MHC class II molecules only in the thymi of MHC class II deficicent mice, thus permiting the selection of $CD4^+$ T cells and release in to a periphery lacking MHC class II ligands. They reported that spMHC recognition by naïve $CD4^+$ T cells was essential for their steady state survival, since the absence of the MHC class II expression in the periphery yielded half-lives of 3-4 wks compared to the long lived wildtype cells. The use of replete mice also, excludes the possibility that the effects observed are due to expansion of the peripheral pool due to LIP rather than spMHC TCR triggered cell survival. More recently studies performed by (Martin et al., 2006; Martin et al., 2003) provide further support to the view that TCR spMHC interaction can support peripheral naïve $CD4^+$ T cell survival. Unexpectedly, they found that, in $A_\beta^{-/-}$ mice classically used as MHC class II deficient hosts in the large majority of $CD4^+$ T cell survival studies, the A_α chain and the E_β chain could associate to form a hybrid $A_\alpha E_\beta$ MHC class molecule that has the capacity to support the LIP of naïve $CD4^+$ T cells in lymphopenic hosts. They also found that classical and hybrid MHC class II expression was not essential for the long-term survival of naïve $CD4^+$ T cells in lymphopenic mice, but was required for maintaining peripheral $CD4^+$ T cell survival in replete mice, suggesting that under

lymphopenic conditions non-TCR signals are sufficient for naïve CD4 T cell survival.

Interestingly (Witherden et al., 2000) demonstrated that the decay in naïve T cell survival observed in their model, correlated with abnormally low levels of partial CD3ζ chain phosphorylation (21kDa). *In vivo* the 16kDa CD3ζ homodimer appears to be constitutively tyrosine phosphorylated to 21kDa form in thymocytes and resting peripheral T cells, (van Oers et al., 1993) and appears to be a consequence of TCR ligation (Nakayama et al., 1989; van Oers et al., 1994). It has been suggested that a difference in the induction of the relative ratios of 21kDa ζ and 23kDa ζ chain homodimers maybe an indication of anergic T cells (Madrenas et al., 1995; Sloan-Lancaster et al., 1994). It has also been suggested that the constitutively phosphorylated 21kDa ζ chain may reflect a survival signal (Witherden et al., 2000) or maybe required to prime the TCR for antigenic stimulation (Stefanova et al., 2003). Currently the precise function of the different forms of ζ dimer remains unknown, however the loss CD3ζ chain phosphorylation infers a requirement for kinases for the transduction spMHC/TCR survival signal.

1.3.1.2 The Role of Src Kinases in Naïve αβ T cell Survival

Other studies have directly addressed the requirement for specific signalling molecules for T cell homeostasis. Utilising the tetracycline inducible gene regulatory system Seddon *et al* have further dissected the TCR mediated survival pathway (Seddon et al., 2000; Seddon and Zamoyska, 2002b). They proposed that should a TCR signal be required for peripheral T cell survival, it

maybe mediated in a similar manner as TCR signals that trigger T cell differentiation and activation. These studies investigated whether the initial kinases that facilitated the first steps in the transduction of such TCR signals may also play a role in the propagation TCR signals that are required for peripheral naive T cell maintenance.

Using mice bearing a tetracycline-inducible transgene for the Src family kinase Lck (Lck^{ind}) on an endogenous $Lck^{-/-}$ and/or $Fyn^{-/-}$ background (Legname et al., 2000), Seddon, *et al* demonstrated the essential role for Lck and Fyn in maintaining the long-term steady state survival of naïve T cells. Wildtype levels of either kinase were able to support the survival of the naïve $CD4^+$ and $CD8^+$ T cell subsets. However, in the absence of Lck and Fyn the cells failed to survive. As with the (Witherden et al., 2000) study, it was also found that the defect in long-term steady state survival was associated with a loss of CD3ζ phosphorylation, consistent with a loss of signalling via the TCR. However, Lck and Fyn have been shown to associate with receptors other than the TCR/CD3 complex, such as the IL-2R (Hatakeyama et al., 1991; Kobayashi et al., 1993; Minami et al., 1993), the cell death protein Fas (Atkinson et al., 1996) and adaptors independent of TCR signalling (Parravicini et al., 2002; Wary et al., 1998) and thus it cannot be ruled out that the effects of Lck and Fyn on T cell survival maybe occur independently of the TCR. Furthermore, it is also unclear how Lck and Fyn survival signals are propagated further and the nature of the downstream adaptor proteins involved in the transmission of these TCR mediated survival signals has yet to be elucidated.

1.3.2 Regulation of Lymphocyte Homeostasis by IL-7

It has been well established that in addition to signals evoked by interaction of the TCR with spMHC complexes, cytokines are also essential for naïve T cell survival and homeostasis. Interleukins IL-4, IL-6 and IL-7 can maintain naïve T cell survival *in vitro* (Vella et al., 1997) and blockade of IL-7 with anti-IL-7 antibodies in thymectomised hosts *in vivo* dramatically reduces the numbers of naïve but not memory T cells (Boursalian and Bottomly, 1999). Further analysis using genetic knockouts of IL-4 and IL-6, revealed that neither cytokine was essential for naïve T cell survival, given that normal numbers of naïve T cells were generated in these mice (Kopf et al., 1994; Kuhn et al., 1991). In contrast, IL-7 and IL-7Rα deficient mice showed significantly reduced numbers of peripheral naïve T cells that were largely attributed to survival defects in the early stages of T cell development.

A key study that specifically assessed the role of IL-7 in naïve T cell survival was performed by (Tan et al., 2001) who reported that naïve $CD4^+$ and $CD8^+$ T cells declined dramatically and disappeared within 30d following adoptive transfer into *Il-7*$^{-/-}$ hosts compared to *Il-7*$^{+/+}$ controls. This was supported by a study performed by (Vivien et al., 2001) in which depletion of IL-7 signalling by anti-IL-7R antibodies in normal thymectomized hosts led to a reduction in naïve T cell numbers, that was not observed in the absence of either IL-4 or IL-6, suggesting that IL-7 is indeed essential for naïve T cell survival.

1.3.2.1 IL-7R Signalling

How IL-7 promotes the survival of naïve T cells has yet to be fully determined but regulation of pro and anti-apoptotic survival factors appears to be involved (reviewed in (Jiang et al., 2005)). The IL-7R is composed of two chains, the IL-7Rα chain (CD127) and the common cytokine receptor γ chain (γ_c/CD132/IL-2γ) (Noguchi et al., 1993) which is also a component of the IL-4 (Kondo et al., 1993), interleukin 9 (IL-9) (Kimura et al., 1995), interleukin 15 (IL-15) (Giri et al., 1994), and interleukin 21 (IL-21) cytokine receptors (Asao et al., 2001). The γ_c is widely expressed by most haematopoietic cells, whereas the IL-7Rα chain is restricted primarily to cells of the lymphoid lineage (reviewed in (Mazzucchelli and Durum, 2007)). For the γ_c family of cytokines including IL-7, the initial receptor signals are transduced by the Janus kinase family of tyrosine kinases (Jak) (Nosaka et al., 1995; Suzuki et al., 2000). The cytoplasmic tail of IL-7Rα chain is constitutively associated with the tyrosine kinase Jak1 and the γ_c is associated with Jak3 (Russell et al., 1994). Following engagement of IL-7Rα chain with IL-7, the IL-7Rα subunit recruits the γ_c bringing the cytoplasmic domains bearing Jak1 and Jak3 together, allowing Jak3 to phosphorylate Jak1 increasing its enzymatic activity (Qin et al., 2001). Jak 3 also phosphorylates the tyrosine 449 within the IL-7Rα chain, creating a docking site for the SH2 domain of the transcription factors, signal transducers and activators of transcription protein 1, 3 and 5 (STAT) (Yu et al., 1998).

In T cells the predominant STAT activated by IL-7 and other γ_c cytokines including IL-2 and IL-15 as well as cytokines important for haematopoiesis is

STAT5 (Johnston et al., 1995; Lin and Leonard, 2000). STAT5 exists as two isoforms STAT5a and STAT5b, which associate with Y449 within the IL-7Rα and are rapidly phosphorylated by Jak1 and Jak3 following IL-7 stimulation (Foxwell et al., 1995; Rosenthal et al., 1997; Yu et al., 1998). This leads to their dimerisation and translocation to the nucleus where they regulate the activation of cytokine dependent genes including Bcl-2 (Kim et al., 2003) (Jiang et al., 2004) and Bcl_{XL} (Socolovsky et al., 1999). Early attempts to knockout STAT5a and STAT5b in mice revealed no appreciable defect in T cell development, nor did peripheral T cell numbers appear to be reduced in the absence of these transcription factors, suggesting that the essential substrates of Jak 1 and Jak 3 had yet to be identified. However, more recently it has been found that these mice expressed truncated but partially functional STAT5 protein (Teglund et al., 1998). (Yao et al., 2006) have generated mice in which the entire STAT5 locus was deleted, revealing severe abnormalities in thymic development, such that this gene deletion results in 99% perinatal lethality. Analysis of the fetus at embryonic day 18.5 revealed that *STAT5*$^{-/-}$ precursors could not support the development of any lymphoid lineage, resulting in a SCID phenotype that was more severe than the defects observed in *Il-7R*$^{-/-}$ or $\gamma c^{-/-}$ fetuses. Furthermore, selective deletion of both STAT5 isoforms in T cells, results in a marked peripheral reduction in naïve and memory $CD4^+$ and $CD8^+$ T cells, with the effect appearing most pronounced within the $CD8^+$ compartment, suggesting that STAT5a/b are major mediators of γc survival signals.

IL-7 exerts its affects as a survival factor by protecting IL-7 dependent cells from cell death, in part via STAT5 activation. IL-7 has been reported to mediate such effects by influencing the intrinsic pathway of mitochondrial apoptosis through the regulation of pro and anti-apoptotic Bcl-2 family members **(Fig 1.3)**. The anti-apoptotic Bcl-2 family members, in mammals include Bcl-2, Bcl-$_{XL}$ and myeloid cell leukemia sequence 1 (MCL-1) and display sequence similarity throughout all four of the Bcl-2 homology domains (BH domains 1-4) (reviewed in (Huang and Strasser, 2000)). In contrast there is less homology between the pro-apoptotic family members, and are divided into two further sub groups: the "BH3-domain only" (BH3) members and the "multidomain" members. The BH3 proteins, which in mammals include Bcl-2 antagonist of cell death (Bad), Bcl-2 interacting killer (Bik/Nbk), Blk, Hrk/DP5, BH3 interacting domain death agonist (Bid), Bim, p53-upregulated modulator of apoptosis (Puma) and Noxa share amino acid sequence similarity only within the short BH3 domain. In contrast the multidomain Bcl-2 family members, display sequence homology in BH1-3 domains and include Bcl-2 antagonist/killer (Bak) and Bax (reviewed in (Huang and Strasser, 2000)).

1.3.2.2 Regulation of Apoptosis by Bcl-2 Family Proteins

The first Bcl-2 family member to be characterised was the anti-apoptotic protein Bcl-2, originally cloned as an oncogene from a chromosomal breakpoint in human B cell follicular lymphoma (Tsujimoto et al., 1985) that was later discovered to inhibit apoptosis (Vaux et al., 1988). How anti-apoptotic Bcl-2 family members protect cells from apoptosis is not clearly understood. It is known that in response to apoptotic stimuli such as growth factor withdrawal,

activated multidomain proapoptotic Bcl-2 family members Bak and Bax induce mitochondrial outer membrane permeablisation (MOMP) leading the release of cytochrome *c* into the cytosol. Following its release from the mitochondria, cytochome *c* associates with the cytosolic adapter protein apoptotic protease activating factor (Apaf-1) leading to the ATP/dATP-dependent activation of the cysteine-dependent aspartate-directed proteases termed caspases. These capases (9 and 3) in turn cleave various intracellular polypeptides including essential architectural components of the nucleus and cytoplasm leading to apoptotic cell death (reviewed in (Earnshaw et al., 1999)). How pro and anti-apoptotic Bcl-2 family members regulate MOMP as well as the process that permits the release of cytochrome *c* is still unclear. At present there are two main hypotheses regarding the roles Bcl-2 family members play in mitochondrial apoptosis (reviewed in (Hacker and Weber, 2007)).

The first model, the "direct-binding hypothesis", suggests that BH3-domain only proteins Bim and tBid (the active form of Bid which is produced following cleavage of Bid by caspase 8) bind and activate the multidomain proapoptotic proteins Bax and Bak leading to cytochrome *c* release and cell death (Letai et al., 2002). To reconcile the observation that the anti-apoptotic Bcl-2 homologues, can bind and sequester tBid and Bim in the cytosol, the direct model suggests that the function of other BH3 proteins, such as Bad and Noxa (also known as "sensitisers") is to neutralise anti-apoptotic Bcl-2 homologues and thus prevent them from sequestering the "activators" Bim and tBid, allowing them to activate Bak and Bax. However, this model has been challenged by studies assessing the role another BH3-domain only member

known as Puma. Early studies using isolated outer mitochondrial membranes (Kuwana et al., 2002), tested the activity of BH3 domain peptides generated against the various BH3 proteins. These studies suggested that only Bim and tBid (activated form of Bid) could associate with Bax directly resulting in its activation and cytochrome *c* release (Kuwana et al., 2005; Letai et al., 2002) suggesting that these proteins were the essential activators of apoptosis. However, deletion of Bim along with Puma another BH3 Bcl-2 family member in T cells results in the cells being entirely resistant to cell death, suggesting that Puma is also essential for apoposis induction and calling into question the precise role of tBid (You et al., 2006). Furthermore, although peptides of the BH3 domain of Puma were reported to associate with Bax, this did not lead to cytochrome c release (Cartron et al., 2004) suggesting that Puma does not promote apoptosis by activating Bax and Bak.

The alternative model for the initiation of apoptosis is known as the "displacement model" (Chen et al., 2005; Willis et al., 2005). This model proposes that BH3 proteins activate Bax or Bak indirectly by engaging and inactivating the anti-apoptotic Bcl-2 homologs that work to oppose the pro-apoptotic effects of Bak and Bax. Evidence to support this comes from a recent study performed by (Willis et al., 2007) which appears to contradict the direct model of apoptosis induction. In contrast to studies performed by (Letai et al., 2002) and (Kuwana et al., 2005) this study reported that Bim and Bid BH3 peptides could not associate with native Bax. Following detergent treatment that appeared to mimic an activation step, Bim and tBid could only bind weakly to Bax, with an affinity lower than observed between Bax and pro-survival Bcl-2

homologues. In addition coimmunoprecipitation studies using full length BH3 proteins revealed that while tBid had some functional interaction with Bax, there was no evidence of contact between Bax and Bim. Similarly Bak was unable to associate with any BH3 proteins. Importantly MCL-1, Bcl-2 and Bcl-$_{XL}$ were found to both directly associate and functionally constrain Bax, with MCL-1 and Bcl-$_{XL}$ performing a similar function with Bak. These data suggests that different Bcl-2 homologues bind and sequester Bak and Bax, thus inferring that BH3 proteins induce apoptosis indirectly by occupying the anti-apoptotic Bcl-2 family members allowing the activation of Bak and Bax.

1.3.2.3 Regulation of T cell Survival by IL-7

Until recently it was unclear how IL-7R signals influenced mitochondrial apoptosis. It was initially suggested that IL-7R signals regulated apoptosis by inducing the expression of the pro-survival protein Bcl-2. This was originally suggested by data produced in early studies assessing the manner in which IL-7 potentiates survival in DN1, DN2 and DN3 thymocytes *in vitro* (Kim et al., 1998) as well as human T-ALL cells (Karawajew et al., 2000). These studies showed that IL-7 inhibited apoptosis, and that this effect correlated with increased levels of Bcl-2 expression. This was further reported in an IL-7 dependent thymocyte cell line, which showed that Bcl-2 expression decreased upon withdrawal of IL-7, and was upregulated following re-administration of IL-7 (Kim et al., 2003), suggesting that Bcl-2 was a key pro-survival mediator in cells deprived of IL-7R signals and that IL-7 may regulate Bcl-2 expression. However there is still much controversy regarding whether Bcl-2 is essential for IL-7 mediated survival, given the conflicting results produced in a number of

studies described below. Firstly *Bcl-2*$^{-/-}$ mice only show a modest defect in thymic development (Matsuzaki et al., 1997) and while expression of a human Bcl-2 transgene could partially rescue αβ thymopoiesis, in *Il-7Rα*$^{-/-}$ mice, there was no rescue of γδ or B cell development, nor was there evidence of enhanced *in vitro* or *in vivo* survival of the thymocytes or peripheral T cell subsets (Akashi et al., 1997). Enforced expression of the Bcl-2 transgene in $\gamma_c^{-/-}$ mice produced conflicting results in a number of studies (reviewed in (Di Santo and Rodewald, 1998)). (Kondo et al., 1997a) reported that expression of a Bcl-2 transgene could restore αβ T cell development however (Strasser et al., 1991) found that Bcl-2 expression did not substantially correct T cell development in *γc*$^{-/-}$ mice, although the thymocytes did show enhanced survival *in vitro*.

A study performed by (Rathmell et al., 2001) has added more controversy by reporting that IL-7-mediated survival in mature T cells *in vitro* is Bcl-2 independent, since the levels of Bcl-2 did not change despite ongoing cell death following nutrient withdrawal. While it is difficult to reconcile the large body of data assessing the role of Bcl-2 in IL-7 mediated survival, it is likely that Bcl-2 can mediate part of the survival effects elicited by IL-7, but that other molecules may also be required for IL-7 mediated cell survival. Indeed this does appear to be the case. In addition to regulating the expression of Bcl-2, IL-7 has been reported to upregulate the anti-apoptotic Bcl-2 family member Bcl-$_{xL}$ (Amos et al., 1998). However it appears that Bcl-$_{xL}$ is not a significant contributor to mature T cell survival given that the lifespan of mature T cells

from Bcl-$_{XL}$ deficient mice are normal. In contrast the survival of immature thymocytes, is shortened in the absence of Bcl-$_{XL}$ expression, suggesting it is more essential in maintaining the viability of T cell precursors (Motoyama et al., 1995).

More recently, IL-7 has also been implicated in the regulation of MCL-1 also an anti-apoptotic Bcl-2 family member, found to specifically bind the BH3 proteins Bim and Noxa (Chen et al., 2005; Opferman et al., 2003). Deletion of MCL-1 in mice results in peri-implantation embryonic lethality, such that blastocysts fail to implant in utero (Rinkenberger et al., 2000). Selective deletion of MCL-1 during early lymphocyte differentiation results in increased apoptosis and severely arrests T cell development at the DN3-DN4 stage, and B cell development at the Pro-B cell stage (Opferman et al., 2003). Importantly, ablation of MCL-1 in peripheral T and B cell populations results in their rapid disappearance suggesting a survival role for MCL-1. In addition the cytokines IL-7, IL-15 and IL-2 were found to induce MCL-1 protein expression in mature wildtype T cells *in vitro*, with the effect appearing most pronounced with IL-7 treatment. MCL-1 mRNA was also shown to be upregulated in wildtype T cells following culture with IL-7. Importantly, the survival effects of IL-7 in peripheral T cells, were found to be partially dependent on MCL-1 expression such that the percentage of viable cells *in vitro* were significantly reduced in the absence of MCL-1 expression. It is important to note however that IL-7 mediated survival was not completely abrogated in the absence of MCL-1, inferring that IL-7 survival signals are likely to be transduced by a range pro survival molecules **(Fig 1.3)**.

1.3.2.4 Death Pathways Suppressed by IL-7

Bax

In addition to protecting the cells from cell death, by upregulating anti-apoptotic factors, IL-7R signals also actively suppress the activity of pro-apoptotic proteins. This includes the regulation of the multidomain protein Bax as well as the BH3 proteins Bad and Bim. IL-7 mediated regulation of Bax, was first discovered by data showing that in DN1, DN2 and DN3 thymocytes cultured *in vitro,* IL-7 withdrawal led to a dramatic decline in Bcl-2 levels and concomitant increase of Bax, which could be inhibited by the addition of IL-7 (Kim et al., 1998). Studies performed by (Khaled et al., 1999) using an IL-7 dependent thymocyte cell line, reveal that IL-7 withdrawal induces the translocation of Bax from the cytosol to the mitochondrial membranes. Withdrawal of IL-7, (and IL-3 in an IL-3 dependent cell line) led to a transient rise in intracellular pH, which preceded Bax translocation and appeared to trigger a conformation change in Bax itself. This exposed the membrane targeting domains of Bax, facilitating its integration into the mitochondrial membranes leading to the induction of apoptosis. Indeed further studies have shown that the deletion of Bax in *IL-7Rα*$^{-/-}$ mice can rescue αβ thymopoiesis, however this is only evident in young mice up to 8 weeks of age, with older mice exhibiting a phenotype similar to that of *IL-7Rα*$^{-/-}$ mice (Khaled et al., 2002), presumed to be a consequence of "stress" factors active in the adult but not the younger mice. Although this data suggests that IL-7 does play a role in Bax repression during thymocyte development, selective deletion of Bax and IL-7Rα in peripheral T cells has yet

to be performed; therefore it is unclear if IL-7 has a significant role in regulating Bax activity in peripheral T cells.

Bad

IL-7 has also been implicated in the regulation of the BH3 protein Bad. Bad has been shown to promote cell death, such that overexpression of Bad in mice (Mok et al., 1999) or expression of a constitutively active Bad mutant by replacement of serine residues 112, 136 and 155 with alanine (Datta et al., 2002), results in thymocytes and mature T cells that are highly sensitive to apoptotic stimuli. (Li et al., 2004) using an IL-7 dependent thymocyte cell line, have reported that IL-7 inactivates Bad, by inducing the phosphorylation of serine residues 112, 136 and 155. The phosphorylation of Bad promotes its binding to 14-3-3 proteins retaining it in the cytosol and preventing its translocation to the mitochondria (Datta et al., 2000; Masters et al., 2001). Following IL-7 withdrawal, Bad is dephosphorylated and translocates the mitochondrial membranes, presumably to bind and constrain its putative binding partners Bcl-2 and Bcl_{XL} localised at the mitochondria and endoplasmic reticulum (ER) (Chen et al., 2005). This alternative function of IL-7 appears to occur partly through the activation the PI3K pathway. IL-7 signalling has been shown to activate the lipid PI3K (Dadi et al., 1994). In human T cells it has been reported that the p85 subunit of PI3K can associate with Jak3 and that following engagement of the IL-7R, activated Jak3 tyrosine phosphorylates the p85 subunit leading to PI3K activation (Sharfe et al., 1995). A key target of PI3K is the serine/threonine kinase protein kinase B (AKT/PKB), which in turn

is responsible for Bad phosphorylation in response to IL-7 signalling. Although IL-7 has been shown to activate AKT in IL-7 dependent murine thymocyte cell lines, it is unclear how PI3K activation is linked to AKT activation and Bad phosphorylation, since it is reported that this signalling pathway is initiated by the γc not the IL-7Rα chain (Jiang et al., 2004). To date much of the work regarding IL-7 regulated Bad activity has been in thymocytes and it is unclear whether Bad has a similar pro-apoptotic function in peripheral mature T cells. More work is still needed in this area to understand the role of IL-7R signalling in the PI3K/AKT dependent regulation of Bad activity in T cell survival.

Bim

The activity of the BH3 protein Bim is also regulated by IL-7R signals. Bim exists as 3 isoforms termed Bim_S, Bim_L and Bim_{EL}. While some studies report that Bim_L and Bim_{EL} are bound to microtubular cytoskeletal complexes (reviewed in (Schinzel et al., 2004)) particularly the dynein light chain LC8, a component of the dynein motor complex (Puthalakath et al., 1999), recent studies have shown that in T cells Bim is primarily associated with the mitochondria and already bound to Bcl-2 and Bcl-x_L in both healthy and dying T cells (Zhu et al., 2004). The role of Bim in apoptotic cell death following growth factor withdrawal was first reported by (Bouillet et al., 2001) who showed that *Bim*$^{-/-}$ peripheral $CD4^+$ and $CD8^+$ T cells were resistant to apoptosis induced by cytokine withdrawal. Conclusive evidence for the role of Bim as a T cell death mediator during IL-7 deprivation was demonstrated in mice deficient for Bim and the IL-7Rα chain (Pellegrini et al., 2004). Loss of Bim, increased

thymocyte numbers and reconstituted the periphery of these mice with near normal numbers of functional mature T cells, suggesting that IL-7 is a key regulator of Bim activity. How IL-7 regulates Bim activity is unclear. Studies performed in pro-B cell lines have suggested that Bim maybe in part regulated by PI3K/AKT activity. Bim is transcriptionally regulated by the forkhead transcription factor FOXO3a (Dijkers et al., 2000). In the presence of IL-3, AKT phosphorylates FOXO3a (Brunet et al., 1999), leading to its binding with 14-2-2 proteins and retention in the cytoplasm, blocking its translocation to the nucleus and thus the transcription of Bim. Whether IL-7 serves a similar function in naive T cells is not known, and further work is required to fully understand how IL-7 differentially regulates the activity/expression of anti-apoptotic Bcl-2 family members.

1.3.2.5 Regulation of Glucose Metabolism

As discussed, IL-7 can regulate the cell intrinsic mitochondrial pathway of apoptosis, through it affects on the Bcl-2 family members which prevent the release cytochrome C and activation of cellular caspases. However, the cell intrinsic pathway, has been shown to promote cell death independently of caspase activation (McCarthy et al., 1997; Monney et al., 1998; Xiang et al., 1996). The use of an IL-3 dependent cell line lacking Bak and Bax the pro-apoptotic proteins of the cell intrinsic apoptosis pathway, has revealed that following IL-3 withdrawal the *Bak*$^{-}$*Bax*$^{-}$ cells underwent progressive atrophy. This was characterised by decreased glycolytic rates, loss of glucose transporter GLUT1 and reduced cellular ATP content (Lum et al., 2005) suggesting that in addition to regulating apoptosis, cytokines and growth factors

prevent cellular atrophy by maintaining glucose transport and cellular metabolism. A similar role has been suggested for IL-7. (Rathmell et al., 2001) has shown that IL-7 can restore glucose metabolism in primary T cells undergoing growth factor withdrawal. More recently (Barata et al., 2004) have shown that IL-7 can induce GLUT1 expression and promote glucose uptake in T cell acute lymphoblastic leukaemia cells (T-ALL). Importantly IL-7 was shown to mediate these effects through PI3K and AKT activation. Thus the pro-life effects of IL-7 signalling are multifactoral, functioning to prevent mitochondrial apoptosis through the action of Bcl-2 family members but also to maintain cellular metabolism, which together support the survival of the cell **(Fig 1.3)**.

1.3.3 Induction and Control of Lymphopenia Induced Proliferation

The size of the peripheral naïve T cell pool is regulated by many factors, which crucially influence survival but also proliferation and differentiation. While naïve T cells do not undergo significant proliferation in immunoreplete hosts in the absence of foreign antigen, they do have the capacity to proliferate in response to lymphopenia. Such conditions of T cell depletion may arise naturally during viral or bacterial infections (Okada et al., 2000; Tumpey et al., 2000) or artificially as a result of ionising irradiation or chemotherapy (Campbell et al., 1973). Experimentally, LIP is observed in mice following adoptive transfer of naïve T cells into mice lacking T cells, by whole body irradiation (Oehen and Brduscha-Riem, 1999) or through genetic defects (SCID and $Rag^{-/-}$) (Rocha et al., 1989) (Sprent et al., 1991). Until recently the signals which drive this LIP of

the naïve T cell compartment was unclear, however recent studies have identified the key players in the regulation of such antigen independent expansion.

1.3.3.1 The Role of Self Recognition and IL-7 Signals in LIP

The role of TCR signals in the induction of LIP was originally discovered with the finding that LIP was significantly reduced in hosts lacking their restricting MHC class I or class II molecules (Beutner and MacDonald, 1998; Goldrath and Bevan, 1999; Viret et al., 1999). The evidence that the recognition of self antigens and not foreign antigen was responsible for LIP by naïve T cells came from studies reporting that various naïve TCR transgenic T cells proliferate in T cell deficient hosts in the absence of cognate antigen (Ge et al., 2002; Goldrath et al., 2000; Kieper and Jameson, 1999) and that LIP was even observed in recipients raised in completely germ-free conditions (Kieper et al., 2005). Others studies suggest that the low affinity self peptides that influence positive selection in the thymus also drive LIP in the peripheral naïve T cell compartment (Ernst et al., 1999; Viret et al., 1999). A requirement for cytokine signalling via IL-7 is also essential for the LIP response, given than LIP was shown to be completely abrogated in mice lacking IL-7 expression (Schluns et al., 2000; Tan et al., 2001) *in vivo*.

Further studies have confirmed that naïve T cell LIP is dependent on the availability of specific self ligands. These studies show that the adoptive transfer of TCR transgenic naïve $CD8^+$ T cells into mice expressing a different TCR resulted in the proliferation of the donor population, whereas transfer of

the same cells into a host also expressing the same TCR did not result in their LIP, presumably because the two populations were competing equally for the same spMHC ligands (Moses et al., 2003). This shows that in immunoreplete mice, the competition for specific spMHC ligands restrains LIP of the naïve T cell compartment. More recently it has been reported that the extent of LIP is dependent on the avidity of the TCR for specific self antigen, this is consistent with the finding that TCR transgenic $CD4^+$ and $CD8^+$ T cells exhibiting low avidity TCRs homeostatically proliferate significantly less in lymphopenic hosts than naïve T cells expressing high affinity TCRs (Kassiotis et al., 2003; Kieper et al., 2004). (Ge et al., 2004) went on to report that donor T cells can only proliferate in full hosts expressing different TCRs, if the transferred cells experience stronger TCR-spMHC interactions than the resident T cell population. Since the two populations may be competing for different self ligands, this suggests that under these conditions IL-7 is the limiting factor and that naïve T cells expressing higher affinity TCRs have a survival advantage and are better at competing for the limited IL-7.

The precise role of IL-7 in LIP induction still remains unclear. (Kieper et al., 2004), showed that adoptive transfer of TCR transgenic T cells expressing a high affinity TCR into full hosts containing elevated levels of circulating IL-7 augmented the LIP of donor cells when compared to the same cells transferred to a full host containing physiological levels of IL-7. Similarly while TCR transgenic naïve T cells proliferate in hosts containing cells expressing a different transgenic TCR, the rate of proliferation is still reduced compared to hosts lacking T cells. What this suggests is that while unopposed contact with

self ligands can increase TCR signalling to trigger homeostatic cell division, the level of proliferation is stronger in the presence of elevated levels of IL-7. While this may be evidence of the capacity of IL-7 to amplify homeostatic TCR signals to reach the threshold required for LIP (Sprent and Surh, 2003), this may also suggest that that increased TCR signalling has the capacity to reduce the threshold for IL-7R signalling required for LIP, such that less IL-7 is required by cells expressing high affinity TCRs, indeed this has been suggested by numerous reports (Fry and Mackall, 2005), although there is still no direct evidence to support this.

Still the question remains how do TCR and IL-7 signals support LIP in lymphopenic conditions but only survival in T cell replete hosts? The answer to this question appears to be the manner in which IL-7 levels are regulated. In mice IL-7 is produced by a variety of different cell types including thymic stromal cells (Wiles et al., 1992), BM cells (Gutierrez-Ramos et al., 1993), as well as being detected in the fetal intestine (Murray et al., 1998). In humans IL-7 production appears equally widespread and has been detected in intestinal epithelial cells (Madrigal-Estebas et al., 1997), endothelial cells (Kroncke et al., 1996), peripheral blood DCs (Sorg et al., 1998) and keratinocytes (Heufler et al., 1993) (reviewed in (Jiang et al., 2005)). Although IL-7 production appears to be widespread, how IL-7 levels are regulated as well which cells supply IL-7 to the T cell compartments is still not fully understood. IL-7 production at an RNA level appears to be constitutive (Mazzucchelli and Durum, 2007) and uninfluenced by the size of the T cell compartment, it is therefore held that IL-7 levels are regulated primarily by consumption rather than production and is

known as the dosage effect model (Fry and Mackall, 2001). This model suggests that lymphopenia leads to the accumulation of IL-7 as a result of diminished consumption, an idea supported by the finding that the $CD4^+$ T cell depletion in humans correlates with increased levels of circulating IL-7 (Bolotin et al., 1999; Fry et al., 2001; Napolitano et al., 2001). Furthermore, low doses of IL-7 appears to support survival whereas and high doses of IL-7 induce proliferation in IL-7 dependent cell lines (Kittipatarin et al., 2006) as well as $CD4^+$ recent thymic emigrants (RTE) (umbilical cord RTEs) (Swainson et al., 2007). Therefore it is conceivable that naive T cells transferred into a lymphoid compartment containing excess IL-7 may undergo extensive IL-7 mediated proliferation. Whether conditions of elevated IL-7 result in the enhanced responsivess of the TCR for the low affinity self antigens that drive LIP or alters the signalling machinery leading to proliferation remains to be investigated.

1.3.3.2 Influence of IL7R Signalling on Cell Cycle

The transmission of TCR signals that influence LIP, are mediated by the Src kinase Lck, however how this signal is propagated further by downstream intracellular signalling proteins is unknown (Seddon et al., 2000). The proliferative signal arising at the IL-7R is better understood and is the topic of much recent interest (reviewed in (Kittipatarin and Khaled, 2007)).

Recent reports suggest that IL-7 promotes T cell proliferation by influencing the activity of regulatory factors involved in mammalian cell division cycle. The mammalian cell division cycle is cooperatively regulated by different classes of

cyclin-dependent kinases (Cdks) whose activities are in turn negatively regulated by cyclin-dependent kinase inhibitors (CKIs).

IL-7 has been reported to regulate the activity of p27kip1 which is a CKI belonging to the Cdk interacting protein/Kinase inhibitory protein family (Cip/Kip family) that negatively regulate the activity of the Cdks which are essential for the transition from the first Gap phase (G_1) of the cell cycle to the phase where DNA synthesis is initiated (S phase) (reviewed in (Vidal and Koff, 2000). p27kip1 specifically binds to and inactivates the cyclin dependent kinase 2 (Cdk2) and its cyclin binding partners cyclin E or cyclin A (Cdk2/cyclin complex) leading to G1 cell cycle arrest (Sherr and Roberts, 1999). In quiescent cells (G0 phase), p27kip1 levels are high resulting in low levels of Cdk activity. As the cells enter S phase of the cell cycle, p27kip1 levels decrease and the kinase activity of Cdk2 increases leading to cell division (Kaldis, 2007). IL-7 was originally shown to regulate p27kip1 in primary T-ALL cells (Barata et al., 2001). Culture of the cells with IL-7 induced the downregulation of p27kip1, sequential expression of cyclin D2 and cyclin A, increased the kinase activity of Cdk4, Cdk6, and Cdk2 and allowed cell cycle progression to the S phase. Forced expression of p27kip1 prevented the activation of Cdk2 and cell cycle progression. Later studies performed in an IL-7 dependent thymic cell line also confirm the role of p27kip1 in IL-7 mediated proliferation, but found that IL-7 signals promote the destabilistion p27kip1 leading to its degradation (Li et al., 2006). Unlike previous reports, this study showed the effects of p27kip1 on IL-7 mediated LIP responses. Adoptive transfer of *p27kip1*$^{-/-}$ naïve CD4^{+} or CD8^{+} T cells into *Il-7*$^{-/-}$ mice showed that loss of p27kip1 could partially compensate for

the absence of the proliferative IL-7 signal. However, LIP of the naïve T cells was not maximal in the absence of IL-7 and p27kip1 suggesting that other IL-7 regulated factors may be needed to regulate Cdk2 activity, and fully induce LIP.

Proliferative IL-7 signals have also been reported to affect the activity of Cdc25A a dual-function protein phosphatase. The Cdc25 family of phosphatases, positively regulate cell cycle progression by dephosphorylating and activating the Cdks particulary Cdk2. Overexpression of Cdc25A in rat fibroblast cell lines enhances cell cycle transition to the S phase, thus highlighting its importance in cell division (Blomberg and Hoffmann, 1999). Cdc25A was shown to be responsive to IL-7 signalling in a study in which a degradation-resistant Cdc25A mutant was expressed in an IL-7-dependent T-cell line and peripheral T cells. In these cells the mutant Cdc25A rescued cell survival and proliferation following IL-7 withdrawal and was associated with increased phosphorylation of Cdk2 (Khaled et al., 2005). Importantly the expression of this Cdc25A mutant was sufficient to overcome the inhibitory effects of p27kip1. This data suggests that part of the proliferative effects of IL-7 maybe mediated through the activity of Cdc25A however, whether this occurs in primary T cells and specifically in conditions of lymphopenia have yet to be addressed. This combined data suggests that as with IL-7 survival signals, the proliferative effects of IL-7 is mediated by different overlapping intracellular pathways, which culminate to induce an efficient cellular response.

1.4 T cell Activation

In the periphery, the naïve antigen inexperienced cells traffic through the bloodstream to and from the secondary lymphoid organs where they sample peptide MHC complexes on the surface of antigen presenting cells (APCs). While interaction with self antigens, influences the survival of the cells or leads to the induction of LIP, recognition of foreign antigen, activates the T cell and initiates the cell mediated adaptive immune response against infection.

1.4.1 Major Histocompatability Complex and Antigen Presentation

1.4.1.1 The MHC Complex

T cell activation in the peripheral lymphoid organs is dependent upon the ability of the T cell to recognise antigenic peptides. In order for a T cell to effectively recognise peptide antigen, it must be presented in complex with class I or class II MHC molecules. These proteins are encoded as a large cluster of polymorphic genes in the host genome on human chromosome 6 or mouse chromosome 17. MHC class I molecules present endogenous antigens (viral or bacterial) processed in the cytosol to αβ T cells expressing the CD8 coreceptor. In contrast, MHC class II molecules present peptides to $CD4^+$ subset of αβ T cells that are derived from exogenous antigens internalised by APCs and degraded within endosomes and lysosomes that form part of the vesicular system.

MHC class I molecules are composed of two polypeptide chains. An α chain which spans the cell membrane and a smaller non-covalently associated invariant subunit known as β2-microglobulin, which lacks a transmembrane domain. In contrast the class II MHC molecule is formed by two non-covalently linked membrane spanning α and β polypetide chains, and do not express an equivalent accessory β2-microglobulin subunit. As mentioned MHC class I molecules present viral peptides, given that viruses can inflect any nucleated cell, almost all cells except mammalian red blood cells express MHC class I on their surface. The purpose of $CD4^+$ T cells, also referred to as 'helper" T cells is to regulate the activity of other immune cells, thus MHC class II appears to be selectively expressed on professional antigen presenting cells such as B cells, macrophages and DCs that are specifically specialised for the uptake and presentation of antigenic determinants within the host environment.

1.4.1.2 Antigen Processing

The peptides that induce antigen specific immunity are generated by APCs. These specialised cells process exogenous or endogenous antigen into immunogenic peptides and present the degraded peptides on the cell surface along with costimulatory molecules that stimulate T cell activation. There are three main types of APCs, commonly referred to as "professional" APCs, macrophages, DCs and B cells. For naïve T cell activation, the most important APCs are DCs, since they are distributed in the lymphoid organs, particularly in areas enriched in naïve T cells and where B cells and macrophages are largely excluded (reviewed in (Trombetta and Mellman, 2005)). Recognition of microbial or viral products by TLRs on the surface of the DC leads to the

maturation of the immature DC, leading to the upregulation of costimulatory molecules CD80 and CD86, which assist in the activation of T cells, aswell as producing cytokines including IL-12 and type I and II interferons which activate NK and NKT cells. Thus, the capture and processing of antigen by DCs is essential for the efficient and timely activation of adaptive immune responses.

Antigen processing generally occurs by two different pathways. Typically MHC class I molecules present peptides derived from viruses or bacteria which reside in the cytosol of the infected cell, whereas MHC class II molecules present peptides are generated from bacteria and parasites phagocytosed by APCs and degraded to peptides within the intracellular lysosomal or endosomal vesicles. The boundary between the type of peptides the different classes of MHC can present is not fixed, given that recent reports show that MHC class I molecules can present peptides degraded via the vesicular pathway termed 'cross presentation' and have been shown to be particularly effective in priming CTLs (Shen and Rock, 2006). Similarly MHC class II molecules can present peptides derived from the cytosol (reviewed (Trombetta and Mellman, 2005)), however for the purpose of this introductory chapter I will briefly describe only the classical pathways of antigen presentation.

The cytosolic proteins, destined to become MHC class I ligands, are degraded in the cytosol by the multicatalytic protease complex called the proteaosome. The resultant peptides are transported into the lumen of the ER, for loading onto newly synthesised MHC class I molecules. This is mediated by peptide

transporters located in the ER membrane, termed transporters associated with antigen processing 1 and 2 (TAP1 and TAP2). Within the ER, the MHC complex is assembled. The MHC class I α chain associates with a membrane bound chaperone protein called calnexin until it binds to the β2-microglobulin chain at which point calnexin disassociates from the MHC class I heterodimer. The constructed MHC class I complex then translocates to the TAP proteins where it can be loaded with appropriate peptides. The thiol oxiredoreductase Erp57 (Dick et al., 2002), accessory protein tapasin, (Garbi et al., 2003), and the soluble chaperone protein calreticulin (Wearsch et al., 2004) move the MHC class I molecule to the TAP complex. Tapasin forms a bridge between the MHC class I heterodimer and TAP allowing the MHC complex to get into close proximity to the TAP proteins and leads to its loading with antigenic peptides. Once a stable MHC class I:peptide complex is formed, it leaves the ER via the Golgi apparatus, and is expressed on the cell surface (reviewed in (Cresswell et al., 2005)).

The MHC class II antigen presentation pathway differs from the MHC class I pathway, such that loading of the newly synthesised MHC class II molecules occurs within acidified endosomes. This begins with the synthesis and assembly of the MHC class II molecule in the ER, with a chaperone protein termed the invariant chain (Ii) (Cresswell, 1996; Sant and Miller, 1994), which occupies the peptide binding groove preventing the binding of the MHC class II complex with peptides or partly folded proteins and delivers the MHC class II proteins to low pH endosomal compartment. Following entry into the

endosome, the Ii chain is cleaved leaving a short peptide fragment called class II associated invariant chain peptide (CLIP) within the peptide binding groove. Antigens endocytosed by APCs are degraded to short peptides, and delivered to the MHC class II containing endosomes, where following catalytic removal of CLIP by a MHC class II-like molecule human leukocyte antigen HLA-DM, are loaded onto the MHC class II complex (Denzin and Cresswell, 1995; Sherman et al., 1995). The resulting MHC class II:peptide complex are then trafficked to the cell surface.

1.4.2 Peripheral TCR Signalling

1.4.2.1 Co-stimulatory Molecules

At the cell surface, recognition and engagement of the TCR with antigenic peptides presented in the context of appropriate MHC, leads to the activation of the T cell. For the instigation of a full pattern of differentiation leading to T cell proliferation and acquisition of effector function, the T cells must also receive a second stimulatory signal generated by co-stimulatory and accessory proteins. These include interaction of CD28 with its ligands CD80 and CD86 expressed on the surface of APCs (Jenkins et al., 1991; Shahinian et al., 1993) and CD40L expressed on activated $CD4^+$ T cells with CD40 expressing APCs (Grewal et al., 1995; van Essen et al., 1995).

1.4.2.2 Immunological Synapse

Following engagement of the TCR with cognate peptide/MHC complexes and costimulatory molecules, the surface membrane proteins rapidly redistribute to

form an organised "immunological synapse" (IS) at the interface between the T cell and APC. The IS organizes and segregates TCR signalling components and adhesion molecules into two spatially segregated compartments (Monks et al., 1998). These areas, referred to as supramolecular activation clusters (SMACs), include the central (cSMAC), in which the TCR, associated signaling proteins and APC plasma membranes are in close proximity, and the peripheral (pSMAC), which accumulates adhesion proteins and integrins including lymphocyte function associated–1 (LFA-1) and talin which stabilize the interaction between the T cell and the APC (Monks et al., 1998). Proteins required in the most immediate stages of immune synapse formation are already present at the cell surface and are enriched in specialised membrane domains known as lipid rafts or glycolipid enriched microdomains (GEM). These include Lck (Kabouridis et al., 1997), LAT (Zhang et al., 1998b), CD4 (Fragoso et al., 2003), CD8 (Arcaro et al., 2001), and the TCR (Drevot et al., 2002) on T-cells, and MHC class II proteins on APCs (Anderson et al., 2000). The actin cytoskeleton recruits these lipid rafts and associated signaling molecules (Viola et al., 1999)(Xavier et al., 1998) by facilitating their movement laterally in the membrane to the T cell:APC interface (reviewed in (Taner et al., 2004)).

There is much debate as to the purpose of the IS. Initially, it was proposed that this molecular reorganization enhanced TCR signalling and was important for T cell activation (Grakoui et al., 1999; Kupfer and Singer, 1989; Wulfing and Davis, 1998). More recently, however, (Lee et al., 2002) have shown that recognition of peptide-MHC on APCs by naïve $CD4^+$ T cells triggers TCR

signalling well before the mature IS is formed, arguing that the redistribution of signalling molecules and cytoskeletal components may occur to enhance and sustain signalling by receptors that direct T cell effector functions such as cytokine receptors (reviewed in (Davis and van der Merwe, 2001)). While the precise function of the IS may still be unclear, it is clear that the signalling proteins present within the lipid rafts that cluster at the IS play essential roles in naïve T cell activation and the generation of antigen specific immune responses.

1.4.2.3 TCR Signalling Cascade

As with TCR signals that influence thymic selection, signals resulting from engagement of the TCR with antigenic peptide is dependent on the activation of TCR proximal signalling proteins and adaptors. The earliest event in T cell activation is the activation of Lck and Fyn, through the dephosphorylation of a negative regulatory C terminal tyrosine residue by the phosphatase CD45 (McNeill et al., 2007; Pingel et al., 1999; Seavitt et al., 1999). The clustering of the TCR and coreceptors brings Lck, which is constitutively associated with the cytoplasmic domains of the CD4 and CD8 coreceptors (Barber et al., 1989; Veillette et al., 1988) in close proximity to the CD3 complex. Here it phosphorylates tyrosine residues within the ITAM motifs (Straus and Weiss, 1992; van Oers et al., 1996a). The phosphorylated ITAMs of the CD3ζ homodimer then act as docking sites for the SH2 domains of Zap70 (Isakov et al., 1995; Wange et al., 1993), whose function is to amplify and propagate the TCR signal intracellularly. Following recruitment of Zap70 to the CD3ζ chains, it is activated by the phosphorylation of positive regulatory tyrosine residues

either by Lck, Fyn (Chan et al., 1995; Fusaki et al., 1996; Mege et al., 1996) and/or autophosphorylation by Zap70 itself (Brdicka et al., 2005; Neumeister et al., 1995). Following activation, Zap70 phosphorylates the adaptor proteins LAT (Zhang et al., 1998a) and SLP-76 (Wardenburg et al., 1996) which lack enzymatic activity and direct effector function but act as molecular scaffolds which recruit other proteins that are essential for T cell activation. LAT contains two cysteine residues within its cytoplasmic tail, which once palmitoylated mediates its translocation into lipid rafts (Zhang et al., 1998b). LAT contains nine tyrosine residues which following phosphorylation by Zap70, act as docking sites for downstream SH2-domain containing effector proteins including growth factor receptor-bound protein 2 (Grb2), Grb2-related adaptor downstream of Shc (Gads), phospholipase Cγ1 (PLCγ1), the p85 subunit of PI3K (Zhang et al., 1998a; Zhang et al., 2000), and the guanine nucleotide exchange factor Vav1 (Reynolds et al., 2004). Similarly SLP-76 by virtue of proline rich motifs, SH2 domains and phosphorylated tyrosines can associate with the proteins, non-catalytic region of tyrosine kinase (Nck) (Wunderlich et al., 1999), Tec-family kinase interleukin-2 inducible T cell kinase (ITK) (Su et al., 1999), adhesion and degranulation-promoting adaptor protein (ADAP) (da Silva et al., 1997; Musci et al., 1997) and haematopoietic progenitor kinase 1 (HPK1) (Sauer et al., 2001). Thus LAT and SLP-76 couple TCR stimulation to multiple signalling pathways.

One important protein activated by LAT and SLP-76 is PLCγ1. SLP-76 associates with LAT via GADS and directly binds to the SH3 domain of PLCγ1

(Yablonski et al., 2001). Given that LAT alone is insufficient for PLCγ1 activation, it has been suggested that SLP-76 may aid the recruitment of PLCγ1 to LAT allowing it to be fully activated. Activation of PLCγ1 results in the production of second messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) by cleaving phosphatidylinositol 4,5 bisphosphate (PIP_2) at the plasma membrane. These second messengers act as important mediators of T cell activation.

DAG activates protein kinase C (PKC) and the PKC isoform PKCθ that is essential for T cell activation by activating the transcription factors NFκB and AP1 (heterodimer of c-fos and Jun) (Baier-Bitterlich et al., 1996; Sun et al., 2000a), which are responsible for the transcription of genes involved in T cell differentiation and proliferation. More recently DAG has been shown to also activate the nucleotide exchange factor Ras guanyl-releasing protein (RasGRP). RasGRP promotes the activation of the GTPase Ras by promoting the transition from the inactive GDP-bound Ras to the active GTP-bound conformation. Ras-GTP is responsible for the eventual activation of the MAPKs ERK 1 and 2, by activating serine/threonine kinase Raf. Raf activation leads to the phosphorylation of its targets mitogen-activated protein kinase kinase (MEK) 1 and 2 (Morrison and Cutler, 1997), which phosphorylate and activate ERK1 and ERK2. Activated ERK1 and ERK2 move to the nucleus, where they phosphorylate the Ets family of transcription factors, including Elk1, leading to the heterodimerisation of c-fos and Jun family members to form the transcriptional regulator AP1 which enhances the transcription of the IL-2 gene,

the protein product of which drives T-cell proliferation (reviewed in (Iwashima, 2003; Mor and Philips, 2006)). Alternatively there is a second mechanism for Ras/ERK activation, which is mediated by the binding of Grb2 to LAT to recruit the alternative guanine nucleotide exchange factor son of sevenless (SOS) for the activation of Ras (Buday et al., 1994; Zhang et al., 2000).

The second messenger IP_3 also generated by the hydrolysis of PIP_2 also plays an essential role in T cell activation. Following its generation, IP_3 binds to receptors in the ER membrane, triggering the release of calcium stores into the cytoplasm from the ER (Guse et al., 1993; Imboden and Stobo, 1985). The increase in intracellular calcium triggers the activation of the calcium/calmodulin-dependent phosphatase calcineurin, which dephosphorylates the nuclear factor of activated T cells (NFAT) family of transcription factors. Following dephosphorylation NFAT translocates to the nucleus where it is essential for the induction of cytokine and costimulator gene expression, including IL-2, IL-4, TNFα and CD40L (Crabtree and Olson, 2002; Peng et al., 2001).

In addition to inducing the transcription of cytokines and costimulatory molecules important for the proliferation and differentiation of the activated T cells, TCR signals also regulate actin cytoskeletal reorgansation and integrin function important for conjugate formation. Phosphorylated SLP-76 has been reported to interact with ADAP, which is important for the activation of the intergrins LFA-1 and VLA-4, although the precise manner in which this occurs is unknown. SLP-76 also influences F-actin polymerization. The current model

for TCR mediated cytoskeletal rearrangement proposes that phosphorylated SLP-76 functions as a scaffold, bringing Nck and its binding partners p21-activated kinase 1 (PAK1) and Wiskott-Aldrich syndrome protein (WASP) into close proximity with Vav-1 and Cdc42-GTP. This permits the Vav1 dependent activation of Cdc42, which in turn activates its effector WASP that works to stimulate actin polymerization (Sasahara et al., 2002) (reviewed in (Wu and Koretzky, 2004)).

1.4.3 Effector T cell subsets

1.4.3.1 $CD4^+$ T cells

Following T cell activation, resulting from the ligation of the TCR with cognate antigen, $CD4^+$ T cells differentiate into different functional subsets. Several subsets have been distinguished on the basis of their cytokine profile (reviewed in (O'Garra, 1998)). The most well characterised subsets are the T_H1, T_H2 and the recently characterised T_H17 cells.

The T_H1 cells are responsible for the eradication of intracellular pathogens that primarily infect macrophages (intracellular bacteria, viruses and some protozoa). This is mediated by the secretion of the signature cytokines IFN γ and lymphotoxin, which activate the antimicrobial activity of the macrophage instructing the cell to kill the ingested pathogen. IL-2 and TNFα also produced by the T_H1 subset aid the clearance of the infection by inducing effector T cell proliferation as well recruiting macrophages from the blood stream to the site of infection. Commitment to the T_H1 lineage requires the cytokine IL-12 and the

transcription factors, STAT4, STAT1, and the T-cell-specific T-box transcription factor T-bet, the master regulator of T_H1 differentiation (Mullen et al., 2001; Szabo et al., 2000). The activity of these transcription factors and cytokines lead to the upregulation of factors permitting differentiation to the T_H1 lineage and suppression of T_H2 associated factors.

T_H2 cells secrete the signature cytokine IL-4, but also secrete IL-5, IL-6, IL-10 and IL-13, which drive the proliferation and differentiation of B cells into antibody secreting plasma cells, which in turn can differentiate into long lived memory B cells. Commitment to the T_H2 lineage is requires the cytokine IL-4 and the transcription factors STAT6 and T_H2 lineage specific transcription factor Gata3 which promotes the transcription of the T_H2 cytokine cluster (*Il-4, Il-5*, and *Il-13* genes) while suppressing factors associated with differentiation to the T_H1 lineage (Ouyang et al., 2000; Ouyang et al., 1998; Zheng and Flavell, 1997).

T_H17 cells are a recently described subset of $CD4^+$ T cells that are distinct from the T_H1 and T_H2 subsets (reviewed in (Stockinger and Veldhoen, 2007)). T_H17 cells produce IL-17A, IL-17F, IL-6 and TNFα. In stromal, epithelial, endothelial cells aswell as peripheral blood monocytes IL-17 secreted by T_H17 cells stimulates the production of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNFα, which are thought to mobilise neutrophils to the site of infection (Kolls and Linden, 2004). This IL-17 response is thought to contribute to the local tissue inflammation associated with various autoimmune diseases

including experimental autoimmune encephalomyelitis (EAE) (Uyttenhove and Van Snick, 2006), Myocarditis (Sonderegger et al., 2006) and arthritis (Lubberts et al., 2004; Rohn et al., 2006). However, T_H17 cells also appear to play a protective role in immunity. IL-17 responses are essential for the clearance of the bacterial pathogen *Klebsiella pneumoniae* (Ye et al., 2001) since in the absence of IL-17 mice rapidly develop and die from the lethal infection (Happel et al., 2005; Happel et al., 2003). A similar role has been suggested for IL-17 in the host response against gram-negative bacteria *Bacteroides fragilis* (Chung et al., 2003) and the fungi *Candida albicans* (Huang et al., 2004). Recently the pro-inflammatory cytokine IL-23 has been implicated in T_H17 effector function. Studies performed in wildtype and *IL-23*$^{-/-}$ mice reveal that infection with intestinal bacterial pathogens induce the generation of T_H17 cells in both wildtype and IL-23-deficient hosts, but that only wildtype mice can clear the infection, suggesting an important role for IL-23 in the maintenance of effector function in T_H17 cells (Mangan et al., 2006). While IL-23 may be important in the modulation of T_H17 activity, it does not appear to be required for their development. The differentiation of T_H17 cells from the naïve compartment appears to be dependent the cytokines transforming growth factor β (TGF-β) and IL-6 (Veldhoen et al., 2006) and does not appear to require the activity of transcription factors associated with T_H1 or T_H2 polarization, (Harrington et al., 2005; Park et al., 2005), Instead RORγt has been identified as the transcription factor that conveys commitment to the T_H17 lineage (Ivanov et al., 2006).

More recently a role for $CD4^+$ T cells in the development of CTL effector function (Franco et al., 2000), expansion (Wang and Livingstone, 2003) and memory formation (Bourgeois et al., 2002; Shedlock and Shen, 2003; Sun and Bevan, 2003) has been reported, although it is unclear whether a distinct $CD4^+$ effector subset mediates such activity.

1.4.3.2 Regulatory CD4 T cells

Regulatory $CD4^+$ T cells (T_{Reg}) are a subset of $CD4^+$ T cells that are important for immune regulation, such that they actively suppress activation of other immune cells to self or non-self antigens preventing pathological self-reactivity (Sakaguchi et al., 1995). Many types of T_{Reg} cells have been identified however the most well characterised subset is the naturally arising $CD4^+$ $CD25^+$ T_{Reg} which selectively express the Foxp3 transcription factor (Fontenot et al., 2003; Hori and Sakaguchi, 2004) shown to be important for T_{Reg} development. The essential role T_{Reg} cells play in immune regulation was first demonstrated by (Sakaguchi et al., 1995) who showed that transfer of $CD4^+$ $CD25^-$ cells depleted of the T_{Reg} subset into immuno-competent hosts led to development of various autoimmune pathologies. Similarly the autoimmune syndrome that develops in humans with the lethal immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome characterised by severe autoimmunity is associated with a mutation in Foxp3 gene resulting in the complete absence of T_{Reg} cells in these patients (Wildin et al., 2001). The mechanism by which T_{Reg} cells mediate their suppressive function is still unclear however it has been shown that the suppressive mechanism requires cell-to-cell contact with the target cell and that T_{Reg} cells may specifically inhibit the production of IL-2, thus suppressing the

proliferation of the target T cell (Thornton et al., 2004; Thornton and Shevach, 1998).

1.4.3.3 $CD8^+$ T cells

Activation of naïve $CD8^+$ T cells by cognate antigen and costimulatory molecules leads to their rapid cell cycle progression producing a large number of clonally expanded activated T cells, that will differentiate in effector CTL. When exposed to infected or tumorigenic cells CTL release cytotoxins stored within specialized lytic granules (reviewed in (Russell and Ley, 2002)). These include granulysin, which has antimicrobial activity and induces the apoptosis of target cells, and perforin which polymerises in the plasma membrane of the target cell producing pores (Kagi et al., 1994; Kojima et al., 1994; Smyth et al., 2000). CTL then release lytic enzymes known as granzymes, a set of serine proteases, that can enter target cells via the perforin-formed pore and induce apoptosis by activation of nucleases with cleave DNA through both caspase dependent and independent pathways (Trapani and Smyth, 1993). CTL also produce the antiviral cytokine IFNγ, which aids the clearance of the infection by inducing the increased expression of MHC class I proteins on infected cells, thus increasing the chance that the infected cell will be recognized by a CTL.

1.5 T cell Memory

Following successful clearance of a pathogen by the immune response, many of the effector cells of the adaptive system die. Crucially, however, some survive long term. These cells form the basis of immunological memory, and

have the capacity to protect the host from repeat exposure to specific pathogen, responding with increased speed and efficiency. The accelerated recall responses of memory T cells to re-infection is attributed in part to the increased precursor frequency of antigen specific T cells in immune mice compared with naïve animals (Bousso et al., 1999; Hou et al., 1994; Kaech et al., 2002b). The finding that memory T cells have more lipid rafts with higher proportion of phosphorylated proteins prior to TCR stimulation compared with naïve and effector $CD8^+$ T cells, and appear to more efficiently induce the phosphorylation of LAT, ERK, p38 MAP kinase and JNK suggests that memory $CD8^+$ T cells are better equipped to propagate TCR signals, which may explain the rapid expansion and production of effector cytokines upon rechallenge with antigen (Kersh et al., 2003). Memory cells have also been shown to over express genes that regulate the G1-S transition of the cell cycle compared to naïve T cells (Latner et al., 2004; Veiga-Fernandes and Rocha, 2004). This suggests these cells are pre-charged with factors necessary for cell cycle progression thus allowing the rapid proliferation of memory T cells following antigenic stimulation (Latner et al., 2004; Veiga-Fernandes and Rocha, 2004).

1.5.1 Memory T cell Generation

The mechanisms of memory T cell generation still remain unclear. While some studies report that memory cells are generated along a linear pathway directly descending from differentiated effector cells (Garcia et al., 1999; Kaech et al., 2002a; Opferman et al., 1999) other reports suggest memory T cells might be generated without passing through an intermediate effector stage of

differentiation (Lauvau et al., 2001; Manjunath et al., 2001). More recently the "intersecting model" proposed by (Moulton and Farber, 2006) suggests that memory T cells can be generated from activated precursors at various stages of differentiation and that memory generation occurs via pre-memory intermediates following antigen withdrawal. Memory T cell development is further complicated by the finding that the memory compartment is functionally heterogenous, containing different memory subsets. Early, studies in humans have identified two subsets of memory $CD4^+$ and $CD8^+$ T cells. This is proposed on the basis of cell surface expression of the activation marker CD44, the cell adhesion molecule L-selectin and the chemokine receptor CCR7 (Sallusto et al., 1999). These subsets of memory T cells have been described as 'central memory' (T_{CM}) which are $CCR7^+$ $CD62L^+$ and reside in the blood, spleen and non-lymphoid tissue and 'effector memory' (T_{EM}) which are $CCR7^-$ $CD62L^-$ and reside in the LN, spleen, blood but not non-lymphoid tissue (Sallusto et al., 1999). More recently these subsets have been identified within murine $CD4^+$ memory (Reinhardt et al., 2001) and $CD8^+$ memory T cell compartments (Masopust et al., 2001). Although it is appreciated that these two memory subsets exist, the factors influencing their generation and maintenance are largely unknown.

Memory cells can also be generated in the absence of foreign antigen. In response to T cell lymphopenia naïve T cells can undergo homeostatic proliferation and differentiate into memory cells in response to self antigens and IL-7 signals (Ernst et al., 1999; Goldrath and Bevan, 1999; Kieper and Jameson, 1999; Viret et al., 1999). These memory cells are referred to as

memory-like or memory phenotype cells. While these cells have not been generated by the contact with foreign antigen they do possess characteristics of conventional memory T cells. These include the upregulation of memory cell markers Ly6C and CD44, the ability to produce IFNγ and for $CD8^+$ T cells the acquisition of cytolytic effector function (Cho et al., 2000; Goldrath et al., 2000; Hamilton et al., 2006). Thus it is considered that these memory-like T cells represent the long-lived progeny of naive T cells responding to environmental or self antigens. Interestingly the memory-like $CD8^+$ T cells can be further subdivided on the basis of IL-2Rβ chain (CD122) expression. ~70% of $CD8^+$ memory-like T cells express high levels of CD122. In contrast the remaining 30% of memory-like T cells express low levels of CD122. These cells differ from $CD122^{hi}$ subset, in that they exhibit a more rapid turnover and display signs of an activated phenotype ($CD69^{hi}$ $CD62L^{lo}$ IL-$7R\alpha^{lo}$) (Boyman et al., 2006).

1.5.2 Memory T cell Homeostasis

The homeostatic requirements of memory T cells differ on the basis of the subset and the manner in which the cells are generated. There is a large body of evidence reporting that antigen specific memory $CD8^+$ T cells (Becker et al., 2002b; Goldrath et al., 2002; Schluns et al., 2002) and memory-like $CD8^+$ $CD122^{hi}$ T cells (Burkett et al., 2003; Kennedy et al., 2000b; Lodolce et al., 1998; Tan et al., 2002; Zhang et al., 1998c) are dependent on IL-15 for their proliferation and/or maintenance. Memory-like $CD122^{hi}$ CD8 T cells are absent from *Il-15*$^{-/-}$ or *Il-15R*$^{-/-}$ mice (Kennedy et al., 2000b; Lodolce et al., 1998), and

fail to homeostatically proliferate or survive following adoptive transfer into *Il-15*$^{-/-}$ hosts (Judge et al., 2002). Similarly it has been reported that antigen specific CD8$^+$ T generated in *Il-15*$^{-/-}$ mice infected with lymphocytic choriomeningitis virus (LCMV) (Becker et al., 2002b) or vesicular stomatitis virus (VSV) (Schluns et al., 2002) fail to survive long-term as a result of abrogated homeostatic proliferation and survival.

IL-7 has also been shown to support the survival of CD122^{hi} memory-like CD8$^+$ T cells (Kondrack et al., 2003). Similarly antigen specific CD8$^+$ T cells also require IL-7 for their survival and but not their proliferation (Schluns et al., 2000). In contrast the CD122^{lo} population of memory-like CD8$^+$ T cells do not need γ_c cytokines for their survival or proliferation since they are present in normal numbers in $\gamma_c^{-/-}$ mice (Boyman et al., 2006). In contrast to naïve CD8$^+$ T cells, antigen-specific memory CD8$^+$ T cells and the CD122^{hi} subset of memory-like CD8$^+$ T cells do not require homeostatic TCR signals for their maintenance. Interestingly however the CD122^{lo} subset of memory-like CD8$^+$ T cells do require MHC expression for their survival given that they fail to survive following adoptive transfer to MHC class I deficient hosts (Boyman et al., 2006).

The memory CD4$^+$ T cell population appears to be more heterogenous than the CD8$^+$ pool and thus the homeostatic signals that regulate their survival and homeostatic proliferation are less well characterised than the CD8$^+$ subsets. The requirement for IL-15 in antigen-specific and memory-like CD4$^+$ T cell homeostasis is still unclear. It has been suggested that memory-like CD4$^+$ T cells do not require for IL-15 in their survival or proliferation since they are

persist in *Il-15*$^{-/-}$ mice (Judge et al., 2002). However, IL-15 has been shown to influence the basal proliferation and survival of antigen-specific murine CD4$^+$ T cells (Lenz et al., 2004). The disparity between the cytokine requirements for these CD4$^+$ memory subsets is unknown, but maybe a consequence of the mechanism by which these different subsets are generated. Similarly, the sensitivity of these memory CD4$^+$ subsets to homeostatic IL-7 signals also differ. While memory-like CD4$^+$ T cells, can utilise IL-7 for their survival and proliferation in the absence of TCR signals (Seddon et al., 2003), antigen-specific memory CD4$^+$ T cells require IL-7 signals even in the presence of intact TCR signalling (Kondrack et al., 2003). The loss of signals resulting from reduced TCR/MHC contact also affects the homeostasis of the CD4$^+$ memory subsets. Ablation of TCR signals, has been reported to reduce the basal proliferation of memory-like CD4$^+$ T cells *in vivo* (Polic et al., 2001) while antigen-specific CD4$^+$ memory cells in the absence of MHC class II show impaired expansion and effector function *in vivo* (Kassiotis et al., 2002).

1.6 Thesis Aims

Engagement of the TCR by spMHC ligands is essential for the long-term survival of the peripheral naïve CD4$^+$ and CD8$^+$ T cell compartment. These homeostatic TCR signals have been reported to be transmitted intracellularly by TCR proximal kinases Lck and Fyn. However, these kinases can interact with other receptors, therefore it is unclear whether their survival activities are entirely TCR restricted. The main objective of this thesis was to investigate the

role of Zap70 in naïve T cell survival and homeostasis, given that its signal transduction activity is dependent on its recruitment and activation at the TCR/CD3 complex. Zap70 is essential for the transduction of positive selection signals, thus *Zap70*$^{-/-}$ mice are completely arrested at the DP stage of thymopoiesis and therefore cannot be used to address the role of Zap70 in naïve T cell homeostasis. The aim of this thesis was to generate mice bearing a tetracycline-inducible Zap70 transgene that could be switched on during the crucial thymic selection steps allowing the transmission of positive selection signals and thus reconstitution of the peripheral T cell compartment. The requirement for Zap70 in naïve T cell survival could then be addressed by switching off Tre-Zap70 transgene expression through the cessation of tetracycline treatment.

The second focus of my thesis was to better understand how homeostatic TCR signals and IL-7 cytokine signals synergise to support T cell survival and LIP. While there is evidence that both signals are required for naïve T cell survival and LIP, the manner in which these signals overlap and the precise quantitative role of IL-7R signalling and homeostatic TCR signals in LIP and survival is unknown. The extent to which these signals are required is not clearly understood largely as a result of difficulties in the dissection of defects in survival, homeostatic proliferation and expansion under lymphopenic conditions. Since survival, proliferation and homeostatic expansion can occur at the same time, our aim was to quanititate the contributions of IL-7R and TCR driven signals on naïve T cell survival, LIP and expansion in the same physiological assay and to measure the impact of specific defects within the

TCR or IL-7R pathways have on the survival, proliferation and expansion of the naive T cell pool.

Figure 1.1: Schematic of T cell development.

Haematopoietic stem cells migrate to the thymus where they undergo a series of differentiation steps, leading to the rearrangement of the TCR β, γ and δ loci during the early DN stages of thymocyte development. Following the successful rearrangement of the TCRβ chain, signals through the pre-TCR facilitate further maturation to the DP stage of development. Following successful rearrangement of the TCRα loci, the TCRαβ complex is expressed on the surface of the thymocytes and the cells upregulate the CD4 and CD8 coreceptors to become DP thymocytes. Following positive selection the DP thymocytes downregulate either CD4 or CD8 to become CD4 SP or CD8 SP thymocytes.

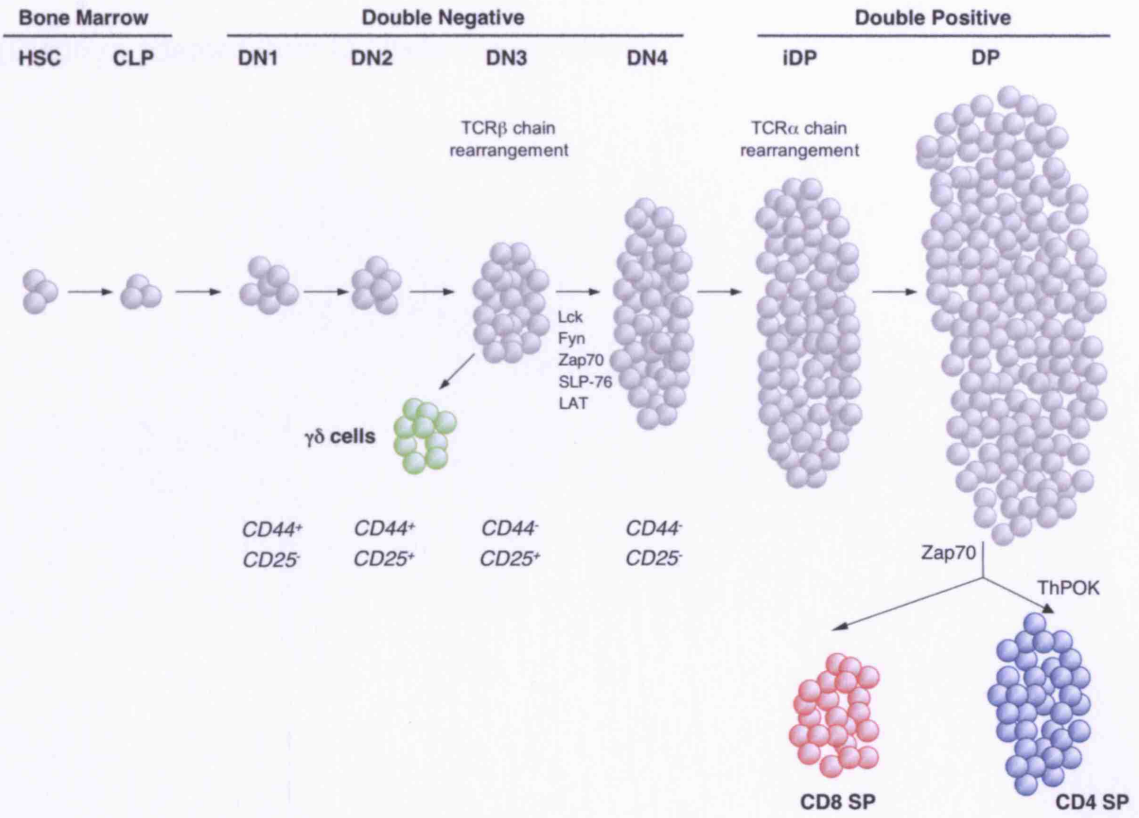

Bone Marrow
Double Negative
Double Positive
HSC
CLP
DN1
DN2
DN3
DN4
iDP
DP
TCRβ chain rearrangement
TCRα chain rearrangement
Lck
Fyn
Zap70
SLP-76
LAT
γδ cells
CD44+ CD25-
CD44+ CD25+
CD44- CD25+
CD44- CD25-
Zap70
ThPOK
CD8 SP
CD4 SP

Figure 1.2: Structure of murine Zap70.

Zap70 is composed of two tandem SH2 domains, followed by a central linker region called interdomain B. This linker connects the SH2 domains with the carboxyl terminal kinase domain. Figure shows the amino acid sequence of interdomain B, and the key tyrosine residues involved in Zap70 signalling. (Diagram adapted from Di Bartolo *et al*, 1999).

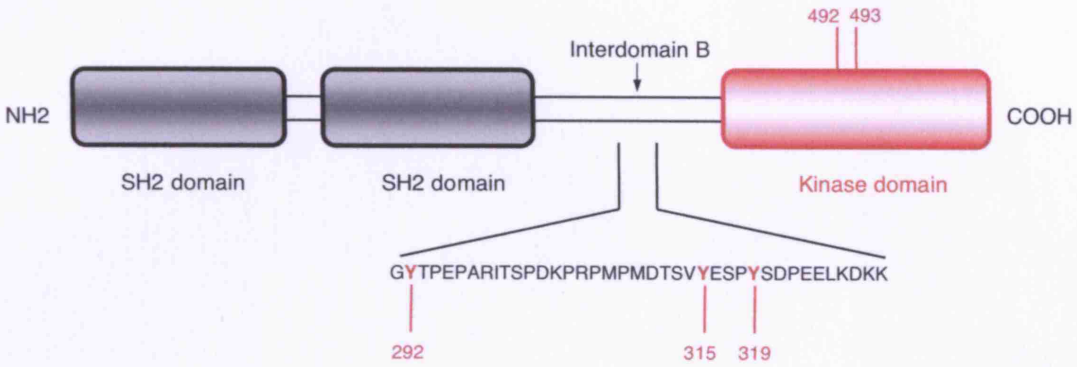

492 493
Interdomain B
NH2
COOH
SH2 domain
SH2 domain
Kinase domain
GYTPEPARITSPDKPRPMPMDTSVYESPYSDPEELKDKK
292
315 319

Figure 1.3: IL-7R T cell Survival Signalling.

A schematic representation of the pro and anti apoptotic proteins and signalling pathways which are activated upon engagement of the IL-7R with IL-7.

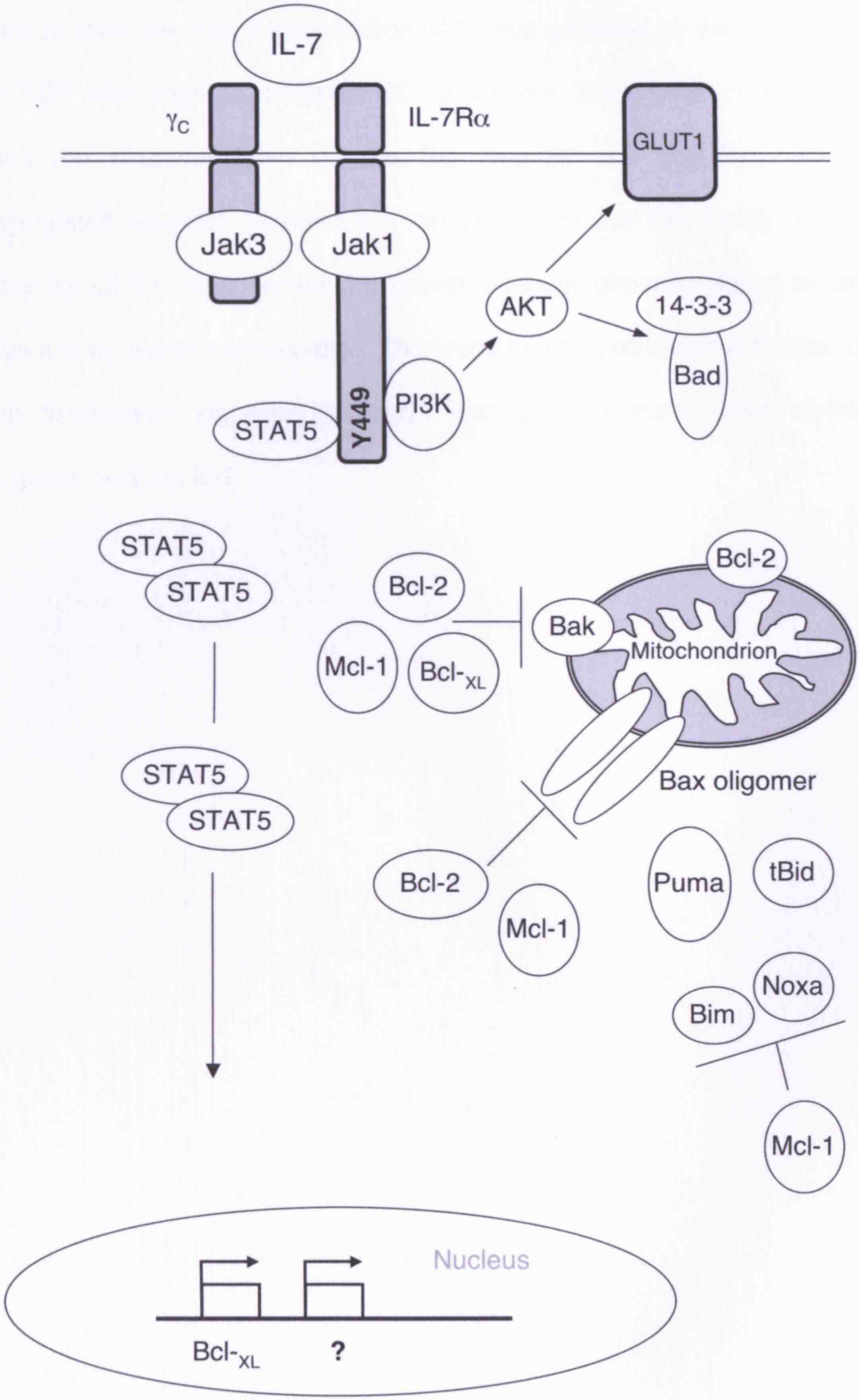
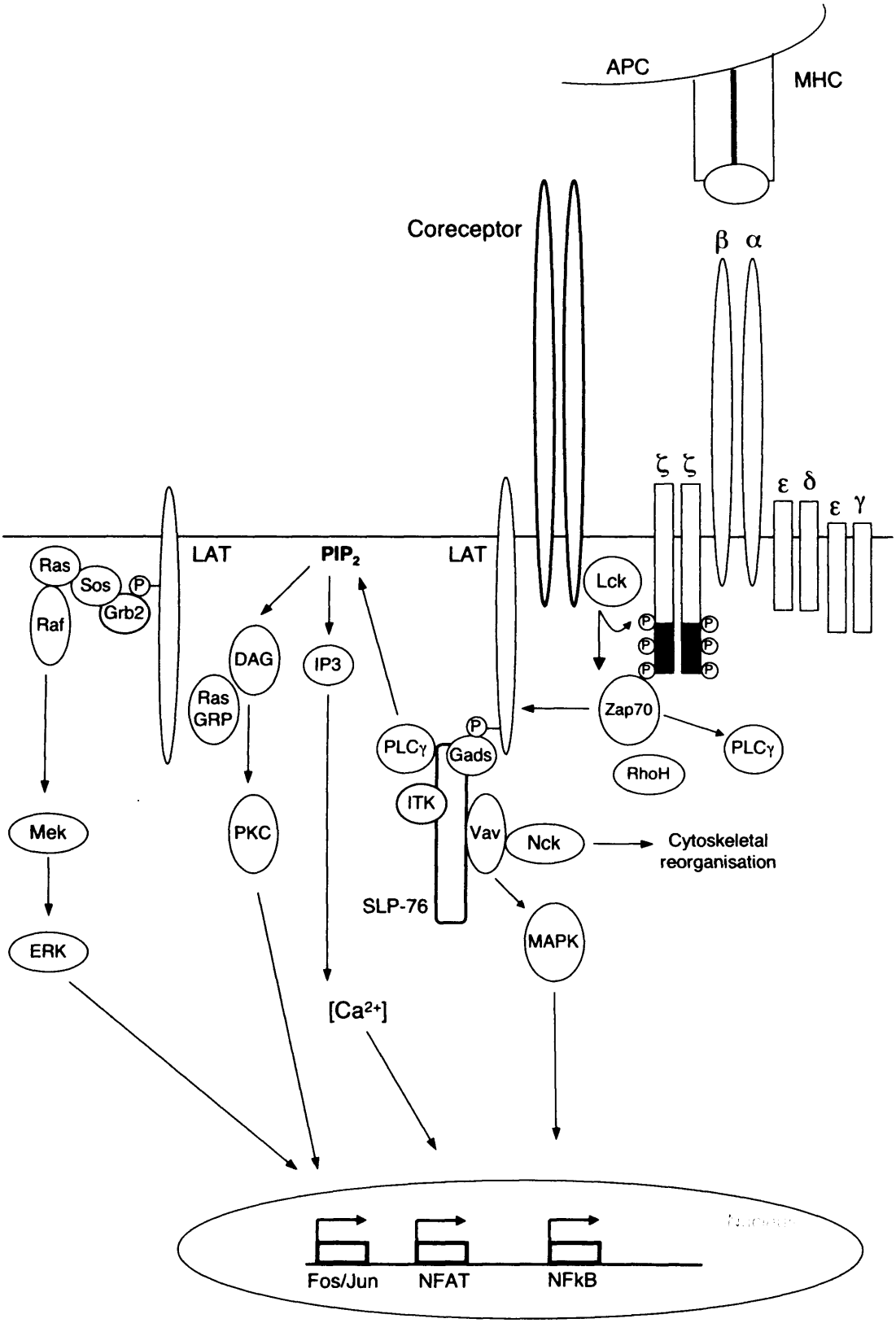

IL-7
γc
IL-7Rα
GLUT1
Jak3
Jak1
AKT
14-3-3
Bad
Y449
PI3K
STAT5
STAT5
STAT5
STAT5
STAT5
Bcl-2
Mcl-1
Bcl-XL
Bak
Bcl-2
Mitochondrion
Bax oligomer
Bcl-2
Mcl-1
Puma
tBid
Noxa
Bim
Mcl-1
Nucleus
Bcl-XL
?

Figure 1.4 Peripheral TCR Signalling.

Diagram to show the signal transduction pathways activated by the interaction of the TCR with cognate antigen/MHC complexes. The ITAMs of the CD3 complex are phosphorylated by the Src kinases Lck and Fyn. These phosphorylated residues facilitate the recruitment of the Syk family kinase Zap70 to the CD3 complex where it is activated though phosphorylation by Lck and Fyn and by autophosphorylation. This leads to the subsequent activation of several downstream signalling pathways leading to the transcription of key target genes such as IL-2.


APC
MHC
Coreceptor
β α
ζ ζ
ε δ
ε γ
LAT
PIP2
LAT
Ras
Sos
Grb2
Raf
DAG
IP3
Ras GRP
Lck
Zap70
PLCγ
Gads
RhoH
ITK
Vav
Nck
Cytoskeletal reorganisation
SLP-76
MAPK
Mek
PKC
ERK
[Ca2+]
Fos/Jun
NFAT
NFkB

Chapter 2

Materials and Methods

2.1 Buffers

Annealing Buffer

100mM potassium acetate, 30mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4 and 2 mM Mg-acetate.

T cell Handling Media

Air buffered Iscove's modified dulbecco's medium (AB-IMDM) (made in house) containing 0.1% (v/v) bovine serum albumin (BSA).

FACS Buffer

Phosphate buffered saline (PBS) (made in house) containing 0.5% (v/v) Sodium Azide and 0.5% (v/v) BSA.

Immunisation Media

Dulbecco's PBS (Invitrogen, UK).

ACK Lysis Buffer

dH_20 containing 150mM NH_4Cl, 10mM $KHCO_3$ and 0.1mM Na_2EDTA.

10 X Lymphocyte Permeabilisation Buffer for Intracellular Staining

1% NP40 (Sigma,UK), 50mM Tris, pH 7.6 and 150mM NaCl.

Human Embryonic Kidney Cell Media (HEK-M)

90% (v/v) Eagle Minimal Essential Medium (alpha modification) (Sigma. UK), 10% (v/v) Tet System Approved Fetal Bovine Serum (FBS) (Clontech BD Biosciences, UK), 4 mM L-glutamine, 100 μg/ml G418 (Sigma. UK), 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulphate and 100 μg/ml hygromycin B (Gibco BRL, UK).

Human Collagen Type 1 Coating Solution

Human Placental Collagen Type 1 (Clontech BD Biosciences, UK) used at 0.1μg/cm^2 in 0.1% (v/v) Acetic Acid.

Tail Vein Injection Media

PBS (Gibco BRL, UK).

2.2 Antibodies used in Flow cytometry

Marker	Clone	Conjugate	Supplier
CD4	RM 4-5	PE/PerCP/APC/APC-Cy7/Biotin	BD
CD4	RM 4-5	FITC	eBioscience
CD8α	53-6.7	PerCP/PE	BD
CD8α	53-6.7	FiTC/APC	eBioscience
CD8α	53-6.7	PE-Texas Red	Caltag laboratories
CD5	53-7.3	FITC/Biotin	eBioscience
CD5	53-7.3	APC	BD
IL-7Rα/CD127	A7R34	PE	eBioscience
TCRβ	H57-597	PE/APC	eBioscience
TCRβ	H57-597	FITC/Biotin	BD

CD25	PC61	PE	BD
CD44/Pgp-1	IM7	FITC/APC	eBioscience
CD44/Pgp-1	IM7	Biotin	BD
CD69	H1.2F3	PE	eBioscience
CD69	H1.2F3	FITC	BD
CD43	1B11	PE	BD
CD122	5H4	PE	eBioscience
CD45.1	A20	PE/APC	eBioscience
Human CD2/LFA-2	RPA-2.10	PE	BD
I-A^b/Ab^b	25-9-17	Biotin	BD

Table 2.1 Cell surface antibodies used for FACS analysis.

Marker	Conjugate	Supplier
Streptavidin	PE/PerCP	BD
Streptavidin	PE-Texas Red	Caltag

Table 2.2 Secondary reagents used for FACS analysis.

Marker	Clone	Conjugate	Supplier
Bcl-2 and isotype control	3F11 A19-3	PE	BD
Foxp3	FJK-16s	APC	ebioscience

Table 2.3 Intracellular antibodies used for FACS analysis.

2.3 Mice

Mice were used in this study as follows:

Strain	References
C57BL/6J	-
C57BL/6J CD45.1	-
C57BL/10N *Rag1*$^{-/-}$	(Mombaerts et al., 1992)
Il-7$^{-/-}$ *Rag1*$^{-/-}$	(von Freeden-Jeffry et al., 1995)
Il-7$^{+/-}$ *Rag1*$^{-/-}$	-
F5$^{+/+}$ β2m$^{-/-}$ *Rag1*$^{-/-}$	(Smyth et al., 1998)
F5$^{+/+}$ *Rag1*$^{-/-}$	(Mamalaki et al., 1993)
(F5$^{+/+}$ *Rag1*$^{-/-}$ x C57Bl6/J CD45.1) F1	-
F5$^{+/-}$ Tre-IL-7R rtTA.c^{huCD2} *Rag1*$^{+/-}$	(Buentke et al., 2006)
Il-15ra$^{-/-}$ *Rag1*$^{-/-}$	(Lodolce et al., 1998)

Table 2.4 Mice used in study

The above mice were bred and housed at the NIMR animal facility under SPF conditions, in accordance with Home Office regulations. Genotyping of the mice was performed by a combination of flow cytometric analysis on peripheral blood lymphocytes and PCR using mouse tail DNA. The F5$^{+/-}$ Tre-IL-7R rtTA.c^{huCD2} *Rag1*$^{+/-}$ mice express a doxycycline (dox) inducible IL-7Rα transgene and therefore this strain was maintained on dox feed (1mg/g). All the other mouse stains listed above were maintained on normal feed.

2.4 Cell Lines

Human transformed primary embryonal kidney derived cell line (HEK 293 Tet-On™) that stably expresses the reverse tetracycline controlled transactivator (Clontech BD Biosciences, UK).

2.5 Cell Culture

HEK 293 Tet-On™ were cultured on Human Collagen Type 1 (0.1μg/cm^2) coated 175cm^2 plastic flasks in 15-30ml HEK-M containing all supplied growth supplements and incubated in a 95% humidified chamber with 5% CO_2 at 37°C. Media was replaced every 48-72 h. At approximately 80% confluency cells were passaged using 10% (v/v) trypsin solution. Briefly, cells were washed in serum free HEK-M, incubated with 3ml 10% (v/v) trypsin, neutralised with an equal volume of HEK-M, centrifuged (1200 rpm, 5 min) and resuspended in fresh HEK-M and re-plated.

2.6 Generation of the Tre-Zap70 and Tre-huβ-globin-Zap70 Mice:

2.6.1 Initial Subcloning Steps

To control the expression of Zap-70 in our model, we chose to use an inducible method of gene expression using the tetracycline responsive gene induction system (Furth et al., 1994; Gossen and Bujard, 1992; Gossen et al., 1995). The original tetracycline inducible plasmid only contained the cloning sites

EcoRI and PstI, therefore cDNA encoding a polylinker MCS was designed. The reverse and forward oligonucleotides encoded the restriction sites Pst-I, Cla-I, EcoRI, Nsi-I, SnaBI and an EcoRI-like site in which the GAATT**C** was replaced with an GAATT**G** **(Fig 2.1)**. The oligonucleotides were annealed in annealing buffer and incubated at 95°C for 5 min. Following incubation the mixture was cooled at room temperature (RT) for 1 h. The cDNA encoding the polylinker MCS was then subcloned into a plasmid (downstream of the heptameric Tet operon containing tetracycline responsive elements linked to a minimal CMV promoter (Gossen and Bujard, 1992) and upstream of a 3' sequence containing an intron, a splice sequence and a polyadenylation signal from SV40 (Legname et al., 2000). Using restriction sites within the polylinker, further fragments encoding an internal ribosome entry site (IRES) linked to a huCD2 reporter lacking its cytoplasmic tail (huCD2tailless) and (thus any signalling capacity) (IREShuCD2tailless) (a gift from Dr O Williams, UCL) and a human β-globin sequence (huβg) (a gift from Dr D Kiousis, NIMR) were subcloned upstream of the 3' SV40pA intron sequence. cDNA encoding the Mus musculus Zap-70 transgene (I.M.A.G.E clone 4925739) was inserted into the construct downstream of the huβg sequence and upstream of the IREShuCD2tailless reporter. Two constructs were generated in this manner Construct 1 Tre-Zap70 lacking the β-globin intron and Construct 2 Tre-huβg-Zap70 containing the intronic cDNA as shown in **Fig 2.2**.

2.6.2 Generation of Founders

The Tre-Zap70 construct 1 and Tre-huβg-Zap70 construct 2 were prepared for injection by digestion of the plasmid vectors using Xba I and Nae I restriction enzymes. The injection fragments were separated from the remnants of the bacterial vectors using agarose gel electrophoreses. The remaining DNA was gel extracted using a gel extraction kit (Qiagen, UK) and further purified using Elutip columns (Schleicher and Schuell, Germany). Transgenic mice were generated by microinjection (performed by Mauro Tolaini) of the constructs into the pronuclei of fertlized oocytes from crosses of (CBA xB10) F1 mice. Screening was performed by a combination of southern blotting and PCR. The Tre-Zap70 construct generated 4 founders from 18 mice and from 2 rounds of injections. The Tre-huβg-Zap70 construct generated 2 founders from 7 mice in total also from 2 rounds of injections. These initial founders were intercrossed with mice expressing the reverse tetracycline transactivator (rtTA.C^{huCD2}) (*see **Chapter 3 Fig 3.3 A and B)*** and subsequently backcrossed onto an endogenous *Zap-70*$^{-/-}$ background *(see **Chapter 3 Fig 3.3 C)***. The rtTA.C^{huCD2} mice (Legname et al., 2000) bear the reverse tetracycline transactivator (rtTA) domain under the control of human CD2 regulatory elements (Zhumabekov et al., 1995), restricting expression of the transgene in a tissue specific manner to cells of lymphoid origin. In the presence of dox, the rtTA fusion protein is able to bind to the tetracycline responsive elements within the TetO/$pCMV_{min}$ promoter region and initiate transcription of the flanking transgenes as shown in **Fig 2.3**. Following removal of dox either from the feed or drinking water of the mice, transcription of the transgenes is terminated and remaining Zap70 protein

is degraded. Mice were fed dox in food (1 mg/g) throughout pregnancy and before weaning. After weaning, the offspring were fed dox-containing food to maintain Tre-Zap70 transgene expression

2.6.3 *In vitro* Testing of Inducible Tre-Zap70 and Tre-huβg-Zap70 Constructs

2.6.3.1 HEK 293 Tet-On™ Preparation

18 h prior to the transfection the HEK 293 Tet-On™ cells were cultured *(as described in the Cell Culture section 2.1)* and collected by centrifugation (1200rpm, 5 min). The cells were then seeded in 6 well plates coated with Human Collagen Type 1 (0.1μg/cm^2) in 5ml of HEK-M at a density of 0.2 x10^6 cells/well ensuring that at the time of transfection there would be a 70 – 80% confluent cell monolayer. Cells were incubated overnight with 5% CO_2 at 37°C to allow the cells to adhere to the collagen matrix.

2.6.3.2 Lipofection

2μg of the tetracycline inducible plasmids were incubated in OptiMem1 GlutaMAX1 (Invitrogen, UK) reduced serum media to a final volume of 200μl. The tubes were inverted 4-6 times to ensure thorough dispersal of the DNA. The tubes were incubated at RT for 5 min wrapped in foil to prevent degradation of the light sensitive buffer. In parallel 18μl of lipofectamine was added to a final volume of 200μl of OptiMem1 GlutaMAX1 media and also mixed thoroughly, wrapped in foil and allowed to equilibrate for 5 min at RT. Following the incubation the DNA-OptiMem solutions were mixed into the

lipofectamine-OptiMem solutions. The tubes were incubated for 30 min at RT allowing the formation of artificial liposomes containing the DNA constructs.

During the DNA-lipofectamine incubation the HEK 293 Tet-On™ cells were prepared for the transfection. The growth medium was removed and the cells were washed with serum free HEK-M. 1.5ml of fresh serum and antibiotic free OptiMem1 GlutaMAX1 media was added to each well and incubated for 10 min at 37°C.

Following both the DNA-lipofectamine and cell incubations the DNA-lipofectamine mixture was added to the wells and incubated at 37°C with 5% CO_2 for 5 h. Dox (Sigma, UK) was added to dox ON wells at a final concentration of 2μg/ml per well to maintain the expression of the huCD2tailless and enhanced green fluorescent protein (EGFP) reporters during the incubation period. Following the incubation, the media containing the DNA-lipofectamine mix was removed and 5 ml of HEK-M supplemented with dox (2μg/ml per well) was added. The cells were incubated for 24 h at 37°C with 5% CO_2.

Briefly, cells were washed in serum free HEK-M, harvested using collagenase (200U/ml) (Gibco BRL, UK) neutralised with an equal volume of HEK-M, centrifuged (1200 rpm, 5 min) and resuspended in fresh HEK-M. The expression of huCD2 and EGFP on the surface of the cells were analysed using 4 Colour Flow Cytometry.

2.7 Cell Isolation

2.7.1 LN and Spleen

The cervical, axillary, brachial, mesenteric and inguinal LN and spleen were removed from mice. Cell suspensions were made in ice cold AB-IMDM containing 0.1% (v/v) BSA using 75μm nylon mesh. Cells were washed in ice cold AB-IMDM, centrifuged (1200 rpm, 4 min), resuspended in fresh AB-IMDM 0.1% (v/v) BSA and kept on ice for the remainder of the assay. Cells were counted using the Casy-1 cell counter (Scharfe System, Germany).

2.7.2 Peripheral Blood

Approximately 100μl of blood was collected from the tail vein of experimental mice, and mixed in 100ul of heparin (Sigma, UK). The blood samples were then lysed in 2ml of ACK lysis buffer for 3 min or until the solution appeared transparent (but no-longer than five min). 2 ml of ice cold FACS buffer was added to the cells to dilute the ACK lysis buffer, centrifuged (1200 rpm, 4 min). The cells were washed again in a further 2 ml of FACS buffer, centrifuged (1200 rpm, 4 min) and ready for cell staining as below.

2.8 Four Colour, Five & Six Colour Flow Cytometry

Antibodies were diluted as per manufacturers guidelines in ice cold FACS buffer. Cells were incubated on ice at a density of $1x10^6 - 5x10^6$ with 100μl of the primary antibody cocktail of directly conjugated fluorescent antibodies and/or biotinylated antibodies for 1 h. The cells were covered with foil to

prevent degradation of the antibodies. The cells were washed briefly with FACS buffer, centrifuged (1200 rpm, 4 min) and if required incubated on ice with the appropriate secondary antibody or conjugate for a further 30 min. Cells were again washed with FACS buffer, centrifuged (1200 rpm, 4 min) and resuspended in 100-300μl of FACS buffer. Cell suspensions were strained through 40μm cell strainers prior to flow cytometric analysis.

Flow cytometric analysis of cell surface and intracellular proteins was performed using 4 colour analysis on the FacsCalibur (Becton Dickinson) and 5/6 colour analysis using the BD-LSR flowcytometer (Becton Dickinson). Unless otherwise stated a minimum of 10,000 total events were collected. Data was analysed using Flowjo software (Tree Star, USA).

2.8.1 Intracellular staining

For Bcl-2 detection, $1x10^6$ cells were washed and stained for cell surface markers, and then fixed with 3% paraformaldehyde for 30 min on ice in the dark. The cells were washed and resuspended in 100μl of permeabilisation buffer for exactly 3 min. 100μl of FACS buffer was added to the cells to stop the reaction. The cells were resuspended in 20μl of PE-conjugated anti-mouse monoclonal Bcl-2 antibody (clone 3F11) or PE-conjugated IgG monoclonal isotype control (BD Pharmingen) and incubated for 30 min on ice in the dark. The cells were washed in 1ml of FACS buffer and resuspended in 100μl of FACS buffer and the samples were acquired on the BD-LSR flowcytometer (Becton Dickinson).

For Foxp3 detection the preparation of the cells ($1x10^6$) was performed using the APC anti-mouse Foxp3 Staining Set as per the manufacturers guidelines (ebioscience), and samples were acquired on the FACScalibur (Becton Dickinson).

2.9 Target Cell Preparation For Adoptive Transfers

Cells were harvested from donor mice LN and cell suspensions were produced as previously described in ***Section 2.7.1 LN and Spleen Cell Isolation.*** The cells were labelled with 1: 10 dilution (10μl of beads per 10^7 total cells in 90μl of AB-IMDM) of CD8a (Ly-2) directly conjugated MACS microbeads for 20 min on ice. The cells were washed in FACS buffer and centrifuged (1200 rpm, 4 min) and resuspended in 1ml of AB-IMDM. The labelled cells were then magnetically sorted into CD8 positive and CD8 negative fractions using the autoMACS cell sorter (Miltenyi Biotec, Germany).

The cells were washed and resuspended at a concentration of $1x10^7$ cells in warm Dulbecco's Phosphate Buffered Saline Solution (PBS) (Gibco, UK). The cell dye Carboxy-fluorescein diacetate, succinimidyl ester (CFSE) (Sigma, UK) was diluted to a concentration of 4μM in warm Dulbecco's PBS and added rapidly to the cell solutions giving a final concentration of 2μM. Cells were incubated at 37°C for 10 min, washed twice in excess AB-IMDM and resuspended in BSA free AB-IMDM. The cells in a total volume of 250μl were then transferred via tail vein injection into recipient mice. 3 to 5 mice per experimental group were sacrificed at selected time points following transfer of

the cells. (LN and spleens were analysed for surface expression of TCR, CD8, CD44, I-A^b and CD5. The proliferation of the cells was assessed using CFSE).

2.10 Radiation Bone Marrow Chimeras

2.10.1 Bone Marrow Extraction

Bone Marrow (BM) was extracted from the Tibia and Fibia of adult dox fed (1mg/g) Tet-Zap70 mice and C57BL/6 CD45.1 mice using a 24 gauge needle and a 5 ml syringe. BM was isolated via flushing of the handling media through the bone. The extracted BM was washed twice, centrifuged (1200 rpm, 4 min), resuspended in 5ml of handling media and strained though a 40μm cell strainer (BD Falcon, UK).

2.10.2 Dynal Bead Depletion and Injection of Hosts

For negative depletion, cell suspensions were incubated with biotinylated TCR antibody (1:500 dilution) on ice for 30 min in handling media (1ml/10^8 cells). Samples were washed and resuspended in 4mls PBS. Handling media was not used as it contains biotin and would interfere with the dynal bead binding reaction. Dynal beads (M280, Dynal, Norway) were added at a 1:1 ratio of beads to cells for 30 min at 4^0C with constant rotation. TCR expressing cells bound to the dynal beads (negative fraction) were removed using a magnetic Dynal bead separator (Dynal, Norway). This step was repeated twice with an intermittent wash step to remove excess beads. The sorted cells were then resuspended in handling media and counted.

24 h prior to injection with BM derived stem cells *C57Bl/10 Rag1*$^{-/-}$ mice were irradiated for 5 minutes with 500 Rads (Caesium source) The mice were treated with irradiated water supplemented with 0.02% v/v Baytril® (Sigma, UK) for 6 weeks. $10x10^6$ T cell depleted BM cells were transferred by intravenous injection into the tail vein of the irradiated mice. The mice were then divided into two groups, a control group maintained on normal feed, and an experimental group maintained on dox fed (3mg/g). The mice were left for 9 weeks to enable reconstitution of the lymphoid compartment. These mice were used in experiments described in ***Chapter 4 Sections 4.2.1, 4.2.2 and 4.2.3***.

2.11 Calculation of Absolute and Adjusted Cell Numbers.

In order to calculate T cell recoveries from different hosts the following calculations was used.

Cell number counted by Casy-1 cell counter	***= A***
Lymphocyte gate of total live cells	***= B***
T cell subset gate	***= C***
Absolute number of specified T cell subset	***= A x B x C***

Analysis of CFSE labelled populations included the following calculations to determine precursor population size, mean division and predicted expansion **(EF)** of the population observed from the profile of cell division (CFSE profile).

Frequency of cells (**F**) that have divided **x** times. $= F_{(x)}$

Frequency of cells *(f)* divided x times excluding expansion $f_{(x)} = F_{(x)} / 2^x$

The predicted expansion (**EF**) from division profile is given by

$$EF = \Sigma F_{(0\text{-}x)} / \Sigma f_{(0\text{-}x)}$$

Mean division of the precursor population $= \Sigma f.x / \Sigma f$

2.12 Western Blotting

2.12.1 Western Blotting Solutions

Cell Lysis Buffer

50mM Tris pH7.5, 150mM NaCl, 1mM NaOVa, 5mM EDTA, 10mM NaF, 10mM NaPyrophosphate, 1% NP40 and 1 complete mini protease inhibitor cocktail (Roche, UK).

Protein Loading Buffer

187.5mM Tris-HCl, 6% (w/v) SDS, 30% (w/v) glycerol, 150mM DTT and 0.03% (v/v) Bromophenol blue.

PBS-Tween

PBS and 0.001% (v/v) Tween20.

Transfer Buffer

10mM CAPS buffer pH 11.

Membrane Blocking Solution

3% (w/v) Skimmed milk powder (Marvel) dissolved in PBS, 0.05% (v/v) Tween20

Antibody Solutions

Primary antibodies: were all diluted in PBS containing 1% (v/v) BSA and 0.05% Azide

Zap70 (BD, UK) diluted 1:1000 and incubated for 1 h at RT.

p44/42 MAPK kinase (pan-Erk) (Cell signaling, UK) diluted 1:1000 and incubated for 1 h at RT.

Actin (I-19) (Santa Cruz Biotechnology, Germany) diluted 1:1000 and incubated overnight at 4°C.

Reagents

Bradford Reagent (Sigma. UK)

Protease Inhibitor Cocktail Tablets Complete, Mini, EDTA-free (Pancreas extract, Chymotrypsin, Thermolysin (Metalloprotease) and Trypsin 0.02mg/ml, Papain 0.33mg/ml) (Roche, UK).

SDS (Sigma, UK)

Azide (Sigma, UK)

Polyvinylidene diflouride (PVDF) membranes (Millipore, UK)

Pre-cast 10% SDS-PAGE mini-gels (Invitrogen, UK)

2.12.2 Western Blotting Protocol

Cells were washed twice in PBS (Gibco) and lysed (pipetting up and down) in a large volume of cell lysis buffer for 30 min at 4°C. The cells were then centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was decanted into a separate clean eppendorf tube. Protein content was quantified at this stage using the Bradford method. Bradford reagent (BioRad, UK) was diluted 1:5 in dH_20 and added to the protein sample (4µl of protein sample to 996µl of Bradford reagent). A calibration curve was made using dilutions of Bradford reagent and BSA solutions. Samples were measured in a spectophotometer at a wavelength of 595nm and the protein content in the samples determined from the absorbance values of the calibration curve. Loading samples were prepared in new vials where equal amounts of protein were mixed with protein loading buffer and denaturated at 100°C for 5 min. 50µg of protein sample (Amersham Bioscienses, UK) were loaded on pre-cast 10% SDS-PAGE mini gels (Invitrogen, UK) and immersed in 1x SDS running buffer (Biorad, UK) and run at a constant 120V for 2 h. Proteins were wet transferred onto PVDF (Millipore, UK) (activated previously in methanol) (Fisher Chemicals, UK) in 10mM CAPS transfer buffer at constant 400mA for 1 h 30 min at 4°C. Membranes were then blocked in 3% skimmed milk solution for 1 h at RT, then washed (2x 5 min) and (1x 15 min) in 1x PBS-Tween solution (Sigma, UK) and immunoblotted with primary antibodies. Immunodetection was performed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (1:5000) (Promega, UK), anti-rabbit IgG (1:10000) (Promega, UK), or anti-goat IgG (1:5,000; Santa Cruz Biotechnology, Germany) as appropriate and developed

by chemiluminescence as per manufacturers guidelines (Amersham Biosciences, UK).

Figure 2.1: DNA Sequence of the Polylinker Primers.

Reverse and forward oligonucleotides (polylinker primers) were generated encoding the restriction sites Pst-I, Cla-I, EcoRI, Nsi-I, SnaBI and an EcoRI-like site in which the GAATT**C** was replaced with an GAATT**G**. The oligonucleotides were annealed together and subcloned into a plasmid containing tetracycline responsive elements linked to a minimal CMV promoter. The restriction sites introduced in the polylinker were used for all further subcloning.

FWD Polylinker Primer

5'**(g)AATT** gG **TACGTA** GC **ATGCAT** GC **GAATTC** GG **ATCGAT** GG **CTGCA (g)**
EcoRI-like SnaBI Nsi-I EcoRI Cla-I Pst-1

RVS Polylinker Primer

5'(cTGCA)G CC **ATCGAT** CC **GAATTC** GC **ATGCAT** GC **TACGTA Cc(AATTc)**
Pst-1 Cla-I EcoRI Nsi-I SnaBI EcoRI-like

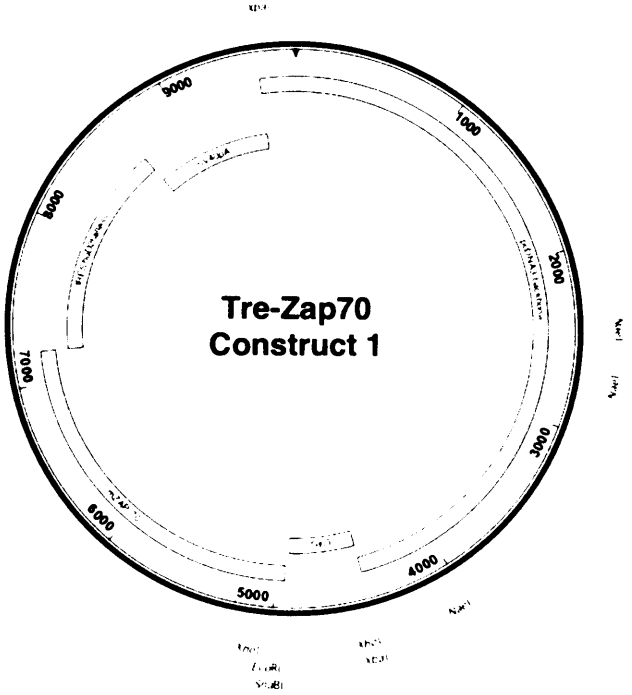
Polylinker Primers Annealed

(g)AATT gG TACGTA GC ATGCAT GC GAATTC GG ATCGAT GG CTGCA(g)
(cTTAA) cC ATGCAT CG TACGTA CG CTTAAG CC TAGCTA CC G(ACGTc)

Figure 2.2: Tetracycline Inducible Plasmid Maps.

A) Tre-Zap70 and **B)** Tre-huβg-Zap70.

A.


1000
2000
3000
4000
5000
6000
7000
8000
9000
Tre-Zap70
Construct 1

B.

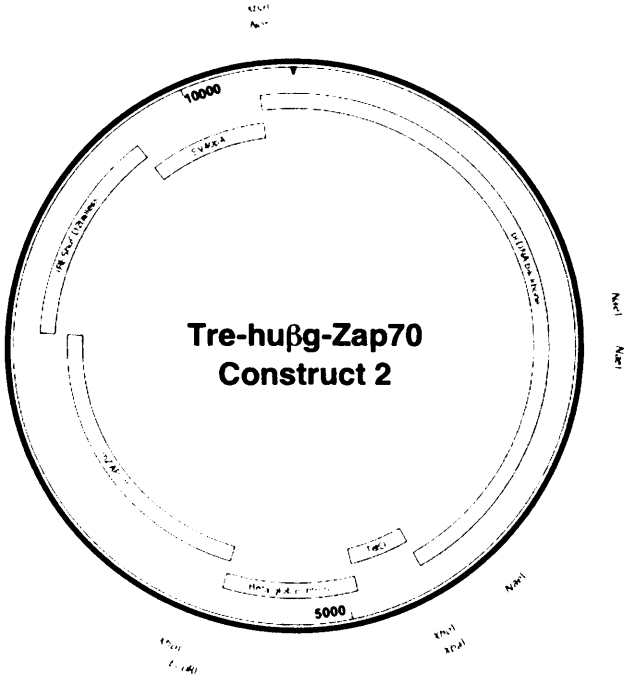
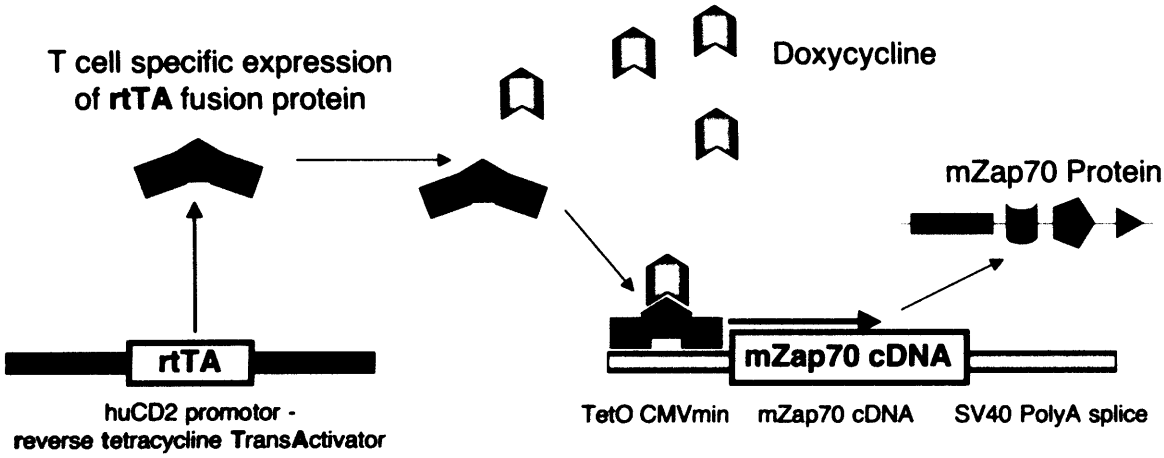

5000
10000
Tre-huβg-Zap70
Construct 2

Figure 2.3: The Tetracycline Inducible System.

Diagram to show how Tre-Zap70 transgene induction is controlled by dox administration. The transgenic T cells express the reverse tetracycline transactivator (rtTA) under the control of the human CD2 promoter. The Tre-Zap70 transgene is under the control of a CMV minimal/TetO promoter containing tetracycline responsive elements (Tre). In the presence of dox the rtTA is able to bind to the Tre within the TetO promoter and induce the transcription of the Zap70 transgene. In the absence of dox, the Zap70 transgene cannot be transcribed and in turn the production of Zap70 protein is terminated.


T cell specific expression
of rtTA fusion protein
Doxycycline
mZap70 Protein
rtTA
mZap70 cDNA
huCD2 promotor -
reverse tetracycline TransActivator
TetO CMVmin
mZap70 cDNA
SV40 PolyA splice

Chapter 3

Generation and Characterisation of Tetracycline Inducible Zap70 Transgenic Mice

3.1 Introduction

It is now well established that stimulation of the TCR by peptide antigens in the context of appropriate MHC complexes, leads to a sequence of tyrosine phosphorylation events within the associated CD3 complex (reviewed in (Kane et al., 2000; Palacios and Weiss, 2004)). This involves the recruitment and activation of proximal kinases and adaptor proteins, which generate a cascade of biochemical events ultimately resulting in T cell activation or differentiation. The earliest stage in the propagation of TCR signals is the activation of cytoplasmic Src family tyrosine kinases Lck and Fyn (Gauen et al., 1994; Straus and Weiss, 1992; van Oers et al., 1996a). Lck and Fyn phosphorylate ITAMs within the ζ homodimer of the CD3 polypeptide complex. The phosphorylated tyrosine residues in turn provide docking sites for the tandem SH2 containing domains of Zap70, a 70kDa cytosolic protein tyrosine kinase (Chan et al., 1992; Wange et al., 1993). Following auto-phosphorylation (Brdicka et al., 2005; Neumeister et al., 1995) and activation by Lck (Chan et al., 1995), Zap-70 interacts with downstream adaptor proteins such as LAT (Zhang et al., 1998a) and SLP-76 (Wardenburg et al., 1996) to facilitate the assembly of a macromolecular signalling complex that includes the signalling

proteins Grb2, Gads, PLC-γ1 (Zhang et al., 1998a; Zhang et al., 2000), Vav (Raab et al., 1997) and Nck (Wunderlich et al., 1999). These adaptor proteins play vital roles in the amplification of signals arising from the TCR/CD3 complex, inferring a crucial role for Zap70 in the propagation of TCR signals.

Zap70 activity is essential for the transmission of TCR signals involved in thymic development. During thymopoiesis, Zap70 kinase activity is essential for the propagation of positive selection signals. Ablation of Zap70 expression partially arrests murine thymocyte development at the DN3-DN4 β-selection checkpoint, however there is no gross reduction in the numbers of DP thymocytes generated. Zap70 deficient mice specifically exhibit a later complete block in positive selection at the DP stage of T cell development (Negishi et al., 1995) and thus lack SP thymocytes. In humans the loss of Zap70 expression either as a result of base pair deletion causing the translation of a truncated form of Zap70 lacking kinase activity (Elder et al., 1994) or insertional mutations within the kinase domain leading to a destablisation of the Zap70 protein (Arpaia et al., 1994) results in an autosomal recessive form of SCID. CD8 SP thymocyte development is completely abrogated in these patients however CD4 SP thymocytes are detected in the periphery of such individuals (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994). The differences in the phenotypes observed in humans and mice exhibiting abrogated Zap70 signalling is attributed to differences in Syk downregulation following the pre-TCR checkpoint and by the finding that if expressed at sufficiently high levels, Syk can compensate for Zap70 during

positive selection (Gong et al., 1997). Pre-TCR signals downregulate Syk in mice such that only low levels of Syk remain at the DP stage of thymopoiesis and are insufficient for the transmission of positive selection signals in the absence of Zap70 expression (Chu et al., 1999). In humans however, the down-regulation of Syk expression is incomplete following pre-TCR signalling. Syk expression remains high within the DP compartment (Chu et al., 1999) and appears to compensate for the loss of Zap70, allowing the selection of some human $CD4^+$ thymocytes.

Genetic studies performed in human cell lines also suggest an important role for Zap70 in mature T cell activation. (Williams et al., 1998), have shown that a Jurkat cell line deficient for Zap70 expression (P116), displayed severe defects in tyrosine phosphorylation, intracellular calcium mobilisation and IL-2 promoter driven transcription following stimulation with anti-CD3. Importantly all the signalling defects exhibited in the absence of Zap70 were restored following transfection with a catalytically active full length Zap70 cDNA. Similarly, the $CD4^+$ T cells that can be identified in the periphery of patients lacking Zap70 expression or function (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994), fail to produce IL-2 and are unable to proliferate in response TCR mediated stimulation by antigens or mitogens, confirming the importance of Zap70 signalling in T cell activation.

While it is understood that TCR signals evoked by recognition of peptide/MHC complexes play important roles in T cell function at different stages in the lifespan of a T cell, it is still unclear if and how TCR signals influence peripheral

naïve T cell survival. In recent years TCR ligation by spMHC complexes in the peripheral lymphoid compartments, similar to those that influence thymic selection, have been reported to support the long-term steady state survival of naïve T cells (Ernst et al., 1999; Muranski et al., 2000). Furthermore the TCR proximal kinases Lck and Fyn have also been shown to play an important role in naive $CD4^+$ and $CD8^+$ T cell survival (Seddon et al., 2000). Given the importance of Zap70 expression in T cell antigen receptor signalling, we wished to determine whether Zap70 was also essential for the transmission of signals required for the steady state survival of the peripheral naïve T cell compartments. Ideally we would need to monitor the survival of naïve T cells lacking Zap70 expression. However as mentioned previously Zap70 deficient mice exhibit a complete block in thymopoiesis at the DP stage in the thymus (Negishi et al., 1995) and lack mature peripheral T cells. Therefore, we could not use this mouse model to study the role of Zap70 in T cell homeostasis. For this reason we wished to generate mice that express Zap70 in a conditional manner, using the tetracycline responsive gene regulatory system (Furth et al., 1994; Gossen and Bujard, 1992; Gossen et al., 1995). This is a dual transgenic system in which expression of a reporter transgene is under the control of tetracycline responsive elements (Tre), while a second transgene expresses the driver construct, a rtTA (Gossen and Bujard, 1992) whose transcriptional activity is required for the induction of the reporter transgene and is regulated by tetracycline or its derivative dox. Using this system would permit Zap70 expression to be maintained during the crucial thymic selection steps in a dox dependent manner, allowing reconstitution of the peripheral lymphoid

compartment. The survival of the mature T cells lacking Zap70 could then be followed in these mice, by ablating Zap70 expression through the removal of dox and subsequent degradation of the Zap70 protein.

The aim of the experiments detailed in the following chapter were to generate transgenic mouse lines capable of expressing a tetracycline inducible Zap70 transgene, to functionally characterise the induction of Zap70 expression within the T cell lineage and to assess the ability of the inducible Zap70 protein to restore normal thymocyte selection, development and ultimately maturation of peripheral $CD4^+$ and $CD8^+$ T cell subsets.

3.2 Results

3.2.1 Generation of Inducible Constructs

Reporter Tre-Zap70 constructs were made by subcloning a full-length Mus musculus Zap70 cDNA (I.M.A.G.E clone 4925739) into a construct containing an upstream 5' heptameric Tre linked to a CMV minimal promoter sequence (Gossen and Bujard, 1992) with a downstream IREShuCD2tailless reporter and an intronic SV40 polyadenylation signal (Tre-Zap70 construct 1) shown in **Fig 3.1 A** *(also see **Chapter 2 Materials and Methods Section 2.6**)*. Previous tetracycline inducible transgenics generated using this system exhibited a ~10 fold reduction in protein levels of the inducible transgene in peripheral T cells compared with expression in the thymus (Buentke et al., 2006; Seddon et al., 2000). Other groups employing the tetracycline inducible system however,

have achieved better peripheral expression of the transgene compared to our models. The constructs employed in these studies differ from ours by the inclusion of an intronic sequence derived from the β-globin gene, between the Tre and the transgene cDNA (Labrecque et al., 2001; Witherden et al., 2000). Inclusion of an intervening generic intronic sequence has been reported to facilitate mRNA accumulation and augment gene expression in transgenic mice (Choi et al., 1991; Clark et al., 1993). Therefore, in an effort to improve peripheral expression of the Zap70 transgene, we made a second construct containing a similar intronic sequence (huβg) between the TetO/CMV minimal promoter and the Zap70 cDNA (Tre-huβg-Zap70 construct 2) shown in **Fig 3.1 B**.

3.2.2 *In-vitro* Construct Validation

The constructs were tested for their inducible functionality *in vitro* by transfection into a human transformed primary HEK 293 Tet-On™ cell line, stably expressing the rtTA (HEK rtTA). The cells were cultured in the presence and absence of dox (2μg/ml) in the culture supernatants. A tetracycline inducible Tre-EGFP construct was used as a positive control (pBiEGFP). Detection of surface huCD2tailless (hereon abbreviated to huCD2) and EGFP reporter expression was performed by FACS analysis. **Fig 3.2** shows the expression of huCD2tailless and EGFP on the HEK rtTA cells 24 h following transfection with Tre-Zap70, Tre-huβg-Zap70 and pBiEGFP constructs. In the absence of dox, all three constructs produced some low level dox independent expression of the huCD2 or EGFP reporters, suggesting a degree of leakiness

in reporter expression. In the presence of dox, all three constructs showed a substantial increase in the frequency of cells expressing the respective reporters. The Tre-huβg-Zap70 construct showed a ~5 fold increase in the frequency of cells expressing the huCD2 reporter compared to the poor expression of huCD2 in the Tre-Zap70 transfectants.

3.2.3 Initial Screening of Inducible Founders

Having confirmed the constructs were functional, both constructs were microinjected into fertilized oocytes from (CBA x B10) F1 mice to generate the initial founders required for the subsequent breeding steps. Four Tre-Zap70 founders and two Tre-huβg-Zap70 founders were generated. The expression of the Zap70 transgene was tested, by intercrossing of these founders with rtTA.C^{huCD2} transgenic mice. These mice express the rtTA under the control of a huCD2 locus control region (LCR) that targets expression of the transgene to the T cell lineage (Legname et al., 2000). The litters generated from this breeding were fed dox food (1mg/g) for three days and the thymi of these mice were analysed for surface huCD2 reporter expression.

We examined the huCD2 reporter expression on T lymphocyte gated CD4 SP, CD8 SP and DP thymocytes from the founding transgenic lines **(Fig 3.3)**. Of the four Tre-Zap70/rtTA.C^{huCD2} transgenic founders generated **(Fig 3.3 A)**, only three lines B, C and D were established. Utilizing huCD2 expression as a reporter of Zap70 transgene expression revealed that only line D showed high levels of huCD2 expression in the CD4 SP, CD8 SP and DP compartments,

possibly reflecting integration of the Zap70 transgene into an unfavourable region of the murine genome for lines B and C. Tre-huβg-Zap70/rtTA.C^{huCD2} line A **(Fig 3.3 B)** failed to breed, however line B was established and showed high levels of huCD2 expression in all the thymic compartments shown. Comparison of the huCD2 expression between Tre-huβg-Zap70/rtTA.C^{huCD2} line B and Tet-Zap70/rtTA.C^{huCD2} line D showed that Tet-Zap70/rtTA.C^{huCD2} mice exhibited a higher frequency of thymocytes expressing the huCD2 reporter compared to Tet-huβg-Zap70/rtTA^{huCD2} mice. This difference in Zap70 transgene expression was further exacerbated following breeding of lines B and D onto an endogenous Zap70 deficient background shown in **Fig 3.3 C**. Although the inclusion of the extra intronic β-globin sequence upstream of the Tre-Zap70 transgene, showed a promising augmentation of huCD2 expression in the *in vitro* HEK rtTA assay **(Fig 3.2)**, this was not seen *in vivo*, at least for the lines generated. For this reason we selected the Tre-Zap70.D transgenic line for further studies.

3.2.4 Testing Transgene Induction *In – vivo*

To facilitate the study of Zap70 in peripheral T cell homeostasis we generated Tre-Zap70.D rtTA.C^{huCD2} *Zap70*$^{-/-}$ mice, so that the dox inducible transgene was the only source of Zap70 expression in the mice. We first checked the fidelity of Zap70 induction to ensure dox independent expression of the transgene was minimal. We examined huCD2 reporter expression on wildtype C57BL/6J (WT) mice, Tre-Zap70.D^{-} *Zap70*$^{-/-}$ littermate controls (lack expression of the Tre-Zap70 transgene and are used as the *Zap70*$^{-/-}$ controls throughout the entire

study), constitutively dox fed (1mg/g) Tre-Zap70.D rtTA.C^{huCD2} *Zap70*$^{-/-}$ mice (heron abbreviated to Tet-Zap70) and Tre-Zap70.D *Zap70*$^{-/-}$ mice lacking the rtTA fusion protein required for the induction of transgene transcription **(Fig 3.4)**. In the absence of rtTA^{huCD2} expression there was no huCD2 expression, confirming that transgene induction was strictly rtTA dependent. Thymocytes from these mice were negative for huCD2 expression and were almost entirely of a DP phenotype, lacking mature cells, a phenotype identical to that of the Zap70 deficient mice. In contrast, dox fed Tet-Zap70 mice not only showed good thymic expression of huCD2, but also showed the presence of mature SP thymocytes whose generation is strictly Zap70 expression dependent.

3.2.5 Western Blotting: Detection of Zap70 Protein

To determine the level of Zap70 protein in the thymus following induction of the Zap70 transgene (Tre-Zap70), we compared the expression of Zap70 in the thymi of WT mice, *Zap70*$^{-/-}$ and Tet-Zap70 mice maintained either on normal feed or dox feed (1mg/g) during gestation and post weaning **(Fig 3.5)**. Zap70 expression in the thymus of dox fed Tet-Zap70 mice was comparable to that seen in WT mice **(Fig 3.5 A)**. Zap70 expression was not detected in Tet-Zap70 mice in the absence of dox feeding, confirming that the tetracycline inducible transgene was the only source of Zap70 in the Tet-Zap70 mice. Surprisingly, the Zap70 protein detected in the dox fed Tet-Zap70 mice was significantly heavier (~5.5kb) than the WT Zap70 protein. Sequencing of the Tre-Zap70 construct 1 used to generate the Tet-Zap70 mice revealed a second in frame ATG start sequence upstream of the Tre-Zap70 transgene. This start codon

was found between the remaining SnaBI and EcoRI restriction sites of the MCS originally introduced into the tetracycline inducible plasmids to aid the subsequent subcloning steps ***(see Appendix).*** Initiation of protein synthesis from this alternative start sequence was found to add 51 amino acids to the amino terminus of the original Zap70 protein, increasing its molecular weight to 75.48 kDa.

The SnaBI and EcoRI restriction sites were used to introduce the huβg intronic sequence into the second Tre-huβg-Zap70 construct, and therefore the alternative start codon was not present in Tre-huβg-Zap70 construct ***(see Appendix)***. To confirm this, we compared the expression of Zap70 protein in thymi of dox fed Tet-huβg-Zap70 and WT control mice **(Fig 3.5 B)**. Importantly the size of the Zap70 protein detected in the Tet-huβg-Zap70 mice was comparable to endogenous Zap70, inferring that the increase in size observed in Tet-Zap70 mice was attributed to the addition of 51 amino acids as a result of the alternative start codon.

3.2.6 Characterisation of Thymocyte Development in Tet-Zap70 Mice

We next monitored the ability of the Tre-Zap70 transgene to reconstitute thymic selection and T cell maturation in the Tet-Zap70 mice. Pregnant Tet-Zap70 mice were fed dox in food (3mg/g) throughout pregnancy and post-weaning offspring were constitutively fed dox-containing food (1mg/g) to maintain Zap70 expression. At 5-12 wks of age Tet-Zap70, Tre-Zap70.D$^-$ *Zap70*$^{-/-}$ littermate

controls (heron abbreviated to *Zap70*$^{-/-}$/KO) and aged matched C57BL/6J WT control mice (hereon abbreviated to WT) were culled and the size and phenotype of the thymi determined. Thymocytes were analysed for the expression of the coreceptors CD4 and CD8, TCR and huCD2 by FACS. Thymi from dox fed Tet-Zap70 mice contained both CD4 SP and CD8 SP thymocyte populations suggesting that T cell development was restored by expression of the inducible Zap70 transgene. The frequency of mature CD4 SP and CD8 SP thymocytes observed in Tet-Zap70 mice were consistently greater than the *Zap70*$^{-/-}$ controls but reduced in comparison to the WT control (**Fig 3.6 A and B**). Calculation of the absolute number of mature TCR^{hi} CD4 and TCR^{hi} CD8 SP thymocytes (**Fig 3.6 C**) also revealed an increase compared to the *Zap70*$^{-/-}$ controls, but were in both cases reproducibly lower than that of the WT. The percentage and absolute number of DP and DN thymocytes appeared normal. Likewise there did not appear to be any bias towards the development of the single positive CD4 or CD8 thymocytes given that the overall ratio of mature CD4 SP to CD8 SP was not dissimilar from WT ratios shown in **Fig 3.6 D.**

Comparison of huCD2 reporter expression between individual Tet-Zap70 mice revealed significant variegation of Zap70 transgene expression **(Fig 3.7 A).** As a consequence, this variation also resulted in increased variability within the percentages and absolute numbers of mature thymocytes generated in Tet-Zap70 mice. To more fairly assess the ability of the Tre-Zap70 transgene to restore thymic selection, we examined the efficiency of positive selection only amongst DP thymocytes capable of expressing the Tre-Zap70 transgene.

Therefore we analysed the thymi of Tet-Zap70 mice on the basis of huCD2 expression but also TCR upregulation. This is because a small percentage of the mature T cells appeared to have partially downregulated huCD2 reporter expression, but maintained high levels of TCR expression and so were included in the analysis. Positively gating on induced cells (TCR^{hi} or huCD2 positive) (gating shown in **Fig 3.7 B**) excluded the huCD2 negative Zap70 negative DP thymocytes that could not undergo positive selection. Using this gating revealed that the percentage of mature SP cells generated in the Tet-Zap70 and WT mice were virtually identical **(Fig 3.7 C)**. This observation suggests that the frequency of SP thymocytes, generated from induced DP thymocytes expressing the Tre-Zap70 transgene is comparable to the WT controls and appears to indicate that thymic selection progresses in a manner comparable to WT mice **(Fig 3.7)**.

We next examined the development of the DN thymocytes through the DN1-DN4 stages of thymopoiesis. DN3-DN4 progression during T cell development requires signalling through the pre-TCR, the activity of which requires the expression of Zap70 and its associated family member Syk (Cheng et al., 1997). To determine whether induction of the Tre-Zap70 transgene could alleviate the partial block in DN3-DN4 development observed in mice deficient for Zap70 (Negishi et al., 1995) we analysed the development of the DN sub-populations by assessment of the cell surface expression of CD44 (phagocyte glycoprotein Pgp1) (Godfrey et al., 1993) and CD25 (IL-2Rα) (Pearse et al., 1989) **(Fig 3.8)**. The frequencies of DN subsets generated in Tet-Zap70 mice

resembled the pattern found in Zap70 deficient mice, exhibiting a partial block in DN3-DN4 progression. This partial arrest in thymopoiesis suggests that the Zap70 transgene may not be effectively switched on by the DN3 – DN4 stage of development.

To more closely examine positive selection and transmission of the TCR signals required for thymocyte maturation, we examined TCR and CD5 surface expression on thymocytes from dox fed Tet-Zap70 mice. TCR expression levels vary throughout the thymocyte development and its upregulation is associated with thymocyte maturation during thymopoiesis. TCR expression on the majority of thymocytes present within the DP compartment is low. These thymocytes either express TCRs that interact poorly with spMHC ligands and fail to engage signals that would maintain their survival, leading to cell death by neglect or they express TCRs that bind too well to self ligands and are deleted by negative selection. The small frequency of DP thymocytes that bind to self peptide ligands with an avidity that promotes their positive selection to the CD4 or CD8 lineages, begin to upregulate TCR expression which reaches maximal levels during maturation to CD4 SP and CD8 SP lineages. CD5 surface expression has been linked to TCR signalling such that CD5 expression has been shown to increase with a rise in TCR signalling strength, that may reflect the avidity of TCR ligation by selecting sp/MHC ligands in the thymus (Azzam et al., 1998).

We analysed the expression TCR and CD5 on the different thymocyte subsets from representative WT and Zap70 deficient individuals **(Fig 3.9 A)** to observe

the extent to which Zap70 influences thymocyte maturation. Interestingly WT DP thymocytes exhibited a biphasic profile of CD5 expression with the majority of the DP thymocytes expressing intermediate levels of CD5 expression, which appeared upregulated ($CD5^{hi}$) on a small sub-population of DP thymocytes (Azzam et al., 2001; Azzam et al., 1998) and was further upregulated on the CD4 and CD8 SP populations. Similarly TCR expression paralleled the increases observed in CD5 surface levels, showing the highest levels of expression on the SP thymocytes. TCR expression was consistently reduced on *Zap70*$^{-/-}$ DP thymocytes compared to WT DP thymocytes, indicating that Zap70 expression is required for transduction of maturation signals. Similarly CD5 surface expression was also low on *Zap70*$^{-/-}$ DP thymocytes compared to DP thymocytes generated in WT mice, reflecting reduced TCR signalling in the absence of Zap70 expression.

We next examined TCR and CD5 expression by thymocytes from dox fed Tet-Zap70 mice. Since huCD2 expression should report Zap70 expression we reasoned that huCD2 positive thymocytes should resemble WT thymocytes and huCD2 negative thymocytes should share phenotypic similarities with *Zap70*$^{-/-}$ thymocytes. We therefore compared the expression of TCR and CD5 on the huCD2 negative Tet-Zap70 thymocytes with *Zap70*$^{-/-}$ thymocytes and huCD2 positive thymocytes with WT thymocytes (**Fig 3.9 B**). Importantly in huCD2 positive Tet-Zap70 DP thymocytes, CD5 and TCR expression was similar to the WT control, indicating that the Tet-Zap70 protein successfully rescues selection and maturation within the DP compartment. CD4 SP thymocytes expressing the huCD2 reporter showed patterns of TCR and CD5

expression, which were similar to the WT control and consistently higher than the *Zap70*$^{-/-}$ control. Likewise the CD8 SP compartment showed WT levels of TCR expression but CD5 expression was reduced compared to thymocytes expressing endogenous Zap70. The phenotype of the huCD2 negative DP thymocytes was similar to the *Zap70*$^{-/-}$ thymocytes, showing lower levels of both TCR and CD5 expression, indicating that the huCD2 negative DP thymocytes either lack or exhibit reduced Zap-70 expression. Although the majority of SP thymocytes fell within the huCD2 positive gate, a small fraction of huCD2 negative CD4 SP and CD8 SP thymocytes were detectable. This small frequency of CD4 SP and CD8 SP thymocytes showed low levels of CD5 but WT levels of TCR expression. These populations may represent mature SP thymocytes that have downregulated the huCD2 reporter following maturation, a phenomenon associated with the use of the tetracycline inducible gene regulatory system (Buentke et al., 2006; Legname et al., 2000).

3.2.7 Stability of Tre-Zap70 Transgene Expression Through Development

Previous in-house studies utilising the tetracycline gene regulatory system have reported problems with the maintenance of transgene expression during thymocyte maturation (Buentke et al., 2006; Legname et al., 2000), therefore we wanted to examine how Tre-Zap70 transgene expression was maintained in the different thymocyte and peripheral T cell compartments. We compared the expression of the huCD2 reporter in cells at different stages of T cell maturation. DP, SP and peripheral TCR^{hi} CD4^{+} and TCR^{hi} CD8^{+} subsets were

analysed for the expression of CD4, CD8, TCR and huCD2 to determine whether Zap70 expression was equivalent between the different populations. HuCD2 expression was high on the DP thymocytes but was consistently reduced on the CD8 SP thymocytes compared to the CD4 SP population.

The level of huCD2 expression was higher on the SP thymocytes **(Fig 3.10 A)** compared to the peripheral subsets. This suggests huCD2 and Zap70 expression is downregulated following maturation of the SP thymocytes and export of the mature cells from the thymus to the peripheral tissues, as observed in similar tetracycline inducible transgenics (Buentke et al., 2006; Seddon et al., 2000). Although there were differences in huCD2 expression between the CD4 SP and CD8 SP thymocyte subsets, peripheral expression of huCD2 was similar on both mature TCR^{hi} $CD4^{+}$ and $CD8^{+}$ populations, suggesting that the expression of the transgenic Zap70 is not biased toward a particular lineage of lymphocytes **(Fig 3.10 B)**. Although a decline in huCD2 reporter expression is apparent in the comparison between mature peripheral T cells with mature SP thymocytes generated in the thymus, the precise quantification of Zap70 protein in the peripheral tissues has yet to be confirmed by western blot protein analysis.

3.2.8 Characterisation of Peripheral $CD4^{+}$ T cell Subsets in Tet-Zap70 Mice

Analysis of the thymi of the Tet-Zap70 mice suggests that the generation of mature SP thymocytes occurs in a manner broadly comparable to WT. We

next examined the phenotype of the mature peripheral T cells generated in the LN and spleen of constitutively dox fed Tet-Zap70 mice (**Fig 3.11**). Naïve, memory-like and regulatory T cells (T_{Reg}) were identified by surface expression of CD4, TCR and the activation markers CD44 and CD25 by FACS. Naïve $CD4^+$ T cells are here defined as $CD4^+ CD44^{lo} CD25^{lo}$. $CD4^+ CD44^{hi} CD25^{lo}$ cells are defined as memory-like since they have upregulated the cell surface activation marker CD44. T_{Reg} cells are defined as $CD4^+ CD44^{intermed} CD25^{hi}$. The memory-like and T_{Reg} populations generated in the Tet-Zap70 mice, consistently displayed a broader pattern CD44 and CD25 surface staining compared to the WT controls, therefore the gates used to identify the memory-like and T_{Reg} populations in the Tet-Zap70 mice were larger, to include all the cells expressing CD44 and CD25.

Tet-Zap70 mice had similar numbers and percentages of total TCR^{hi} $CD4^+$ T cells compared to WT mice. However, examination of the composition of the mature $CD4^+$ compartment revealed differences. In both the LN and spleen the overall frequency of memory-like and T_{Reg} populations was consistently greater than the WT, and the frequency of naïve lymphocytes was correspondingly reduced in comparison to WT controls. This was also reflected in the total number of T cell subsets recovered from the mice. The absolute numbers of naïve T cells recovered from the Tet-Zap70 individuals were reproducibly reduced (~2.5 fold) compared to WT controls. In contrast the numbers of memory-like and T regulatory cells recovered from Tet-Zap70 individuals were increased (~3 fold and ~2 fold respectively) compared to WT T

cell numbers. The presence of all the $CD4^+$ subsets in the peripheral lymphoid tissues, particularly the $CD44^{hi}$ memory-like cells, implies that the Tre-Zap-70 transgene is functional in the periphery, despite the low levels of transgene expression **(Fig 3.10).**

3.2.9 Normal Thymic Production of $CD4^+$ T_{Reg} Cells but Increased Numbers of Peripheral $CD4^+$ T_{Reg} Cells in Tet-Zap70 Mice

Initial characterisation of the peripheral $CD4^+$ T cell subsets in Tet-Zap70 mice revealed an increase in the frequency and absolute number of TCR^{hi} $CD4^+$ $CD25^+$ T cells. We therefore wished to investigate further whether these cells were true $CD4^+$ regulatory T cells and to try to better understand the cause of the increase in their numbers. Since CD25 the α chain of the IL-2R is also expressed on activated $CD4^+$ T cells (Waldmann, 1986), we wanted to determine whether the increase in $CD4^+$ $CD25^+$ T cells in the periphery of dox fed Tet-Zap70 mice was due to an increase in T_{Reg} cells, activated $CD4^+$ T cells or both. The transcription factor Foxp3 is selectively expressed by $CD4^+$ T_{Reg} cells (Fontenot et al., 2003; Hori et al., 2003), therefore we analysed its expression by $CD4^+$ T cells in Tet-Zap70 mice. T cells from the LNs of WT and constitutively dox fed Tet-Zap70 mice were analysed for the surface expression of CD4, TCR and CD25 and intracellular expression of Foxp3 **(Fig 3.12 A)**. The data shows that Foxp3 expression correlated well with CD25 expression in the mature $CD4^+$ compartment of Tet-Zap70 mice, such that nearly all the $CD4^+$ $CD25^+$ T cells were also $Foxp3^+$. The frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ T_{Reg}

cells was reproducibly increased (~2 fold) in the Tet-Zap70 mice compared to the WT controls, as described earlier in **Fig 3.11**.

We next assessed whether the increase of $CD4^+$ $CD25^+$ T_{Reg} lymphocytes in the peripheral lymphoid tissues of Tet-Zap70 mice was a result of increased production of T_{Reg} cells in the thymus. Thymocytes from WT, *Zap70*$^{-/-}$ and constitutively dox fed Tet-Zap70 mice were analysed for the expression of the CD4, CD8, CD25 and CD44 **(Fig 3.12 B)**. Comparison of the frequencies of developing $CD4^+$ $CD44^{lo}$ $CD25^{hi}$ T_{Reg} populations could not identify any reproducible difference between the WT and Tet-Zap70 individuals. This appears to suggest that thymic T_{Reg} production is normal in Tet-Zap70 mice although confirmation through Foxp3 detection still needs to be performed. Importantly, we could not find any evidence of increased production of T_{Reg} precursors in the thymus of Tet-Zap70 mice that would account for the increase in peripheral T_{Reg} cell numbers.

3.2.10 The Mature Peripheral $CD8^+$ Compartment is Markedly Diminished in Tet-Zap70 mice

We next assessed the phenotype of the mature peripheral $CD8^+$ compartment. Naïve and memory-like $CD8^+$ T cells were identified by the expression of CD8, TCR, CD44 and CD122 (IL-2Rβ), which is also the β-chain of the IL-15R (Giri et al., 1994). Naïve $CD8^+$ T cells are here defined as $CD8^+$ $CD44^{lo}$ $CD122^-$ and memory-like cells are here defined as $CD8^+$ $CD44^{hi}$ $CD122^+$. **Fig 3.13** shows the phenotype and absolute numbers of the peripheral $CD8^+$ T cells recovered

from representative WT, *Zap70*$^{-/-}$ and constitutively dox fed Tet-Zap70 individuals. The mature TCR^{hi} CD8^{+} compartment in the LN (**Fig 3.13 A)** and spleen of Tet-Zap70 mice (**Fig 3.13 B)** exhibited a marked reduction in terms of both percentage and absolute number, showing ~16 fold reduction in cellularity compared to mice expressing endogenous Zap70 (**Fig 3.13 C)**. The mature TCR^{hi} CD8^{+} compartment also differed to WT mice on the basis of composition. The frequency of naïve CD8^{+} T cells recovered from Tet-Zap70 mice was reduced compared to the WT control and showed a ~36 fold reduction in absolute cell numbers. In contrast the percentage of memory-like cells was reproducibly greater than WT controls, although this was not reflected in the absolute cell numbers, since the overall size of the mature Tet-Zap70 CD8^{+} T cell compartment was smaller than the WT. Interestingly the naïve CD8^{+} T cell compartment appeared more reduced than the memory-like population in Tet-Zap-70 mice, which may suggest that the homeostasis of the naïve compartment has a greater dependence on Zap70 expression and hence homeostatic TCR signals than the CD8^{+} memory-like T cell compartment.

3.2.11 Abnormal CD8 Memory Compartment in Tet-Zap70 Mice

Studies have shown that the cytokines IL-7 and IL-15 are essential for the maintenance of memory CD8^{+} T cells (Becker et al., 2002b; Goldrath et al., 2002; Schluns et al., 2002; Schluns and Lefrancois, 2003). These CD44^{hi} memory CD8^{+} T cells selectively express high levels of CD122 (IL-2/IL-15Rβ), and IL-15 has been shown facilitate their survival (Ku et al., 2000) and support their proliferation *in vivo* (Becker et al., 2002a; Judge et al., 2002). We

analysed the expression of CD122 on peripheral $CD8^+$ subsets derived from WT and Tet-Zap70 mice to address whether the memory-like cells shared a similar phenotype. The mean fluorescence intensity (MFI) of CD122 expression on the Tet-Zap70 cells was normalised against control CD122 staining on the WT $CD8^+$ T cells.

Mature LN $CD8^+$ T cells generated in Tet-Zap70 mice showed abnormal CD122 expression (**Fig 3.14**). CD122 levels were consistently reduced (~3.5 fold) on the memory-like T cells generated in Tet-Zap70 mice. Unexpectedly, the naïve Tet-Zap70 $CD8^+$ T compartment also exhibited a reduction in CD122 expression (~1.6 fold). The decrease in CD122 expression on both the naïve and memory-like populations may suggest that IL-2 or IL-15 cytokine signalling is impaired within the peripheral $CD8^+$ compartment of Tet-Zap70 mice.

We next assessed whether the memory-like $CD8^+$ T cells generated in the Tet-Zap70 mice also exhibited abnormalities in the surface expression of other memory-cell markers. We measured the cell surface expression of the activation marker CD44 and the activation associated glycoform of CD43 whose expression has been shown to be upregulated on effector $CD8^+$ T cells, compared to memory $CD8^+$ lymphocytes (Harrington et al., 2000; Onami et al., 2002). Naïve $CD8^+$ T cells are here defined as $CD8^+$ $CD44^{lo}$ $CD43^{lo}$. Memory-like T cells are defined as $CD8^+$ $CD44^{hi}$ $CD43^{lo}$ and effector phenotype $CD8^+$ T cells are defined as $CD8^+$ $CD44^{hi}$ and $CD43^{hi}$. The percentages of naïve, memory-like and effector phenotype cells recovered from the LNs differed greatly between the WT and Tet-Zap70 individuals (**Fig 3.15**). While the

percentage of memory-like cells appeared similar to the frequencies observed in WT mice, the percentage of naïve $CD8^+$ Tet-Zap70 T cells was consistently reduced compared to the WT (~1.6 fold). In contrast the percentage of effector T cells generated in the Tet-Zap70 mice was consistently greater than WT controls (~18.5 fold). This may suggest that the increased frequency of $CD8^+$ $CD44^{hi}$ $CD122^{hi}$ cells detected in the periphery of Tet-Zap70 mice **(Fig 3.13)** may actually be due to increased generation of effector phenotype T cells.

To determine whether the peripheral $CD8^+$ compartment showed evidence of chronic stimulation, we analysed mature LN derived $CD8^+$ T cells for the expression of the activation markers CD25 and CD69 (**Fig 3.16**). Ligation of the T cell antigen receptor leads to the upregulation of CD69, a cell surface glycoprotein shown to be upregulated within 2 hours following *in vitro* or *in vivo* stimulation, (Lopez-Cabrera et al., 1993). CD25 is also expressed on activated lymphocytes but whose upregulation is delayed in comparison with CD69 (Waldmann, 1986). The percentage of T cells triggered to express either CD25 or CD69 alone or both CD25 and CD69 was consistently greater in Tet-Zap70 mice compared to the WT controls, suggesting that this phenotype may be a consequence of increased activation of the naïve $CD8^+$ T cell compartment by either self or environmental antigen.

3.2.12 Zap70 Regulates CD5 expression on peripheral CD4 and CD8 subsets

As previously discussed CD5 is a negative regulator of TCR signalling such that CD5 expression levels are regulated by TCR signals evoked by interaction

with self Ag/MHC, both in the thymus (Azzam et al., 1998) and the peripheral lymphoid tissues (Seddon and Zamoyska, 2002b; Smith et al., 2001b). Modulation of CD5 expression is thought to fine tune TCR responsiveness to antigenic stimulation (Azzam et al., 2001; Wong et al., 2001). In the periphery the expression level of CD5 reflects interaction of the TCR with homeostatic spMHC ligands, which play a role in maintaining the survival of the naïve T cell compartments. We examined the expression of CD5 on the peripheral $CD4^+$ and $CD8^+$ lymphocytes to determine whether the patterns of CD5 surface expression set in the thymus, were maintained in the periphery and whether there were any differences in CD5 expression between the peripheral $CD4^+$ and $CD8^+$ subsets. LN T cells were analysed for the surface expression of CD4, CD8, TCR, CD5, CD25, CD44 and CD122. For the scatter plots the MFI of CD5 expression on the Tet-Zap70 cells was normalised against control CD5 staining on the WT T cells.

While TCR expression was normal in all $CD4^+$ and $CD8^+$ subsets generated in the Tet-Zap70 mice **(Fig 3.17 A)**, CD5 expression was reduced on all the $CD4^+$ and $CD8^+$ subsets compared to WT controls, suggesting reduced homeostatic TCR signalling in Tet-Zap70 T cells. Interestingly, the reduction in CD5 expression was not the same for all the subsets analysed. The reduction in CD5 expression on the naïve $CD4^+$ T cells was consistently greater than observed in the memory-like and T_{Reg} subsets. In contrast, while the Tet-Zap70 $CD8^+$ subsets also exhibited decreased expression of CD5 compared to the same cells from control mice, the reduction was similar between the naïve and

memory-like subsets. Comparison of CD5 expression between the $CD4^+$ and $CD8^+$ subsets revealed that CD5 expression was not significantly different in the naïve $CD4^+$ T cells compared to the naïve $CD8^+$ population, also supporting the suggestion that Tre-Zap70 induction is comparable in the peripheral $CD4^+$ and $CD8^+$ T cells.

3.3 Discussion

TCR signals evoked by recognition of self peptide MHC complexes, have been shown to play a role in the long term survival of the peripheral naïve $CD4^+$ and $CD8^+$ T cell compartments (Ernst et al., 1999; Polic et al., 2001; Takeda et al., 1996; Tanchot et al., 1997; Viret et al., 1999). Recent studies have also implicated the TCR proximal Src Kinase family members Fyn and Lck in the transmission of TCR survival signals evoked by ligation of the TCR with self antigen (Seddon and Zamoyska, 2002b). However, how this TCR survival signal is propagated further by other intracellular signalling proteins is unknown. Zap-70 a key signalling intermediary downstream of Lck and Fyn, is crucial for the transduction of TCR signals influencing thymocyte development and peripheral T cell activation (Arpaia et al., 1994; Negishi et al., 1995) but its role in the transmission of TCR derived survival signals has not been studied. Mice deficient for Zap70 expression (Negishi et al., 1995; Wiest et al., 1997) exhibit a complete block in positive selection and consequently lack mature SP thymocytes and peripheral T cells. Therefore in an effort to address whether Zap70 may have a role in peripheral T cell homeostasis, the present study focused on generating tetracycline inducible transgenic mice, in which

expression of a Zap70 transgene could be switched on in a tetracycline dependent manner. Induced expression of the Tre-Zap70 transgene would restore the transmission of thymic selection signals and thus permit the reconstitution of the peripheral $CD4^+$ and $CD8^+$ T cell compartments. Survival of the mature T cells lacking Zap70 expression could then be measured following cessation of Zap70 transgene induction, through the removal of dox treatment.

The data presented in this chapter outlined the initial generation and preliminary characterisation of the Tet-Zap70 mice bearing a tetracycline inducible Zap70 transgene. The data reported here shows that the expression of the Tre-Zap70 transgene, restores positive selection signals in the thymus of Zap70 deficient mice. Thus allowing the generation and maturation of TCR^{hi} CD4 SP and TCR^{hi} CD8 SP thymocytes, capable of reconstituting the mature peripheral naïve, memory-like and regulatory $CD4^+$ and $CD8^+$ T cell compartments.

Previous mouse models generated in house using the tetracycline inducible gene expression system (Tet-inducible system) have highlighted issues with the stability of transgene expression in the peripheral T cells. Although the transgenes showed good expression in the thymocytes, expression in peripheral T cells was significantly reduced in comparison (Buentke et al., 2006; Legname et al., 2000). This is in contrast to other studies where the authors report good peripheral expression of the tetracycline responsive transgenes (Labrecque et al., 2001; Witherden et al., 2000). In an effort to

improve Zap70 transgene expression in the peripheral T cells, we employed the cloning strategy used by these groups by generating an additional tetracycline inducible construct (Tre-huβg-Zap70) containing an intronic sequence between the Tre and the Zap70 cDNA **(Fig 3.1)**. While initial results *in vitro* **(Fig 3.2)** suggested that inclusion of the human β-globin intron in the inducible Zap70 constructs, increased the frequency of cells capable of expressing the Zap70 transgene, this was not observed *in vivo*. Tet-huβg-Zap70 founders generated from the Tre-huβg-Zap70 construct, failed to show improved peripheral expression of the Zap70 transgene compared with Tet-Zap70 mice lacking the human β-globin intronic sequence **(Fig 3.3)**. This may suggest that the poor expression of the Tre-Zap70 transgene in the peripheral T cells may not be a feature of the Tet construct. One reason for this maybe the rtTA used to drive expression of the transgene. It is possible that the rtTA may be inefficient at driving peripheral expression of the Tre-Zap70 or that the rtTA protein itself may be unstable and degrade before sufficient transgene induction has been achieved.

It is also important to note that the studies using the Tet-inducible system, exhibiting the greatest transgene expression (Labrecque et al., 2001; Witherden et al., 2000) used a model whereby administration of the antibiotic repressed transgene induction (Tet Off). This is in contrast to the system we employed, in which dox administration switched on transgene expression (Tet On). It is therefore possible that the poor peripheral transgene expression observed in the Tet-Zap70 mice (in addition to Tet-Lck (Legname et al., 2000)

and Tet-IL-7R mice (Buentke et al., 2006)) maybe an intrinsic difference between the Tet On and Tet Off gene regulatory systems. It is also possible that the level of inducible transgenic protein detected in the varied Tet-inducible systems may be reflective of the turnover of the specific protein in question. This may account for the differences observed in the time taken for transgene induction to cease in Tet-Off models using similar inducible constructs. Tetracycline controllable OT-1 TCR surface expression persisted for 14 days following cessation of transgene induction by tetracycline treatment (Labrecque et al., 2001). In contrast, tetracycline controlled repression of Eα MHC expression on thymic epithelial cells of Class II MHC deficient mice, persisted for only 2 days following tetracycline administration (Witherden et al., 2000).

The ability of the Tre-Zap70 transgene to restore thymic selection signals within the thymi of Tet-Zap70 mice was extensively examined by assessing transgene induction within the different thymocyte and peripheral T cell subsets generated in dox fed Tet-Zap70 mice. This was firstly assessed in the DN thymocyte population, given that β-selection at the DN3 stage of thymic development requires the transmission of pre-TCR signals by Zap70 and related family member Syk (Cheng et al., 1997). Our data demonstrates that the Tre-Zap70 transgene is unable to rescue thymocyte differentiation following the β-selection checkpoint **(Fig 3.8).** HuCD2 reporter expression indicative of Zap70 expression is undetectable on these subsets (data not shown) and therefore the precise timing of transgene induction is difficult to determine, but suggests it is not fully upregulated until the DP stage of development. However, since Syk

fully compensates for Zap70 in terms of DP cellularity, efficient expression of the Tre-Zap70 transgene during DN thymocyte development does not appear to be required for optimal thymic reconstitution.

Further assessment of huCD2 expression within the DP compartment of dox fed Tet-Zap70 mice, revealed that the efficiency of transgene induction appeared high, illustrated by the large of frequency of DP thymocytes expressing the huCD2 reporter **(Fig 3.3 C and 3.10 A)**. However, huCD2 expression was variable between individuals **(Fig 3.7 A)** and did not appear to be maintained in each thymocyte compartment equally. HuCD2 expression levels were consistently reduced in the mature SP compartments compared to the DP population, suggesting a downregulation of Tre-Zap70 induction following maturation to the CD4 SP and CD8 SP lineages **(Fig 3.10 A)**. Similarly, huCD2 expression was further downregulated in the mature peripheral TCR^{hi} $CD4^{+}$ and $CD8^{+}$ subsets, although the downregulation of transgene expression appeared equivalent between the two populations, suggesting no bias towards either T cell lineage **(Fig 3.10 B)**. Interestingly however, there was evidence of differential transgene induction in the SP thymic compartments of Tet-Zap70 mice. Surface levels of huCD2 were consistently reduced on the CD8 SP thymocytes, compared to the CD4 SP thymocytes generated in Tet-Zap70 mice **(Fig 3.10 A)**. Similarly, CD5 levels were also reduced in the Tet-Zap70 CD8 SP thymocytes compared to WT CD8 SP thymocytes **(Fig 3.9 B)** supporting the suggestion that Tre-Zap70 induction may be reduced within the CD8 SP thymocytes generated in Tet-Zap70 mice. The CD4 SP and CD8 SP subsets are generated from the same population of

DP thymocytes and therefore would be expected to exhibit similar levels of Tre-Zap70 expression. However, there are several possibilities for the generation of this phenotype. The reduced level of huCD2 within the Tet-Zap70 CD8 SP thymocytes but not the CD4 SP thymocytes, may suggest that huCD2 and hence Zap70 expression is downregulated faster in the CD8 SP compartment, and may be an artefact of the reporter employed. It is also possible that the Tet-Zap70 CD8 SP thymocytes are derived from DP thymocytes expressing lower levels of Zap70, suggesting a fundamental difference in the selection signals that govern the fate of DP thymocytes to the $CD4^+$ and $CD8^+$ lineages. Whether WT CD8 SP thymocytes also exhibit reduced levels of Zap70 compared to their CD4 SP counterparts has yet to be formally quanitated. However, examination of CD5 expression, which is dependent on the strength of TCR ligation with spMHC complexes in the thymus (Azzam et al., 1998) suggests that WT CD8 SP thymocytes are selected on the basis of reduced TCR signalling capacity, given that CD5 levels were reproducibly lower on WT CD8 SP thymocytes compared to WT CD4 SP thymocytes **(Fig 3.9 A)**. This is in support of the strength of signal hypothesis regarding CD4-CD8 lineage commitment, which proposes that the duration or strength of TCR signalling influences differentiation to the $CD4^+$ or $CD8^+$ lineages (Itano et al., 1996; Matechak et al., 1996). Short duration signals or weak TCR signals have been suggested to promote the generation of $CD8^+$ lineage, whereas stronger or a longer duration of TCR signalling leads to commitment to the $CD4^+$ lineage (Itano et al., 1996; Liu and Bosselut, 2004; Yasutomo et al., 2000). Currently there is data to suggest that the extent of Lck activation may control cell fate

decisions (Hernandez-Hoyos et al., 2000). Since the CD4 coreceptor molecules isolated from DP thymocytes are associated with more Lck than the CD8 coreceptor, suggests that signalling would be much stronger in response to MHC class II ligands than MHC class I ligands (Ravichandran and Burakoff, 1994; Veillette et al., 1988). If this is the case, the level of Zap70 expression within DP compartment may also influence the strength of signals elicited by TCR ligation with spMHC complexes instructing lineage commitment decisions, given that the transmission of such TCR signals may require Zap70 activity.

Despite the differing levels of Tre-Zap70 transgene induction within the thymocyte subsets, the expression of the Tre-Zap70 transgene in the DP compartment importantly did alleviate the arrest in DP differentiation exhibited in mice deficient for Zap70 expression. TCR and CD5 levels were comparable between WT and Tet-Zap70 DP thymocytes (**Fig 3.9 B)** and the development of the mature TCR^{hi} CD4 SP and TCR^{hi} CD8 SP populations was restored by the Tre-Zap70 transgene (**Fig 3.6 and Fig 3.9 B**) illustrating that the transgenic Zap70 protein can transduce the positive selection signals, required for the differentiation of the DP thymocytes to the $CD4^+$ and $CD8^+$ lineages. This also suggests that although the transgenic Zap70 protein is attached to an additional amino terminal (SH2 termini) peptide ***(See Appendix)***, it does not overtly interfere with the signalling capacity of transgenic Zap70 protein. The Zap70 protein appears to have retained its functionality and is recognised by the cellular machinery involved in the propagation of thymocyte differentiation signals arising at the TCR.

While the data demonstrates the restoration of positive thymic selection following the induction of the Tre-Zap70 transgene **(Fig 3.6, 3.7 and 3.9)**, the efficiency of thymic development in Tet-Zap70 mice appeared reduced compared to mice expressing endogenous Zap70. Tet-Zap70 mice exhibited reduced cellularity within the mature TCR^{hi} CD4 SP and TCR^{hi} CD8 SP thymic compartments **(Fig 3.6 B and C)**. This may occur for several reasons. As previously mentioned, expression of the transgene is high within the thymic compartments of Tet-Zap70 mice, however differing degrees of transgene variegation are apparent between Tet-Zap70 individuals **(Fig 3.7A).** This variegation could potentially reduce the frequency of precursors that can audition for selection and hence ultimately reduce the numbers of DP thymocytes that are positively selected. We also observed that the Tre-Zap70 and huCD2 reporter transgenes were not ubiquitously expressed within the entire DP population, with the Tet-Zap70 DP compartment exhibiting a significant proportion of thymocytes lacking huCD2 expression, further reducing the pool of thymocytes capable of selection **(Fig 3.7 A).** Although it is difficult to quantitate Zap70 expression by western blots analysis, the overall level of protein in the thymi of Tet-Zap70 and WT mice does not appear vastly dissimilar **(Fig 5.5)**. However, it is possible that the profile of Zap70 expression within the DP population of the Tet-Zap70 mice, maybe more heterogeneous on a per cell basis, with a large proportion of cells expressing low levels of the protein compared to WT controls. This has been reported previously in the thymi of Tet-Lck mice also generated using the Tet-inducible system (Legname et al., 2000). This may reduce the number of DP thymocytes capable of

reaching the threshold required for optimal TCR signal transduction and as a consequence decrease the proportion of DP thymocytes capable of positive selection. Detection of intracellular Zap70 on a per cell basis will help to analyse the expression profile of the transgenic Zap70 within the thymic subsets and help evaluate the effectiveness of the Tre-Zap70 transgene in restoring thymocyte development.

In this preliminary study we have analysed the effects of the Tre-Zap70 transgene in positive selection, since Zap70 is essential for the transmission of TCR signals essential for the development of the mature T cell repertoire. However, we have not assessed the potential effect the transgene is having on negative selection within the DP compartment. (Negishi et al., 1995) have shown that Zap70 WT DO10 TCR transgenic thymocytes undergo deletion at the DP stage of development *in vitro* in response to chicken ovalbumin. In contrast Zap70 deficient thymocytes were shown to be resistant to this deletion, suggesting a potential role for Zap70 in negative thymic selection. It is possible that the expression of the Tre-Zap70 transgene may produce changes in thymic selection that are masked by the heterogeneity of the polyclonal TCR repertoire. To investigate this further, the Tet-Zap70 mice are now being bred onto Class I restricted F5 (Mamalaki et al., 1993) and OT-1 TCR transgenic backgrounds (Hogquist et al., 1994). F5 mice express a low affinity TCR compared to OT-1 mice, which express a high affinity TCR as demonstrated by the ability of these cells to homeostatically proliferate in response to peripheral self antigens (Ge et al., 2004). Fixed TCR expression in these monoclonal

transgenics may highlight subtle differences in thymic selection associated with the expression of the Tre-Zap70 transgene.

While αβ T cell development appears to be restored by the Tre-Zap70 transgene, we have not assessed whether the γδ T cell development is affected in Tet-Zap70 mice. Zap70 signalling is also reported to be required for optimal γδ T cell development (Kadlecek et al., 1998), therefore in future experiments we will also investigate whether γδ development proceeds normally in Tet-Zap70 mice.

Examination of the peripheral $CD4^+$ and $CD8^+$ T cell populations revealed both similarities and significant abnormalities compared to mice expressing endogenous Zap70. The presence of TCR^{hi} naïve $CD4^+$ and $CD8^+$ T cells in the LN and spleen of the Tet-Zap70 mice, suggests that the mature CD4 SP and CD8 SP thymocytes generated, are capable of seeding the peripheral compartments **(Fig 3.11 and 3.13)**. The generation of $CD44^{hi}$ memory-like also suggests that the transgene is functional in the periphery despite reduced transgene expression. However, both the TCR^{hi} $CD4^+$ and TCR^{hi} $CD8^+$ populations generated in Tet-Zap70 mice showed a significant reduction in the frequency and absolute number of naïve T cells **(Fig 3.11 and 3.13)**. Hence the periphery of Tet-Zap70 mice may be regarded as partially lymphopenic, with the reduction in cellularity appearing most evident within mature $CD8^+$ compartment. Further evidence of lymphopenia in the periphery of Tet-Zap70 mice was the elevated percentages of mature T cells expressing the activation

marker CD44 **(Fig 3.11 and 3.13)**. This phenotype is likely to reflect cells that have undergone homeostatic expansion in response to the partial lymphopenia (Cho et al., 2000; Ge et al., 2002; Murali-Krishna and Ahmed, 2000b; Oehen and Brduscha-Riem, 1999) possibly as a consequence of the reduction in thymic output.

The mature $CD4^+$ compartment also showed an increase in the numbers of regulatory T cells in the peripheral lymphoid organs **(Fig 3.11 and 3.12 A)**. This increase was not a function of enhanced production of T_{Reg} precursors in the thymi of Tet-Zap70 mice **(Fig 3.12 B)** and therefore implies that the increase was the result of either peripheral expansion or de novo generation of the T_{Reg} population in the periphery. Proliferation of the T_{Reg} population could occur for several reasons. The increased numbers of T_{Reg} cells may occur in response to the partial T cell lymphopenia. Data to support this comes from studies documenting the ability of T_{Reg} cells to divide following transfer into T cell deficient hosts. (Zhang et al., 2005) reported that peripheral lymphopenia augmented T_{Reg} expansion in the presence IL-2 therapy, above the expansion seen with IL-2 alone, suggesting that lymphopenia can modulate T_{Reg} homeostasis. It is also possible however that the increased numbers of T_{Reg} cells observed in the Tet-Zap70 individuals may also occur as a counter-response, to dampen the expansion within the $CD44^{hi}$ memory-like compartment (Shen et al., 2005). The expansion could also reflect a cell autonomous defect associated Tre-Zap70 transgene induction. The underlying cause for the increased proportion of $CD4^+$ T_{Reg} cells in Tet-Zap70 mice is examined more extensively in the subsequent chapter.

The reason for the peripheral lymphopenia, which alters the composition of the peripheral $CD4^+$ and $CD8^+$ subsets, is unclear. The precise quantification of Zap70 protein in the LN and spleen is yet to be performed, but as already mentioned the expression of huCD2 reporting Zap70 expression is much lower in the peripheral lymphoid tissues compared to the thymus **(Fig 3.10)**, showing equal induction of the Zap70 transgene within the mature $CD4^+$ and $CD8^+$ T cell subsets. However, there is a disparity in the reconstitution of the peripheral lymphoid tissues by the $CD4^+$ and $CD8^+$ T cells. The extensive reduction in the cellularity of peripheral naïve and memory-like $CD8^+$ compartments but partial reduction of the mature naïve $CD4^+$ population in Tet-Zap70 mice, suggests that the $CD4^+$ compartment is less sensitive to a reduction in Zap70 expression. The dramatic reduction in the number of mature naive $CD8^+$ T cells in the periphery of Tet-Zap70 mice does not reflect altered thymic generation, since the ratio of CD4 SP versus CD8 SP thymocytes is normal in the thymi of Tet-Zap70 mice **(Fig 3.6 D)**. The data could imply a defect in the egress of the newly generated cells from the thymus, given that reports have shown that Zap70 and LAT are critical for the regulation of β_1 intergrin function by the TCR/CD3 complex. Therefore impaired peripheral expression of Zap70 may have effects on the homing of the mature T cells to the lymphoid tissues (Epler et al., 2000; Goda et al., 2004). The diminished naïve $CD8^+$ compartment may also reflect impaired survival of the lymphocytes within the peripheral lymphoid tissues. This may involve defects in the ability of the cells to receive homeostatic TCR survival signals, for example to engage spMHC presented by DCs or as consequence T cell intrinsic defects associated reduced Zap70

expression. If this is the case, this would suggest Zap70 has a role in the propagation of TCR survival signals. The specific role of Zap70 signals in T cell survival is investigated in more detail in the subsequent chapter.

Interestingly, the reduction in the absolute numbers of mature $CD8^+$ T cells appeared most apparent in the naïve compartment. In contrast the $CD8^+$ memory-like T cell population showed a smaller decline in numbers compared to controls, suggesting that the memory compartment is less dependent on Zap70 expression for its maintenance. This view is consistent with the literature which reports that memory $CD8^+$ T cells do not to require TCR based signals for their continued survival (Murali-Krishna et al., 1999).

The data presented in this chapter also suggests new roles for Zap70 in the regulation of CD5 and CD122 expression in thymocytes and peripheral T cells. CD5 but not TCR expression was consistently reduced on the CD8 SP thymocytes **(Fig 3.9 B)** and all the peripheral $CD4^+$ and $CD8^+$ subsets generated in Tet-Zap70 mice compared to the WT control **(Fig 3.17).** The reduction in CD5 levels also correlated well with the observed downregulation of huCD2 expression **(Fig 3.10)** suggesting that Zap70 may play a role in the transmission of TCR signals that influence CD5 expression. This may have important implications in the peripheral phenotype of Tet-Zap70 T cells. *CD5*$^{-/-}$ T cells are hyperresponsive to TCR stimulation (Pena-Rossi et al., 1999), therefore the reduced expression of CD5 on the peripheral naïve $CD4^+$ and $CD8^+$ T cells may affect the ability of the cells to be activated by antigenic stimulation. The frequency of TCR^{hi} T cells expressing the activation markers

CD25 and CD69 appeared within the normal range for the mature $CD4^+$ T compartment (data not shown). In contrast the $CD8^+$ population showed an increased frequency of activated T cells expressing CD25 and/or CD69 **(Fig 3.16)** and also showed an increase in the percentage of $CD8^+$ T cells expressing the activation associated glycoform of CD43, reported to be upregulated on activated effector memory lymphocytes **(Fig 3.15)** (Harrington et al., 2000). This data is somewhat surprising given that Zap70 expression is crucial for the transmission of TCR signals required for T cell activation (Arpaia et al., 1994) and assists in the recruitment of WASP to the immunological synapse during T cell activation, where it plays a critical role in actin re-organization stabilising contacts between the T cell and the APC (Gallego et al., 1997; Sasahara et al., 2002; Snapper et al., 1998). Hence a partial loss of Zap70 expression would be expected to reduce not increase the capacity of the cells to be activated by antigen. The reduction in CD5 expression at least within the $CD8^+$ T cell compartment may impart a degree of hyperactivity and augment the frequency of cells capable of activation by self or environmental antigen. These data may also suggest that the $CD8^+$ T cell compartment is more sensitive to a reduction in CD5 expression given that the mature $CD4^+$ population appears unaffected by the decline in CD5 surface levels. To confirm these observations, the capacity of the naive $CD4^+$ and $CD8^+$ T cells to be activated *in vitro* will be investigated, to determine whether the threshold for activation is impaired in Tet-Zap70 T cells exhibiting a partial loss of Zap-70 and CD5 expression. The potential significance of the reduced expression of CD5 on the $CD4^+$ and $CD8^+$ memory-like T cells as well as the $CD4^+$ T_{Reg} cells

will also be investigated in future studies. Overall this data suggests that Zap70 may play a role in the regulation of CD5 expression in the thymus and periphery. Interestingly studies have reported that CD5 levels are modulated by the activity of the Src kinase Lck in DN thymocytes (Azzam et al., 1998) and in peripheral naïve $CD4^+$ T cells (Smith et al., 2001a). Since Lck is located upstream of Zap70 in the TCR signalling cascade, it is conceivable that Zap70 may also propagate such signals.

As well as regulating CD5 expression the data presented in this chapter also implies a similar role in the regulation of CD122 expression. The memory-like (TCR^{hi} $CD44^{hi}$) $CD8^+$ T cells generated in Tet-Zap70 mice displayed reduced expression of CD122 suggesting a potential a role for Zap70 activity in the regulation or transduction of signals leading to CD122 upregulation **(Fig 3.14)**. However since Tet-Zap70 mice are partially lymphopenic, the increased proportion of $CD44^{hi}$ lymphocytes is likely to have occurred as a result of increased homeostatic proliferation within the depleted $CD8^+$ T cell compartment. Given that the WT and Tet-Zap70 $CD8^+$ memory-like cells have been generated in different environments, replete versus lymphopenic respectively, it is difficult to compare the two subsets. Further experiments analysing the expression of CD122 on Tet-Zap70 $CD44^{hi}$ cells generated in a replete host would reveal the true role of Zap70 in the regulation of CD122 expression. Focused experiments examining the *in vitro* and *in vivo* generation of antigen specific memory cells from Tet-Zap70 mice also needs to be performed in order to address whether Zap70 has a qualitative role in the generation of memory phenotype cells.

It is also possible that the CD122^{lo} subset of naturally occurring memory-phenotype T cells generated in response to self antigens, which under normal conditions represent ~30% of the memory-phenotype T cell population (Boyman et al., 2006), maybe over represented in Tet-Zap70 mice. This population is reported to be enriched with partly activated cells of a CD43^{hi} and CD69^{hi} phenotype. Similarly we also observed an increased frequency of CD43^{hi} and CD69^{hi} cells with the mature CD8^{+} T cell compartment generated in Tet-Zap70 mice. Therefore it is unclear at present whether these cells represent activated cells, effector cells or the CD122^{lo} subset of memory-phenotype cells. Further analysis of CD44, CD43, CD69 and CD122 on the same cells, would help to determine whether the activated and effector phenotype cells are constituents of the same chronically stimulated CD8^{+} T cell population. The CD8^{+} CD122^{lo} subset of memory-phenotype cells differ from typical effector cells in that they are CD25^{lo}, therefore analysis of CD122 and CD25 expression together would be useful in determining the nature of the cells within the peripheral CD8^{+} T cell compartment generated in Tet-Zap70 mice.

Interestingly CD122 (IL-15Rβ) expression in the peripheral naïve CD8 (TCR^{hi} CD44^{lo}) T cells was also consistently reduced on all the Tet-Zap70 mice analysed **(Fig 3.14)**. This may affect the survival of the naïve CD8^{+} T cells, given that *Il-15*$^{-/-}$ mice exhibit a reduction in the number of naïve CD8^{+} T cells but not naïve CD4^{+} T cells in the secondary lymphoid tissues (Kennedy et al., 2000b). In addition (Berard et al., 2003), have shown that exogenous IL-15 reduces the spontaneous apoptosis of naïve CD8^{+} T cells *in vitro* and observed

that this correlated with and increase of Bcl-2 expression. To dissect the role of IL-15 cytokine signals in the survival of the naïve $CD8^+$ T cells generated in Tet-Zap70 mice future experiments will compare the survival of naïve Tet-Zap70 and WT $CD8^+$ T cells in *Il-15*$^{-/-}$ recipients.

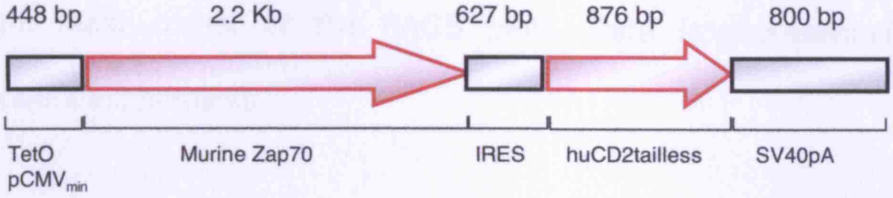
In summary the present study demonstrates that the Tre-Zap70 transgene is capable of restoring positive selection within the DP compartment. Importantly Zap70 successfully transduces the interaction of DP thymocytes expressing the Tre-Zap70 transgene with positively selecting ligands and this activity is sufficient to drive the normal selection and maturation of the thymocyte subsets. Although the Tet-Zap70 protein is attached to a foreign peptide, it is still functional and recognised by the cellular machinery required for the propagation of thymocyte differentiation signals. This data also implies that while the selection signals leading to the generation of mature CD4 SP and CD8 SP cells are restored, the frequency of DP cells expressing Zap70 and therefore capable of differentiation to either the $CD4^+$ or $CD8^+$ lineage is reduced given that the overall cellularity of the CD4 SP and CD8 SP populations in the Tet-Zap70 thymi is reduced. The combined effects of reduced thymic yield, possible heterogenous expression of the transgene in the thymocyte subsets as well as a downregulation of huCD2/Zap70 expression within in the mature peripheral T cells, interestingly results in periphery largely lymphopenic within the mature $CD8^+$ compartment but only partially within the naïve $CD4^+$ compartment. Whether the disparity observed in the cellularity of the peripheral $CD4^+$ and $CD8^+$ compartments reflects differences in survival

associated with a partial loss of Zap70 expression and hence signalling via the TCR is addressed in the following ***Chapter 4***.

Figure 3.1: Tre-Zap70 Inducible constructs

A schematic representation of the tetracycline inducible constructs used for the generation of the Tet-Zap70 and Tet-huβg-Zap-70 transgenic mouse lines. **A)** Tre-Zap70 Construct 1. A polylinker primer encoding a MCS *(see **Materials and Methods Fig 2.1**)* was subcloned into a plasmid downstream of the TetO/CMV_{min} promoter and upstream of a SV40 polyadenylation signal. Further fragments encoding an IREShuCD2tailless reporter (a gift from Dr O Williams, UCL) were subcloned into the plasmid upstream of the SV40pA sequence using imported restriction sites within the polylinker. Further subcloning involved ligation of the cDNA encoding the Mus musculus Zap70 transgene (I.M.A.G.E clone 4925739) into the construct downstream of the TetO/CMV_{min} promoter. **B)** Tre-huβg-Zap-70 Construct 2. The construct was generated as above, but contains an additional intronic sequence. A BamHI-EcoRI fragment excised from the intronic sequence between exon 2 and exon 3 of the human β-globin gene (a gift from Dr D Kioussis) was subcloned into the plasmid downstream of the 5' TetO/CMV_{min} promoter and upstream of the mouse Zap70 transgene. Arrows denote open reading frames.

A. Tre-Zap-70 Construct 1



B. Tre-huβg-Zap-70 Construct 2

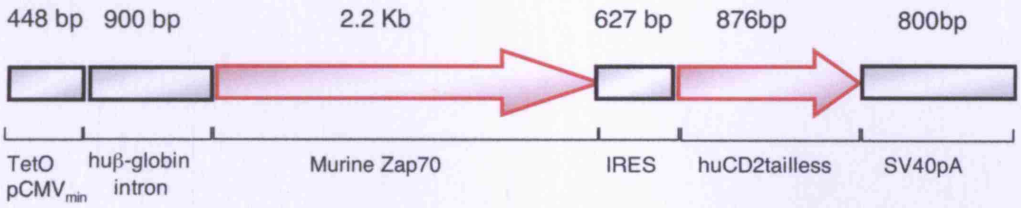


Figure 3.2: *In-vitro* testing of inducible mouse Zap70 DNA constructs

A HEK cell line stably expressing the rtTA was transiently transfected with the dox inducible plasmids and cultured with and without dox (2μg/ml). **A)** Untransfected negative control. **B)** pBiEGFP positive control. **C)** Tre-Zap70 construct 1 and **D)** Tre-huβg-Zap70 construct 2. The expression of huCD2tailless and EGFP reporters were measured by FACS analysis 24 h post transfection. Percentages of reporter positive or negative cells is indicated in the right hand corner of the FACS plots. Data is representative of 2 independent experiments.

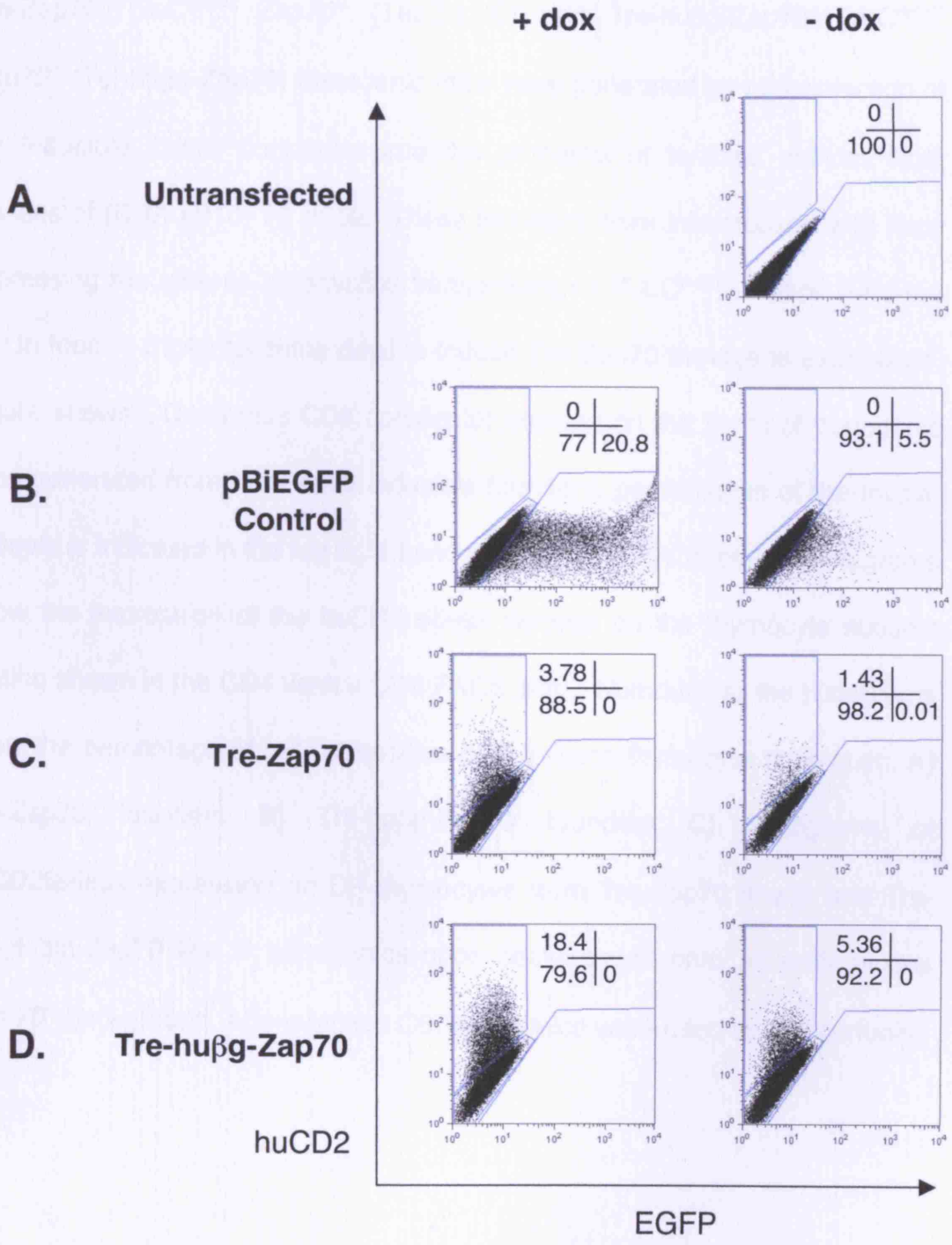
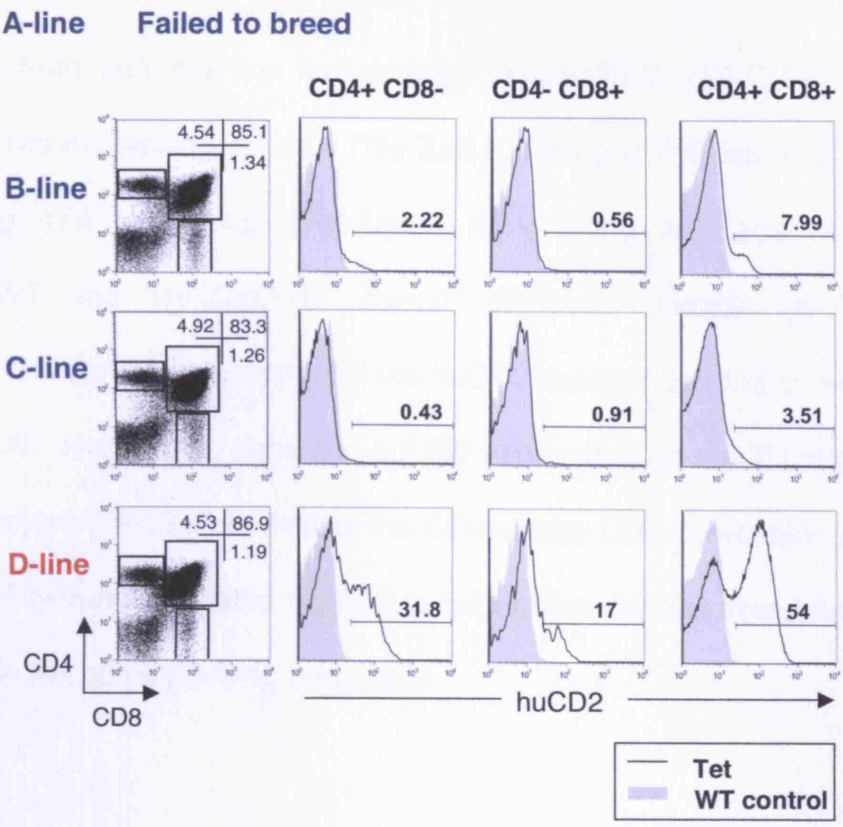

+ dox
- dox
A.
Untransfected
0
100 0
B.
pBiEGFP
Control
0
77 20.8
0
93.1 5.5
C.
Tre-Zap70
3.78
88.5 0
1.43
98.2 0.01
D.
Tre-huβg-Zap70
18.4
79.6 0
5.36
92.2 0
huCD2
EGFP

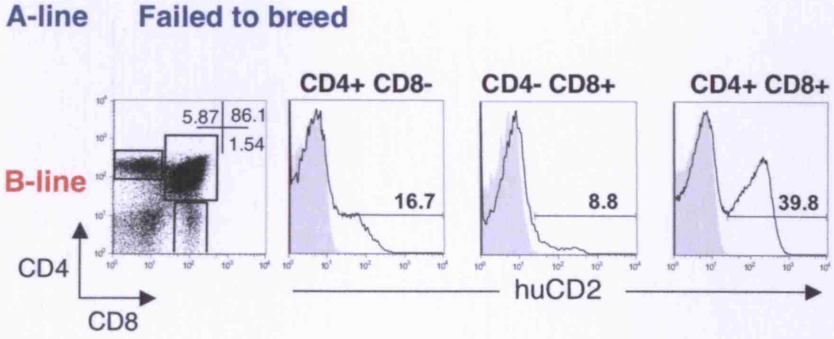
Figure 3.3: Founders generated from microinjections of Tre-Zap70 and Tre-huβg-Zap70 constructs.

Tre-Zap70/rtTA.C^{huCD2} *Zap70*$^{-/-}$ (Tet-Zap70) and Tre-huβg-Zap70/rtTA.C^{huCD2} *Zap70*$^{-/-}$ (Tet-huβg-Zap70) transgenic mice were generated by microinjection of the inducible Zap70 constructs into the pronuclei of fertlized oocytes from crosses of (CBA xB10) F1 mice. These founders were intercrossed with mice expressing the reverse tetracycline transactivator (rtTA.C^{huCD2}). Mice were fed dox in food (1 mg/g) for three days to induce Tre-Zap70 transgene expression. Figure shows CD4 versus CD8 coreceptor staining on the thymi of transgenic lines generated from the Zap70 inducible founders, percentages of the thymic subsets is indicated in the top right hand corner of the FACS plots. Histograms show the expression of the huCD2tailless reporter on the thymocyte subsets (gating shown in the CD4 versus CD8 FACS plot). Numbers on the histograms show the percentage of huCD2 positive cells in each thymocyte population. **A)** Tre-Zap70 founders **B)** Tre-huβg-Zap70 founders **C)** histograms of huCD2tailless expression on DP thymocytes from Tre-Zap70 line D and Tre-huβglobin-Zap70 line B transgenics once backcrossed onto an endogenous *Zap-70*$^{-/-}$ background. Age-matched C57BL/6J mice were used as WT controls.

A. Tre-Zap70 Founders



B. Tre-huβg-Zap70 Founders



C.

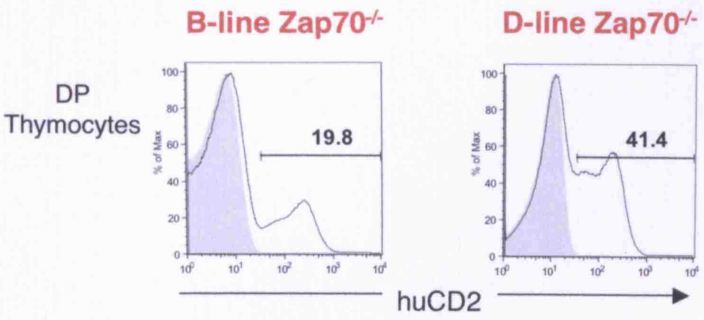


Figure 3.4: Tre-Zap70 transgene induction is dependent on rtTA expression.

Thymocytes from 7wk old dox fed (1mg/g) Tet-Zap70.D rtTA.C^{huCD2} *Zap70*$^{-/-}$ transgenic mice expressing the rtTA (Tre-Zap + rtTA) and Tet-Zap70.D *Zap70*$^{-/-}$ mice lacking rtTA expression (Tet-Zap – rtTA) along with aged matched C57BL/6J WT and Tre-Zap70.D^{-} *Zap70*$^{-/-}$ littermate controls (KO) were analysed for the surface expression of the huCD2 reporter and the coreceptors CD4 and CD8. Histograms show the huCD2 expression on the T lymphocyte gated thymocytes. FACS plots display the CD4 versus CD8 coreceptor staining on the total T lymphocyte gated thymocyte population. Subset frequencies are shown next to the corresponding population.

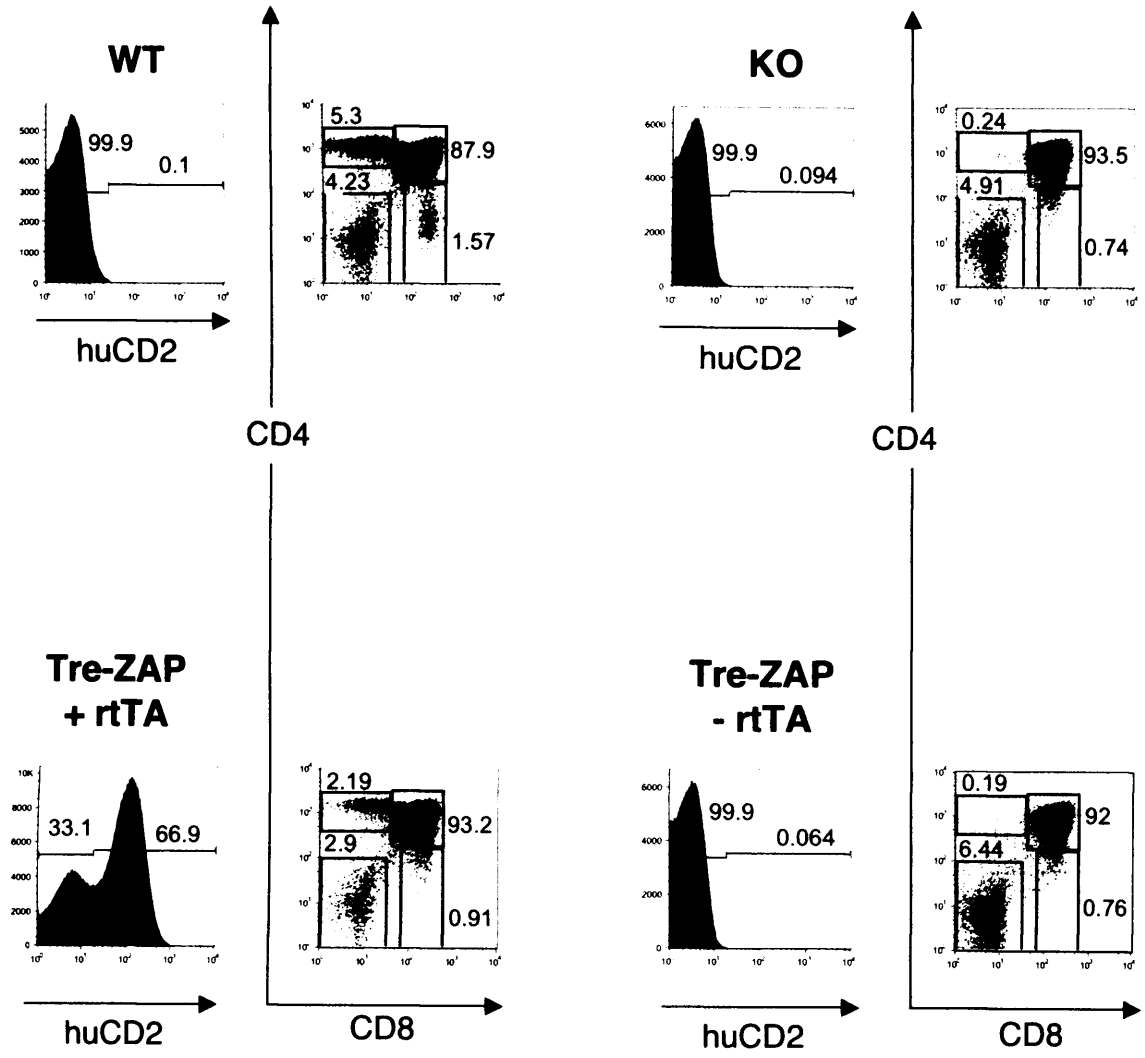
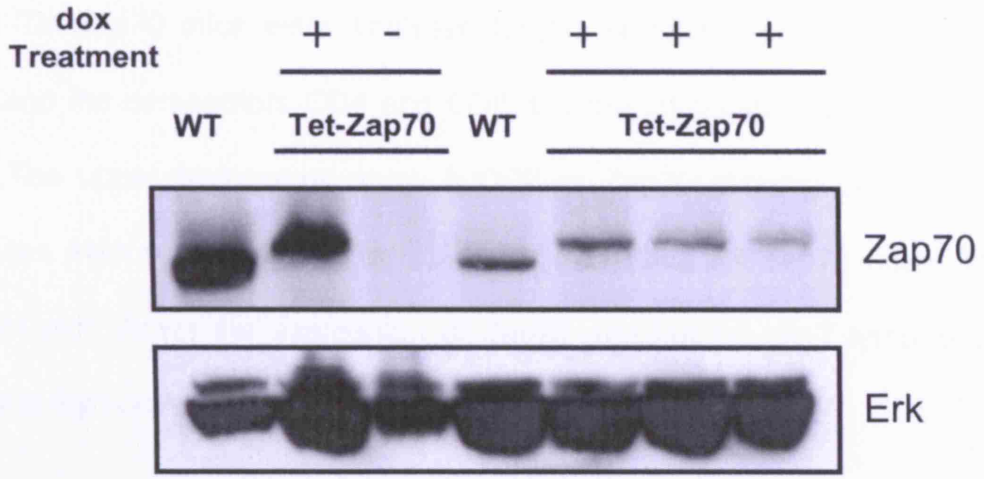

WT
99.9
0.1
huCD2
5.3
87.9
4.23
1.57
CD4
KO
99.9
0.094
huCD2
0.24
93.5
4.91
0.74
CD4
Tre-ZAP
+ rtTA
33.1
66.9
huCD2
2.19
93.2
2.9
0.91
CD8
Tre-ZAP
- rtTA
99.9
0.064
huCD2
0.19
92
6.44
0.76
CD8

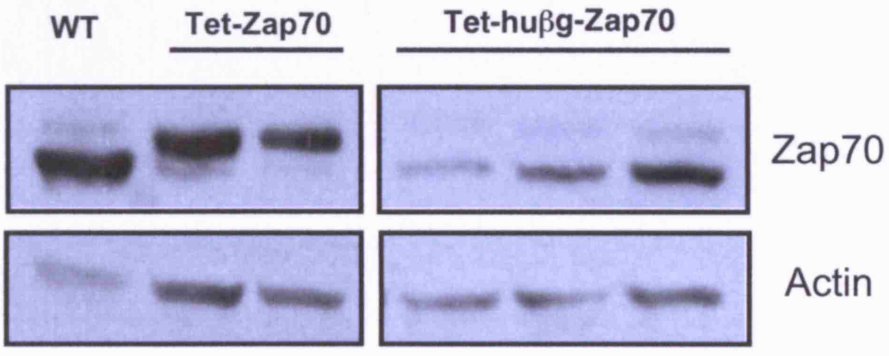
Figure 3.5: Zap70 protein expression in Tet-Zap70 mice.

Total cell lysates were prepared from the thymi of WT mice, Tet-Zap70 **(A)** and Tet-huβg-Zap70 mice **(B)**, on or off dox (1mg/g) as indicated **(A)**. 50μg of each protein sample were separated on 10% SDS-PAGE mini gels and western blotted with anti-Zap70 antibodies. Blots of thymus lysates were probed for actin or Erk as loading controls. Data is representative of two independent experiments. (Western blot was kindly produced by Ana Silva).

A.


dox
Treatment
+ -
+ + +
WT
Tet-Zap70
WT
Tet-Zap70
Zap70
Erk

B.


WT
Tet-Zap70
Tet-huβg-Zap70
Zap70
Actin

Supplemental Figure: added following viva examination. Intracellular Zap70 expression in Tet-Zap70 mice.

The thymi of 5-12 wk old WT, Zap70 deficient and constitutively dox fed (1mg/g) Tet-Zap70 mice were analysed for the surface expression of TCR, huCD2 and the coreceptors CD4 and CD8 and the intracellular expression of Zap70. The upper histograms show huCD2 or Zap70 staining on the total thymocytes from representative WT, *Zap70*$^{-/-}$ (KO) and Tet-Zap70 mice. The lower dot plot shows the expression of Zap70 and huCD2 on T lymphocyte gated total thymocytes from a representative Tet-Zap70 mouse.

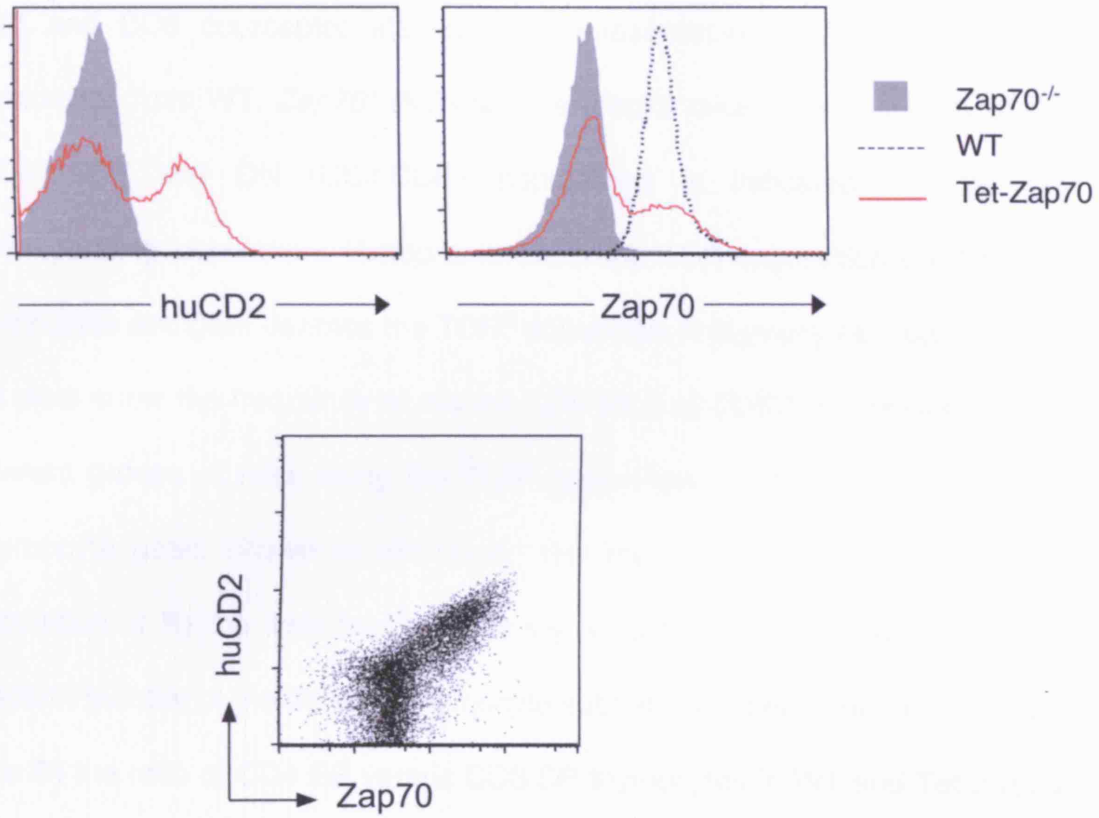
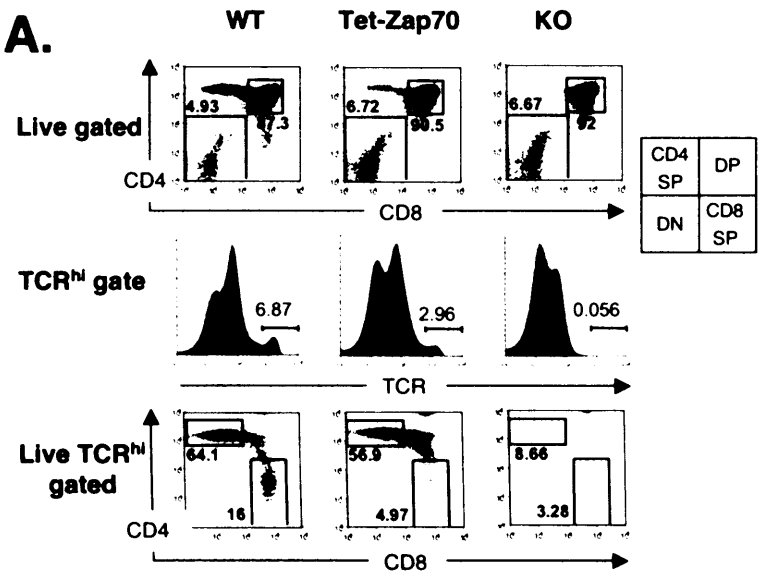
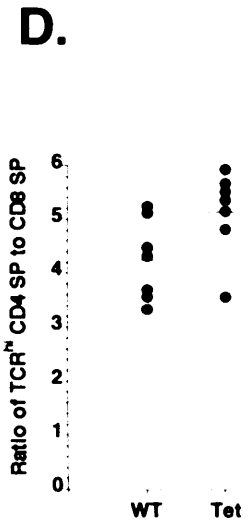

huCD2
Zap70
Zap70-/-
WT
Tet-Zap70
huCD2
Zap70

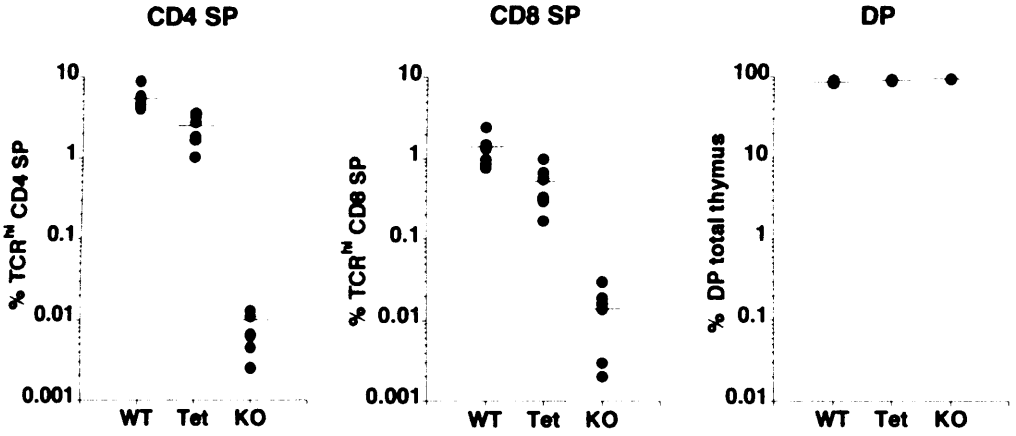
Figure 3.6: Thymocyte development is restored in Zap70 deficient mice following induction of the Tre-Zap70 transgene.

The thymi of 5-12 wk old WT, Zap70 deficient and constitutively dox fed (1mg/g) Tet-Zap70 mice were analysed for the surface expression of TCR, huCD2 and the coreceptors CD4 and CD8. **A)** The upper dot plots shows the CD4 and CD8 coreceptor staining on representative T lymphocyte gated thymocytes from WT, *Zap70*$^{-/-}$ (KO) and Tet-Zap70 mice. The frequency of DP ($CD4^+CD8^+$) and DN ($CD4^-CD8^-$) populations is indicated next to the corresponding population. Histograms show the TCR expression on the total thymocytes and gate denotes the TCR^{hi} population of thymocytes. Lower row of dot plots show the frequency of mature CD4 SP and CD8 SP generated in the different groups of mice using the TCR^{hi} gate shown in the TCR histograms. Thymocyte gates shown in the upper and lower panels were used in the calculation of **B)** the total frequency of thymic subsets generated and **C)** the absolute number of the different thymocyte subsets in the each of the individual mice **D)** the ratio of CD4 SP versus CD8 SP thymocytes in WT and Tet-Zap70 mice. Group sizes were as follows WT n = 7, Tet-Zap70 n = 7 and *Zap70*$^{-/-}$ n = 6 pooled from three independent experiments.





B. Percentages of Total Thymus



C. Thymus Absolute Cell Numbers

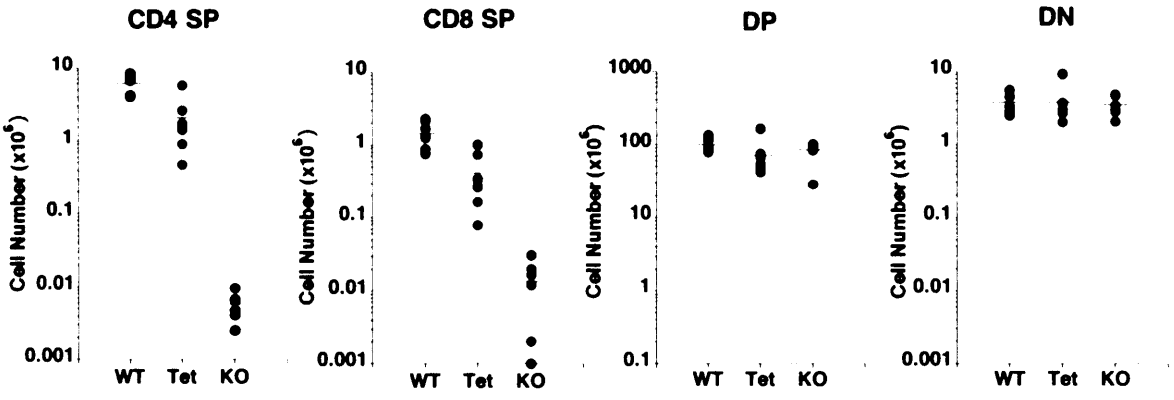
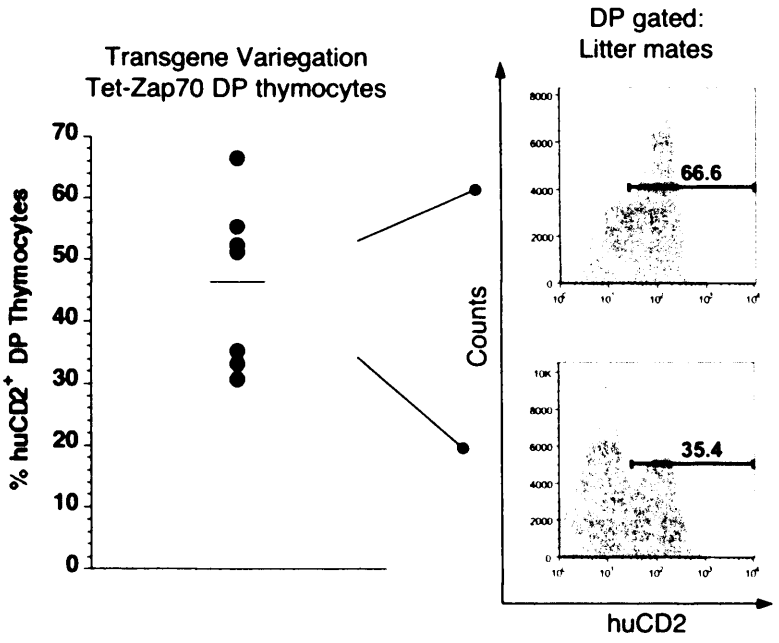


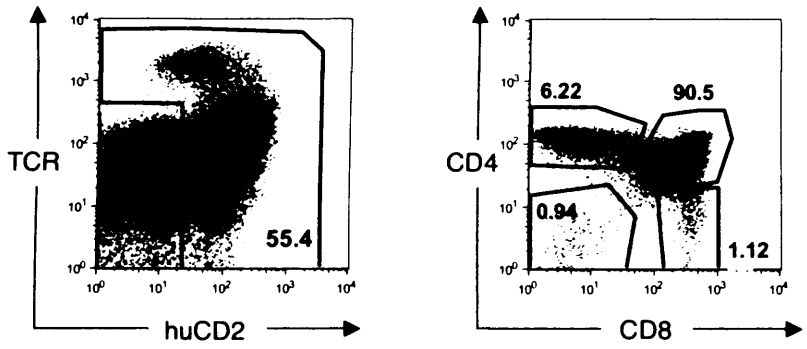
Figure 3.7: Variegation of Tre-Zap70 transgene expression.

The thymi of 5-12 wk old WT and constitutively dox fed (1mg/g) Tet-Zap70 mice were analysed for the surface expression of huCD2, TCR and the coreceptors CD4 and CD8. **A)** Scatter plot shows the frequency of T lymphocyte gated huCD2 positive $CD4^+CD8^+$ DP thymocytes in all the individual Tet-Zap70 mice analysed. Histograms show the profile of huCD2 staining on T lymphocyte gated DP thymocytes from a pair of Tet-Zap70 littermates analysed on the same experimental day. Numbers shown in the histogram FACS plots reflect the percentage of cells positive for huCD2 expression. **B)** shows FACS profiles of TCR versus huCD2 reporter expression on T lymphocyte gated total thymocytes from a representative Tet-Zap70 mouse. TCR^{hi} huCD2 positive gate indicates the populations of thymocytes that show evidence of transgene expression (induced thymocytes). Right hand side FACS plot shows the CD4 versus CD8 coreceptor expression on the TCR^{hi} huCD2 positive gated thymocytes. Percentages of the thymocyte subsets are indicated next to the corresponding populations. **C)** shows scatter plots of the total frequency of CD4 SP and CD8 SP thymocytes generated in all the WT and Tet-Zap70 individuals. Calculation of Tet-Zap70 thymocyte frequencies was performed on the induced cells only. Group sizes were as follows WT n = 7 and Tet-Zap70 n = 7 pooled from three independent experiments.

A.



B. Gating of induced Tet-Zap70 thymocytes



C. Percentage of Induced Tet-Zap70 CD4 SP and CD8 SP

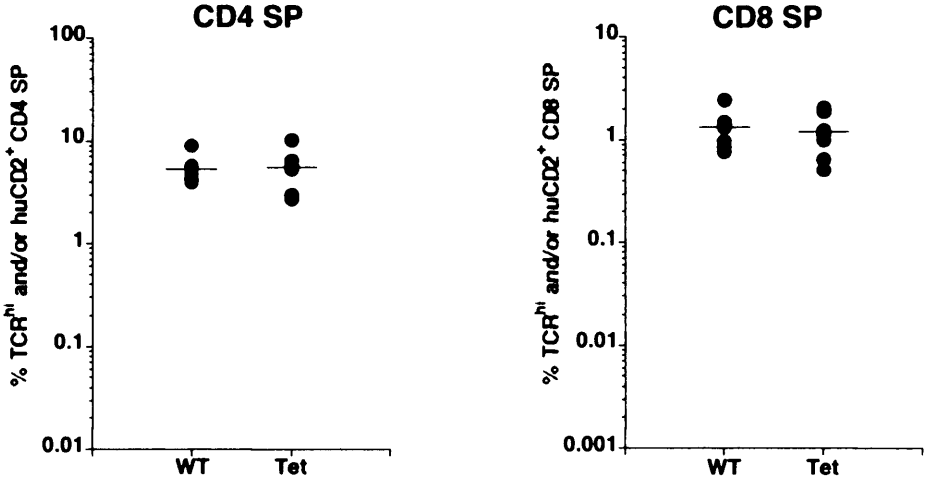
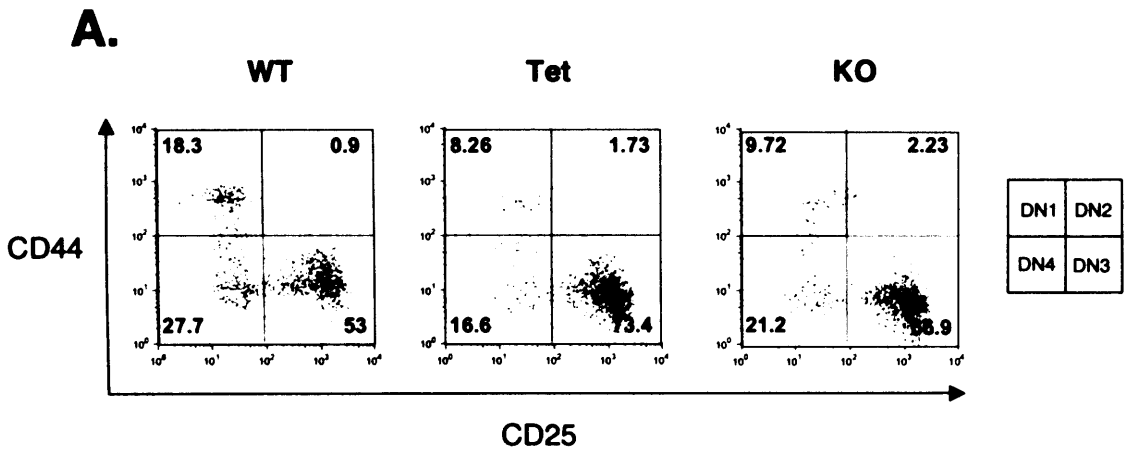


Figure 3.8: Tre-Zap70 transgene induction occurs during the later stages of thymopoiesis and cannot rescue DN thymocyte development.

Thymocytes from 5-12 wk old WT, Tet-Zap70 and Zap70 deficient mice were analysed for the expression of the coreceptors CD4 and CD8, CD44 and CD25 to assess the development of the DN1-4 subpopulations. **A)** shows dot plots of CD44 versus CD25 on the T lymphocyte gated $CD4^-$ $CD8^-$ coreceptor negative (DN) populations from representative mice. Numbers represent the percentage of cells in each gated region. **B)** shows the total frequency of DN1-DN4 subsets in the different groups of mice using the DN gates shown in CD44:CD25 FACS plots. Group sizes were as follows WT n = 7, Tet-Zap70 n = 7 and $Zap70^{-/-}$ n = 6 pooled from three independent experiments.


A.
WT
Tet
KO
18.3
0.9
27.7
53
8.26
1.73
16.6
73.4
9.72
2.23
21.2
56.9
CD44
CD25
DN1
DN2
DN4
DN3

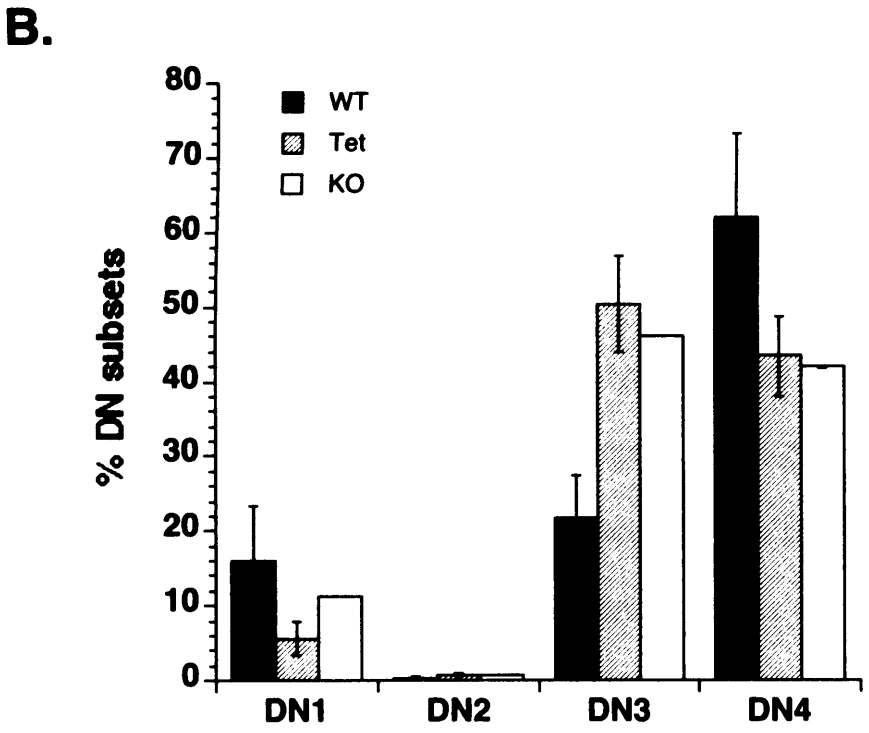

B.
% DN subsets
WT
Tet
KO
0
10
20
30
40
50
60
70
80
DN1
DN2
DN3
DN4

Figure 3.9: Tre-Zap70 transgene restores positive selection in Zap70 deficient mice.

Thymocytes from 5-12 wk old constitutively dox fed (1mg/g) Tet-Zap70 mice and aged matched WT and Zap70 deficient controls were analysed for the surface expression of CD8, CD4, TCR, CD5 and huCD2. **A)** shows FACS plots of CD4 versus CD8 coreceptor staining on representative T lymphocyte gated WT and Zap70 deficient thymi (KO). Histograms show the TCR and CD5 expression on the CD4 SP, DP, CD8 SP and DN thymocyte subsets shown in the CD4:CD8 FACS plots. Zap70 deficient and WT histograms are overlaid. (Dotted line = *Zap70*$^{-/-}$ thymocytes and solid line = WT thymocytes). **B)** shows histogram of huCD2 reporter expression in the thymus of a representative Tet-Zap70 mouse, gates denote huCD2 positive and huCD2 negative populations. The dot plots show the CD4 and CD8 coreceptor expression on the huCD2 positive and negative populations using the gates shown in the huCD2 histogram. Far right histograms show the TCR and CD5 expression on the huCD2 positive and huCD2 negative thymocyte subsets indicated in the CD4:CD8 FACS profiles. (Shaded histograms represent Tet-Zap70 thymocytes, dotted lines indicated Zap70 deficient thymocytes and the solid lines indicate WT thymocytes). Numbers represent the frequency of cells within each gated region. A total of n = 7 WT, n = 7 Tet-Zap70 and n = 6 Zap70 deficient mice were analysed.

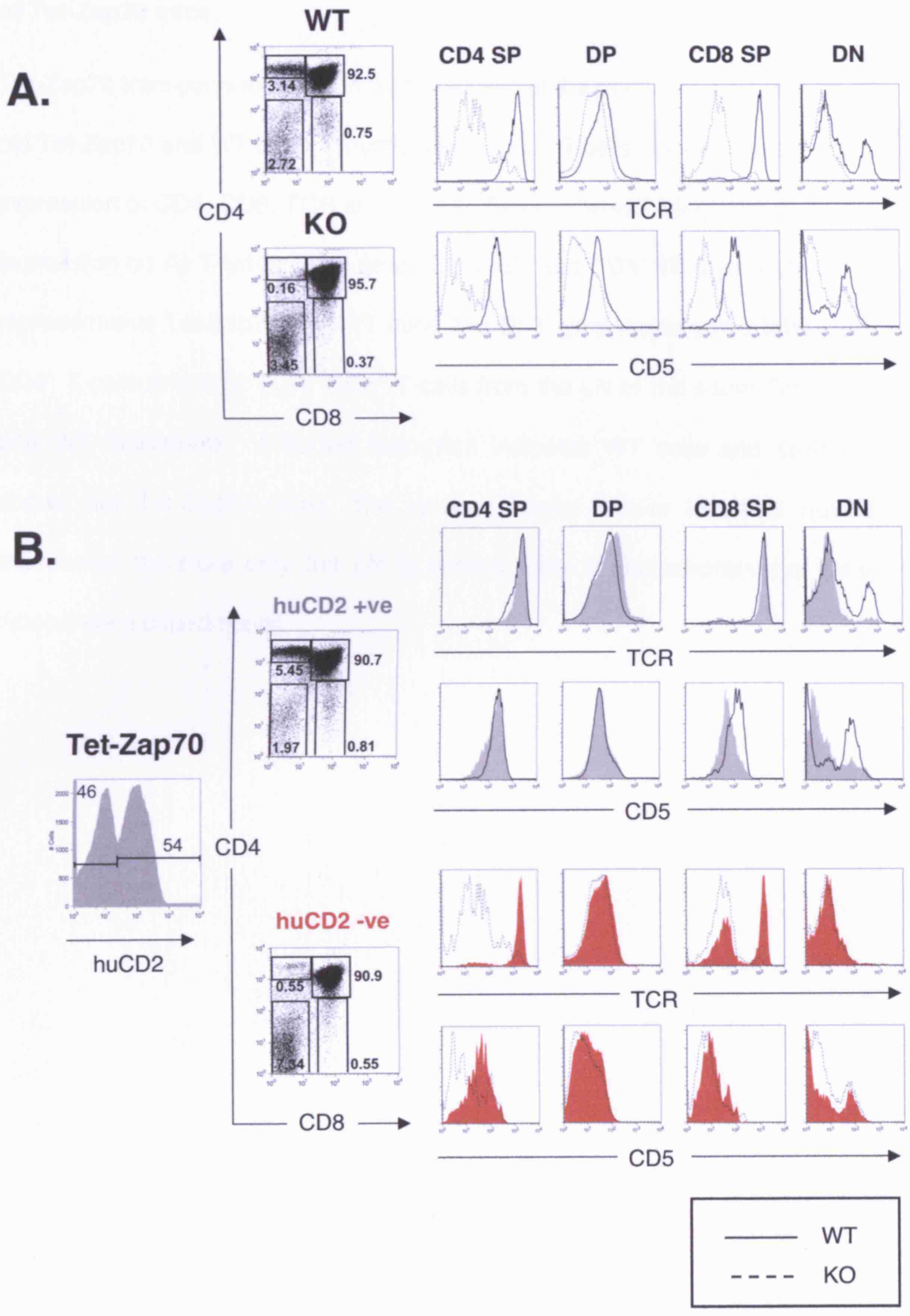
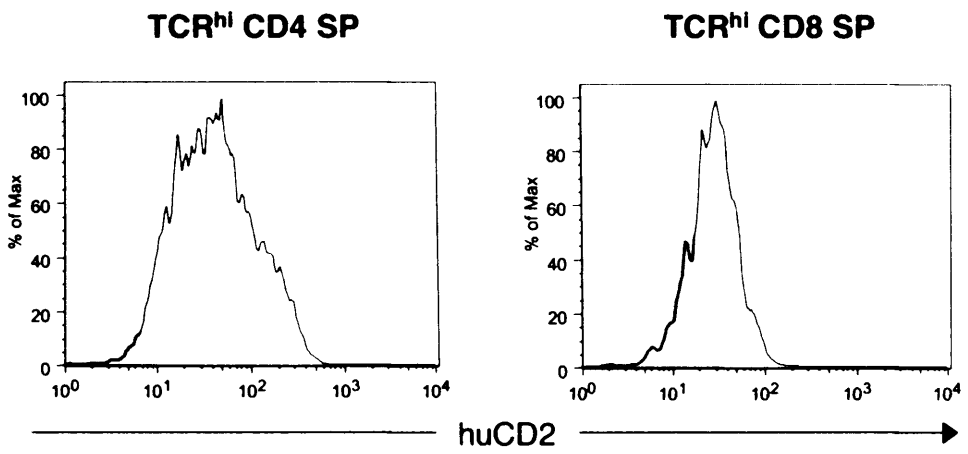

A.
WT
92.5
3.14
0.75
2.72
KO
0.16
95.7
3.45
0.37
CD4
CD8
CD4 SP
DP
CD8 SP
DN
TCR
CD5
B.
Tet-Zap70
46
54
huCD2
huCD2 +ve
5.45
90.7
1.97
0.81
huCD2 -ve
0.55
90.9
7.34
0.55
CD4
CD8
CD4 SP
DP
CD8 SP
DN
TCR
CD5
TCR
CD5
WT
KO

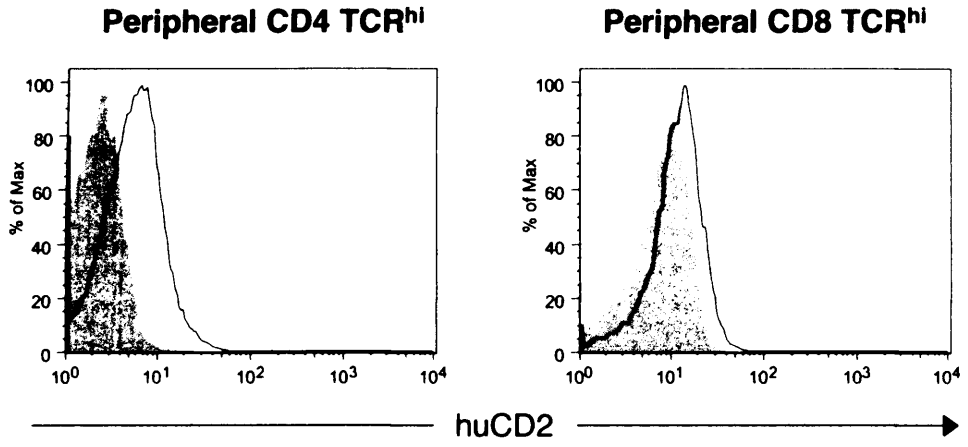
Figure 3.10: Tre-Zap70 transgene expression in the thymi and periphery of Tet-Zap70 mice.

Tre-Zap70 transgene expression was analysed in the thymi and LNs of 5-12 wk old Tet-Zap70 and WT mice. Thymocytes and LN T cells were analysed for the expression of CD4, CD8, TCR and huCD2. Figure shows histograms of huCD2 expression on **A)** T lymphocyte gated $CD4^+$ SP and $CD8^+$ SP thymocytes from representative Tet-Zap70 and WT mice and **B)** T lymphocyte gated total TCR^{hi} $CD4^+$ T cells and total TCR^{hi} $CD8^+$ T cells from the LN of the same Tet-Zap70 and WT individuals. (Shaded histogram indicates WT cells and solid line shows the Tet-Zap70 cells). The spleen showed similar levels of huCD2 expression therefore only the LN is shown. Data is representative of three independent experiments.

A.


TCR^hi CD4 SP
TCR^hi CD8 SP
% of Max
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
huCD2

B.


Peripheral CD4 TCR^hi
Peripheral CD8 TCR^hi
% of Max
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
huCD2

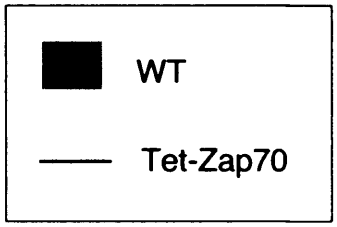
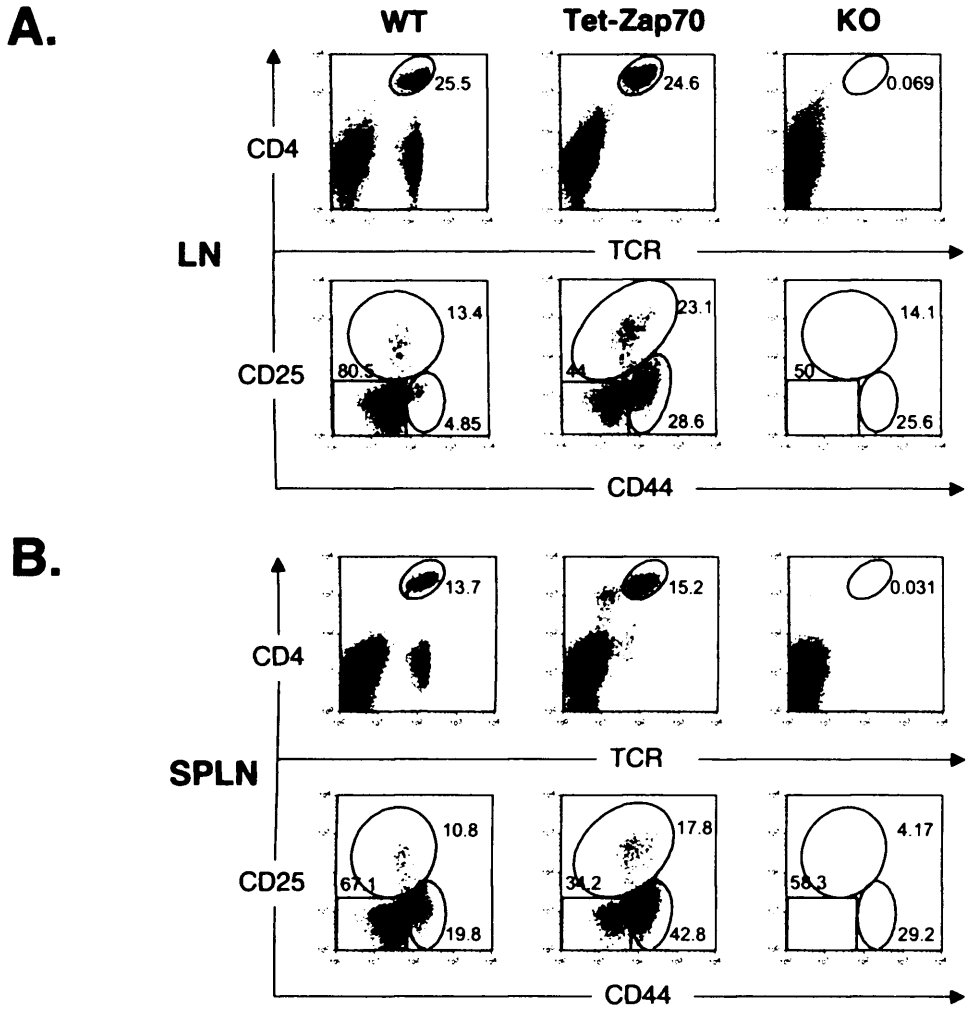

WT
Tet-Zap70

Figure 3.11: Peripheral phenotype of CD4 lineage T cells in Tet-Zap70 mice.

T cells from the LN and spleen of 5-12 wk old WT, Zap70 deficient and Tet-Zap70 mice were analysed for the expression of the coreceptors CD4 and CD8, TCR, CD44 and CD25. The upper panels of FACS plots show CD4 versus TCR expression on the T lymphocyte gated T cells, gate denotes the $CD4^+$ TCR^{hi} population of T cells. The lower panels show the CD44 and CD25 expression on the $CD4^+$ TCR^{hi} cells shown in the upper FACS plots. **A)** shows the phenotype of $CD4^+$ T cells recovered from the LNs and **B)** shows the phenotype of $CD4^+$ T cells recovered from the spleen of representative mice. The numbers show the frequency of cells in each T cell gate that were used to calculate **C)** of the absolute number of cells in each of the peripheral $CD4^+$ TCR^{hi} T cell subsets in each of the individual mice. Group sizes were as follows WT n = 7, Tet-Zap70 n = 7 and $Zap70^{-/-}$ n = 6 pooled from three independent experiments.


A.
WT
Tet-Zap70
KO
LN
CD4
TCR
CD25
CD44
25.5
24.6
0.069
13.4
80.5
4.85
23.1
44
28.6
14.1
50
25.6
B.
SPLN
CD4
TCR
CD25
CD44
13.7
15.2
0.031
10.8
67.1
19.8
17.8
34.2
42.8
4.17
58.3
29.2

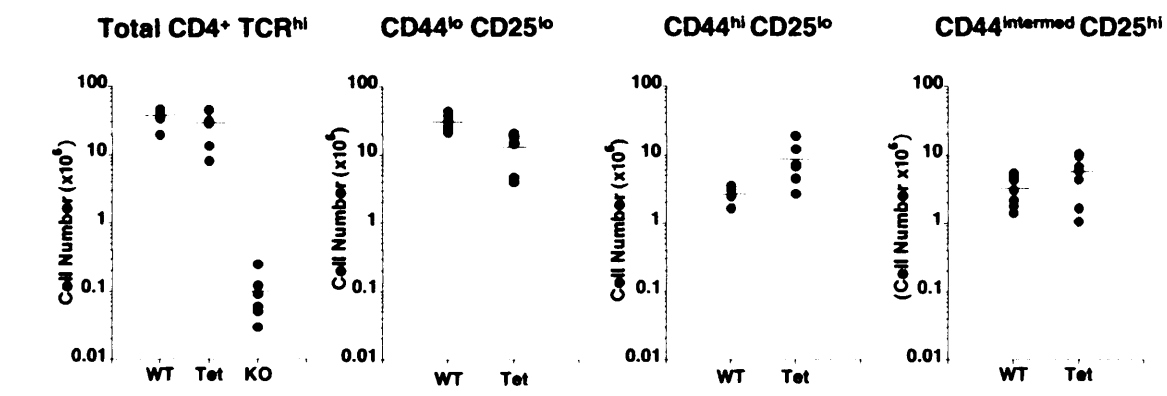
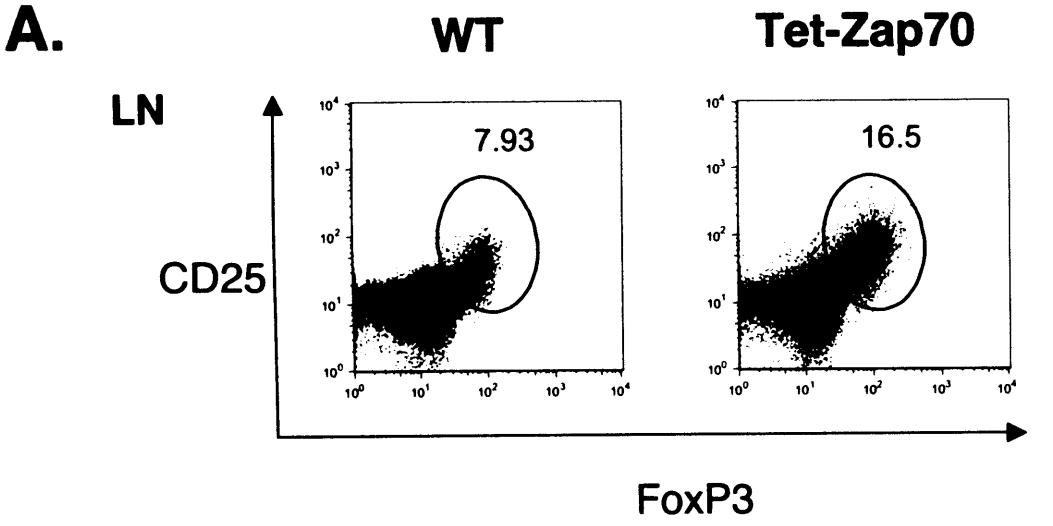

C. Periphery Absolute Cell Numbers
Total CD4+ TCRhi
CD44lo CD25lo
CD44hi CD25lo
CD44intermed CD25hi
Cell Number (x10^6)
100
10
1
0.1
0.01
WT
Tet
KO

Figure 3.12: Normal production of $CD4^+$ regulatory T cell precursors but elevated numbers of peripheral $CD4^+$ regulatory T cells in Tet-Zap70 mice.

A) LN T cells from 5-12 wk old WT and Tet-Zap70 mice were assessed for the surface expression of CD4, CD8 and CD25 and the intracellular expression of Foxp3. FACS plots show the expression of CD25 and Foxp3 on live gated $CD4^+$ T cells from representative individuals. The frequency of the $CD4^+$ $CD25^+$ $Foxp3^+$ T regulatory cells is indicated next to the corresponding population. The data is representative of 2 independent experiments. **B)** Thymocytes from the same WT and Tet-Zap70 mice along with a representative Zap70 deficient mouse were analysed for the expression of CD4, CD8, CD44 and CD25. Upper FACS plots show the CD4 versus CD8 coreceptor staining on T lymphocyte gated total thymocytes from the different mice, gate denotes the CD4 SP thymocyte population. The lower panel shows FACS plots of CD25 and CD44 expression on the CD4 SP gated thymocytes shown in the upper dot plots. The numbers reflect the percentage of T_{Reg} precursors within the CD4 SP population of each individual mouse. The data is representative of 3 independent experiments.


A.
WT
Tet-Zap70
LN
7.93
16.5
CD25
FoxP3

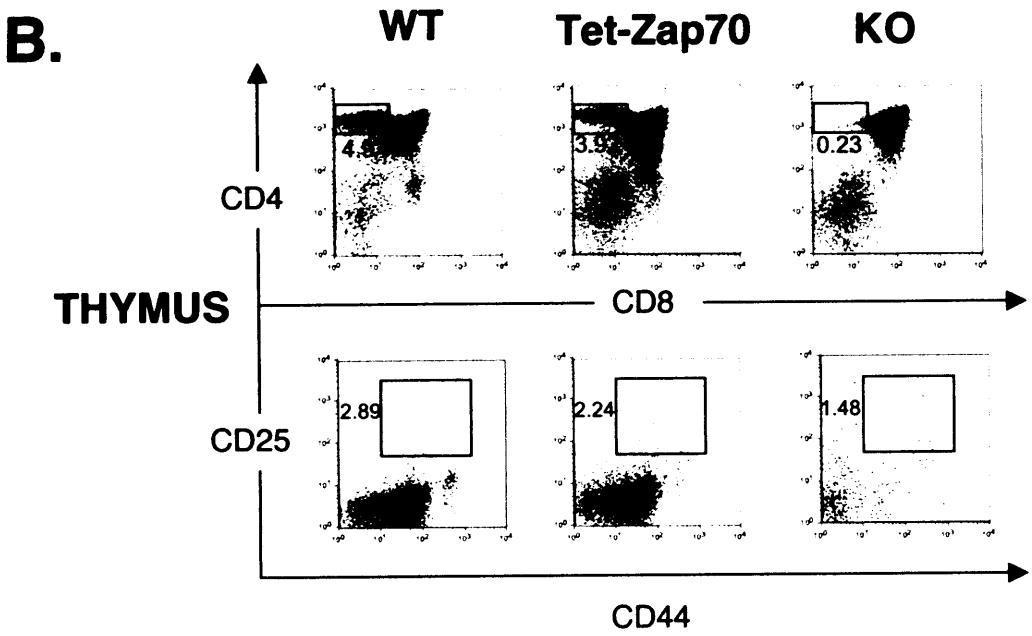
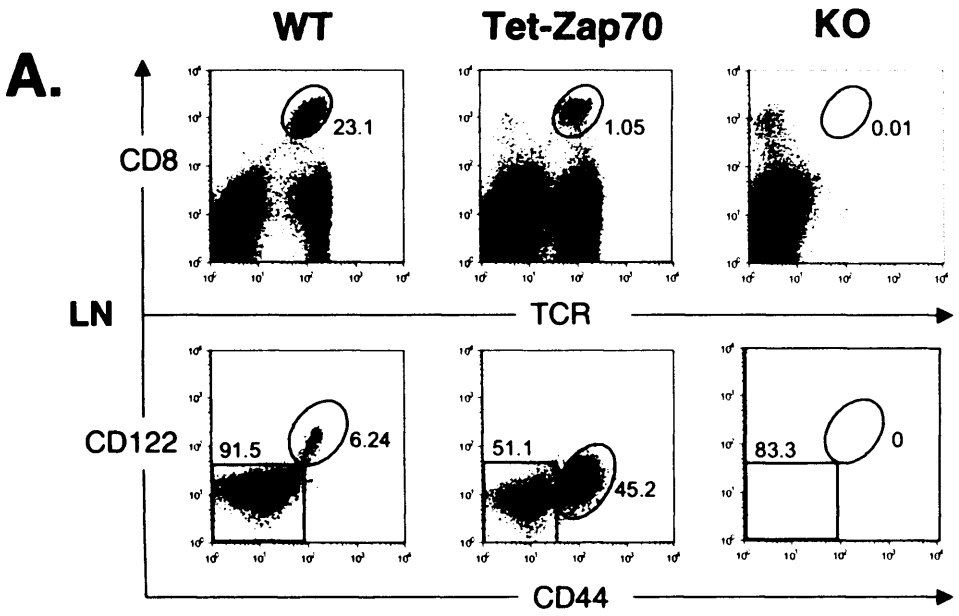
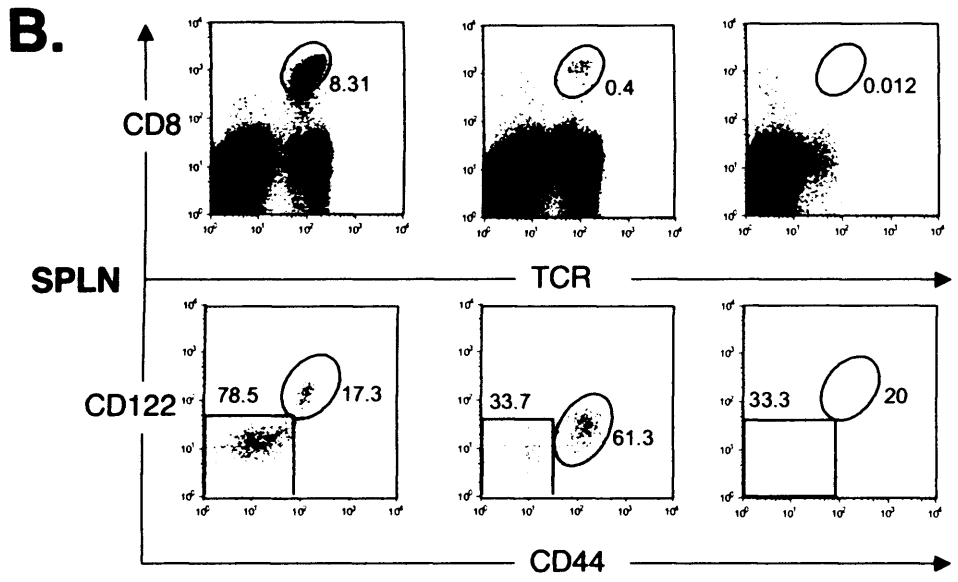

B.
WT
Tet-Zap70
KO
CD4
4.9
3.9
0.23
THYMUS
CD8
2.89
2.24
1.48
CD25
CD44

Figure 3.13: Peripheral phenotype of CD8 lineage T cells in Tet-Zap70 mice.

T cells from the LN and spleen of 5-12 wk old WT, Zap70 deficient and Tet-Zap70 mice were analysed for the expression of the coreceptors CD4 and CD8, TCR, CD44 and CD122. The upper panels of dot plots show CD8 versus TCR expression on the live gated cells, gate denotes $CD8^+$ TCR^{hi} T cells. The lower dot plots show the CD44 and CD122 expression on the $CD8^+$ TCR^{hi} gated cells shown in the upper FACS plots. **A)** shows the phenotype of mature $CD8^+$ T cells recovered from the LNs and **B)** shows the phenotype of mature $CD8^+$ T cells recovered from the spleen of representative mice. The numbers show the frequency of cells in each T cell subset that were used to calculate **C)** the absolute number of peripheral $CD8^+$ TCR^{hi} T cell subsets in each of the individual mice. Group sizes were as follows WT n = 7, Tet-Zap70 n = 7 and $Zap70^{-/-}$ n = 6 pooled from three independent experiments.


A.
WT
Tet-Zap70
KO
CD8
23.1
1.05
0.01
LN
TCR
CD122
91.5
6.24
51.1
45.2
83.3
0
CD44


B.
CD8
8.31
0.4
0.012
SPLN
TCR
CD122
78.5
17.3
33.7
61.3
33.3
20
CD44

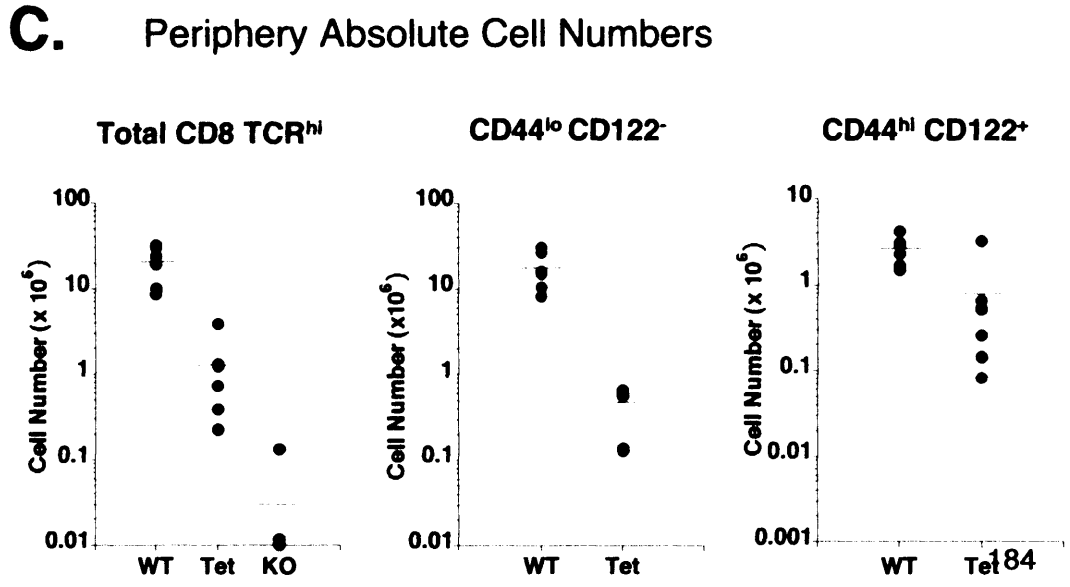

C.
Periphery Absolute Cell Numbers
Total CD8 TCRhi
CD44lo CD122-
CD44hi CD122+
Cell Number (x 10^6)
100
10
1
0.1
0.01
0.001
WT
Tet
KO

Figure 3.14: Reduced expression of CD122 on peripheral naïve and memory-like CD8 lineage T cells in Tet-Zap70 mice.

LN T cells from WT and constitutively dox fed (1mg/g) Tet-Zap70 mice of 5-12 wks of age were analysed for the surface expression of CD4, CD8, TCR and CD122. **A)** histograms show representative CD122 profiles on live gated $CD8^+$ TCR^{hi} $CD44^{lo}$ (naïve) and $CD8^+$ TCR^{hi} $CD44^{hi}$ (memory-like) lymphocytes. Shaded histograms indicates WT CD122 expression and solid lines indicates Tet-Zap70 CD122 expression. **B)** Scatter plot shows CD122 expression on individual mice pooled from 3 independent experiments (Tet-Zap70 n = 7, WT C57BL/6J n = 3 and Zap70 deficient controls n = 6), expressed as a percentage of WT levels (maximum). There was no significant difference between the expression of CD122 on $CD8^+$ T cells collected from the LN or spleen therefore only LN is shown.

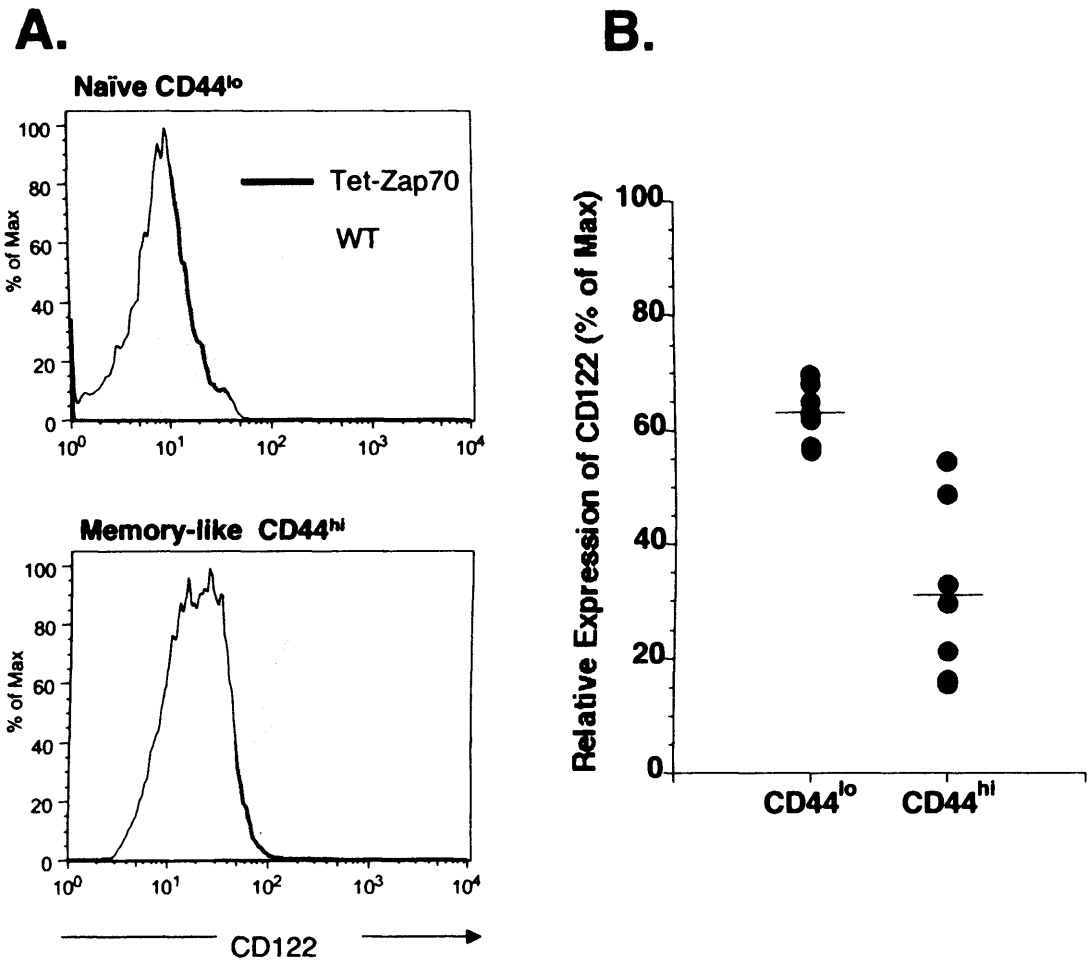
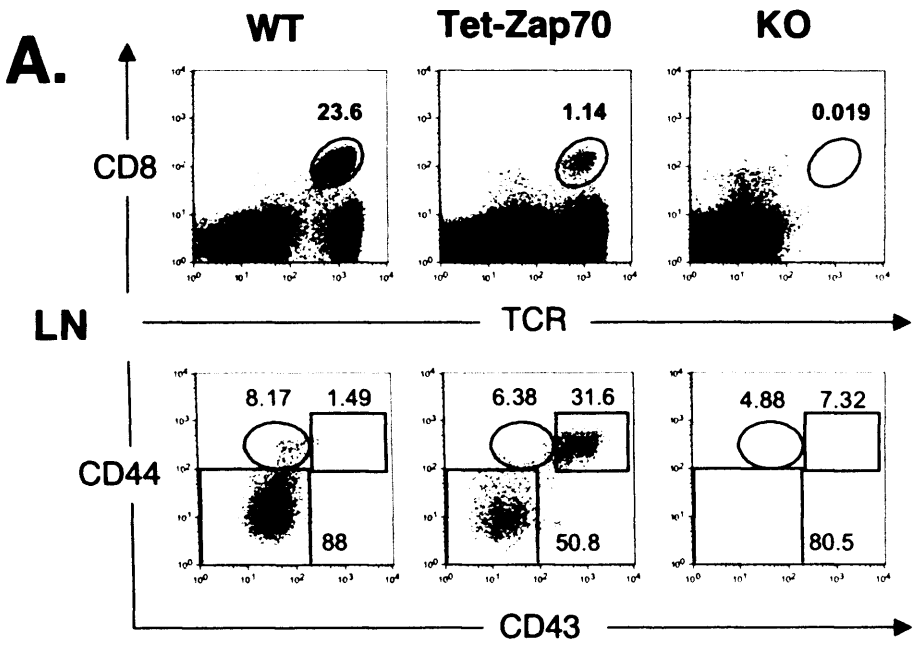

A.
Naïve CD44lo
Tet-Zap70
WT
% of Max
Memory-like CD44hi
CD122
B.
Relative Expression of CD122 (% of Max)
CD44lo
CD44hi

Figure 3.15: Increased proportion of $CD8^+$ effector phenotype T cells in Tet-Zap70 mice.

A) T cells from the LN of 5-12 wk old WT, Zap70 deficient and constituitively dox fed (1mg/g) Tet-Zap70 mice were analysed for the expression of CD8, TCR, CD44 and CD43. The upper panels of dot plots show CD8 versus TCR expression on the peripheral $CD8^+$ T cells and the lower panels show the CD44 and CD43 (activation associated glycoform) expression on the $CD8^+$ TCR^{hi} cells shown in the upper FACS plots. Gates denote $CD44^{lo}$ $CD43^{lo}$ (naïve), $CD44^{hi}$ $CD43^{lo}$ (memory) and $CD44^{hi}$ $CD43^{hi}$ (effector) T cells. The percentage of cells in each gated region is shown next to the corresponding populations. **B)** Scatter plots show the frequency of mature $CD8^+$ $CD44^{hi}$ $CD43^{lo}$ and $CD44^{hi}$ $CD43^{hi}$ in individual mice pooled from 3 independent experiments using the gates indicated in the FACS plots above. Group sizes were as follows Tet-Zap70 n = 7, WT C57BL/6J n = 3 and Zap70 deficient controls n = 6.


A.
WT
Tet-Zap70
KO
23.6
1.14
0.019
CD8
LN
TCR
8.17
1.49
6.38
31.6
4.88
7.32
CD44
88
50.8
80.5
CD43

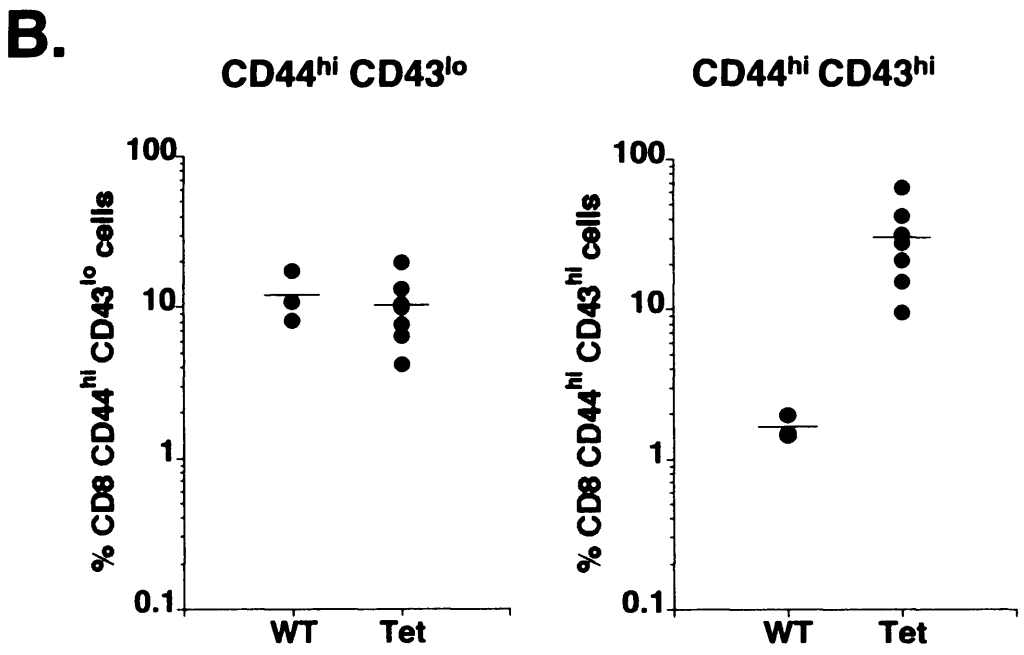
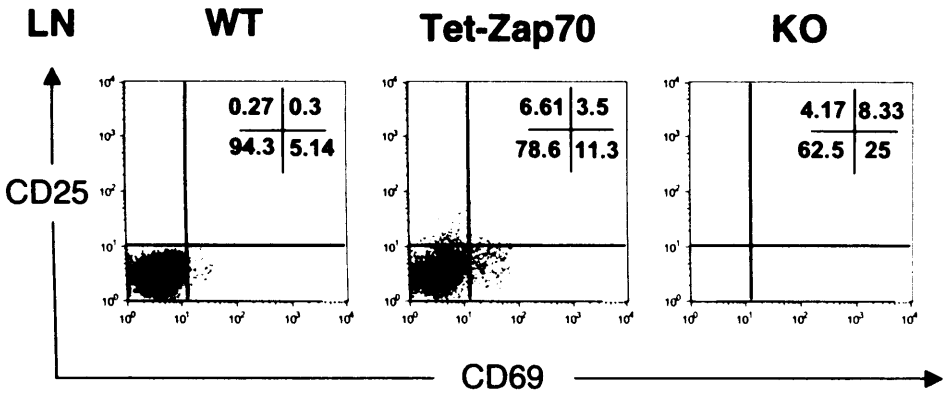

B.
CD44hi CD43lo
CD44hi CD43hi
100
10
1
0.1
% CD8 CD44hi CD43lo cells
% CD8 CD44hi CD43hi cells
WT
Tet
WT
Tet

Figure 3.16: Increased proportion of CD8$^+$ T cells with an activated phenotype in Tet-Zap70 mice.

A) T cells from the LN of 5-12 wk old WT, Zap70 deficient and Tet-Zap70 mice were analysed for the expression of CD8, TCR, CD69 and CD25. The FACS plots show CD25 and CD69 expression on the CD8$^+$ TCR^{hi} gated peripheral T cells from representative individuals (gating shown in **Fig 3.16**). The percentage of cells is shown next to the corresponding quadrant in the upper right hand corner of the FACS plot. **B)** scatter plots show the frequency of mature CD8$^+$ TCR^{hi} T cells expressing CD25 and/or CD69 in individual mice pooled from 3 independent experiments using the gates indicated in the FACS plots above. Group sizes were as follows Tet-Zap70 n = 7, WT n = 3 and Zap70 deficient controls n = 6.

A.


LN
WT
Tet-Zap70
KO
CD25
CD69
0.27
0.3
94.3
5.14
6.61
3.5
78.6
11.3
4.17
8.33
62.5
25

B.

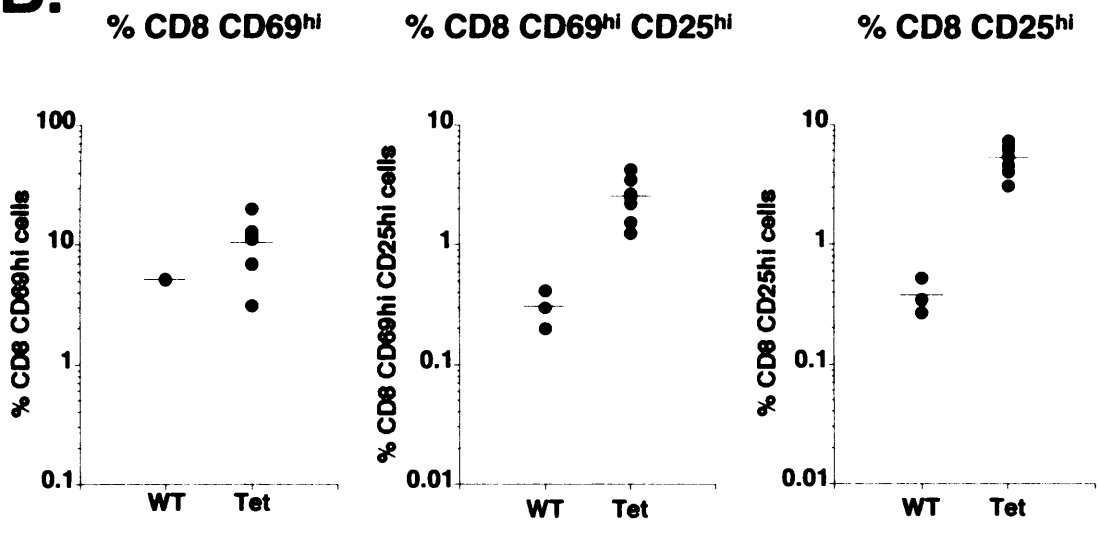
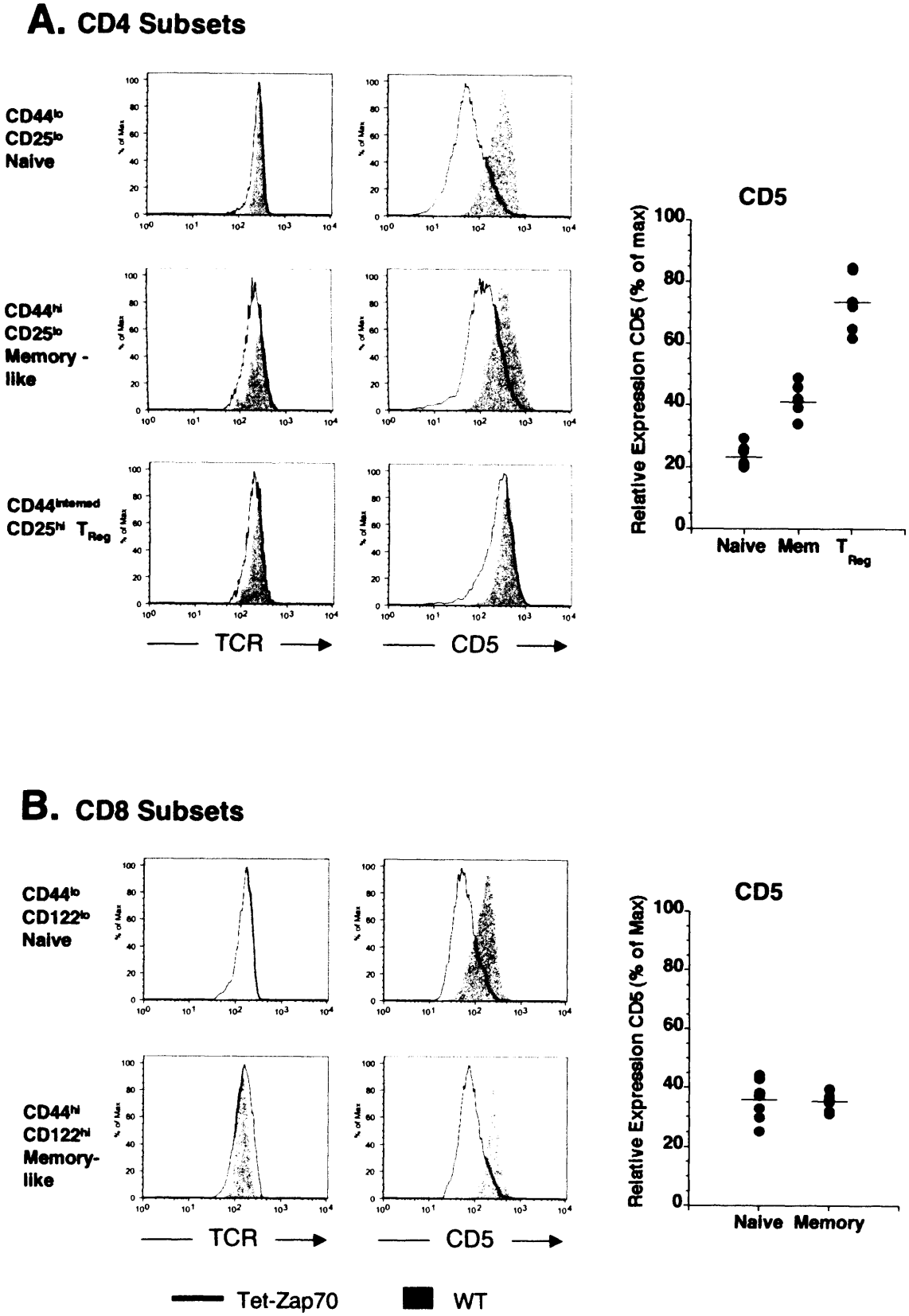

% CD8 CD69hi
% CD8 CD69hi CD25hi
% CD8 CD25hi
% CD8 CD69hi cells
% CD8 CD69hi CD25hi cells
% CD8 CD25hi cells
100
10
1
0.1
0.01
WT
Tet

Figure 3.17: Decreased CD5 surface expression in peripheral $CD4^+$ and $CD8^+$ T cells in Tet-Zap70 mice.

T cells from the LN of 5-12 wk old WT and Tet-Zap70 mice were analysed for the expression of CD4, CD8, TCR, CD5, CD25, CD44 and CD122. Histograms show TCR and CD5 surface levels on T lymphocyte gated **A)** $CD4^+$ TCR^{hi} naïve, memory-like and T regulatory phenotype T cells (gating shown in **Fig 3.11 A**) and **B)** $CD8^+$ TCR^{hi} naïve and memory-like T cells (gating shown in **Fig 3.13 A**). Scatter plots show surface CD5 expression on the mature $CD4^+$ and $CD8^+$ T cell subsets generated in individual WT and Tet-Zap70 mice pooled from 3 independent experiments, expressed as a percentage of WT levels (maximum). T cells recovered from the spleen of WT and Tet-Zap70 mice showed similar levels of TCR and CD5 expression therefore only LN data is shown. Group sizes were as follows Tet-Zap70 n = 7 and WT C57BL/6J controls n = 7.


A. CD4 Subsets
CD44lo CD25lo Naive
CD44hi CD25lo Memory - like
CD44Intermed CD25hi TReg
% of Max
TCR
CD5
CD5
Relative Expression CD5 (% of max)
Naive
Mem
TReg
B. CD8 Subsets
CD44lo CD122lo Naive
CD44hi CD122hi Memory-like
TCR
CD5
Tet-Zap70
WT
CD5
Relative Expression CD5 (% of Max)
Naive
Memory

Chapter 4

The Role of Zap70 in Naïve T cell Homeostasis

4.1 Introduction

In peripheral T cells the transduction of signals by the TCR can instigate many different programs of immunological activity, including T cell activation, differentiation and proliferation. However less is known about the precise TCR signals required for the survival of peripheral naïve T cells. In recent years the view that the survival of naïve T cell populations circulating through peripheral lymphoid tissues, reflected a cell intrinsic activity requiring no overt external signal has been challenged. A number of studies have proposed that the peripheral naïve T cell pool is maintained at a constant size in part via the transmission of environmental signals through the TCR. However, there still is much controversy over whether TCR signals triggered by the recognition of sp/MHC ligands sustain the prolonged steady state survival of the naïve peripheral lymphoid compartment. There have been several studies addressing this question that either support (Brocker, 1997; Nesic and Vukmanovic, 1998; Polic et al., 2001; Takeda et al., 1996; Tanchot et al., 1997; Viret et al., 1999; Witherden et al., 2000) or refute (Dorfman et al., 2000a; Dorfman and Germain, 2002; Germain et al., 2002) the requirement of spMHC/TCR interactions. More recent studies examining the role of TCR associated kinases in the transmission of T cell survival signals suggest a TCR signal is required for T cell survival. These studies have reported a role for Lck

and Fyn expression in the survival of naïve T cells, since ablation of these kinases in peripheral naïve $CD4^+$ and $CD8^+$ lineages gradually resulted in their decay (Seddon et al., 2000). In resting thymocytes and LN T cells the CD3ζ chains are basally phosphorylated (21kDa) and are associated with Zap70 (van Oers et al., 1994; van Oers et al., 1993). In the absence of Lck and Fyn expression, not only was there a decline in naïve T cell survival but also a loss of CD3ζ chain phosphorylation (Seddon and Zamoyska, 2002b) and Zap70 association with the CD3ζ chain (Witherden et al., 2000), suggesting that the of phosphoCD3ζ and Zap70 complex may be providing a survival signal. However it is important to note that the observed relationship between impaired T cell survival and the absence of signalling via the phospho CD3ζ and Zap70 complex is purely correlative since it is not known whether Zap70 expression is required for T cell survival.

Given that Lck and Fyn have been shown to associate with other receptors such as the IL-2R (Hatakeyama et al., 1991; Kobayashi et al., 1993; Minami et al., 1993) and adaptors independent of TCR signalling (Parravicini et al., 2002; Wary et al., 1998) the effects of Lck and Fyn on T cell survival may not be fully restricted to TCR activity. The aim of this study was to examine the role of Zap70 in the survival of peripheral T cell compartments, since it is primarily associated with the ζ homodimer of the CD3 complex and more specifically reflects signals received via the TCR.

4.2 Results

4.2.1 Development of Peripheral Tet-Zap70 cells in Replete Mixed BM Chimeras

Examination of the peripheral T cell subsets in dox fed Tet-Zap70 mice revealed that the CD4 SP and CD8 SP thymocytes generated were capable of seeding the peripheral $CD4^+$ and $CD8^+$ compartments. However there appeared to be abnormalities in the reconstitution of the peripheral T cell subsets. The absolute number of naïve $CD4^+$ and $CD8^+$ T cells were reduced compared to controls whereas the frequency of memory-like ($CD44^{hi}$ $CD25^{lo}$) and $CD4^+$ T_{Reg} cells ($CD44^{intermed}$ $CD25^{hi}$ $Foxp3^+$) appeared considerably elevated. It was unclear whether this abnormal phenotype reflected a response of the T cells to the partially lymphopenic environment resulting from reduced thymic output in Tet-Zap70 mice, or was a result of T cell intrinsic defects associated with the non-physiological expression levels of the Tre-Zap70 transgene. To investigate this we generated mixed BM radiation chimeras from WT and Tet-Zap70 BM, to assess the development of the Tet-Zap70 cells in normal thymic and peripheral compartments that would be provided by cells of WT origin. In addition we compared the competitive fitness of the Tet-Zap70 and WT cells generated in the chimeras, to determine if expression of the Tre-Zap70 transgene affected development and/or maintenance of the Tet-Zap70 cells in these chimeras.

Sub-lethally irradiated C57BL/6J *Rag1*$^{-/-}$ hosts were injected intravenously with a mixture of T cell depleted BM inoculum, derived from Tet-Zap70 mice that express the allotypic marker CD45.2 and WT C57BL/6J CD45.1 mice at a ratio of 10:1 Tet-Zap70:WT cells. The Tre-Zap70 transgene is not ubiquitously expressed in the DP compartment of Tet-Zap70 mice, and therefore a 10:1 mixture of Tet-Zap70 BM and WT BM was used, to ensure a significant representation of Tet-Zap70 derived T cells in the chimeric hosts. Mice were fed dox, (3mg/g) during the reconstitution to maintain the expression of the Tre-Zap70 transgene. A control group of chimeras were not administered dox to confirm the generation of mature Tet-Zap70 cells was dox dependent. Chimeric mice were allowed to reconstitute for 18 weeks. Analysis of the thymocyte subsets generated from the Tet-Zap70 and WT BM progenitors in control chimeras reconstituted in the absence of dox treatment, revealed that huCD2 expression could not be detected in the thymi of control mice **(Fig 4.1)**. Similarly we could not detect any CD45.2 positive mature SP thymocytes derived from the Tet-Zap70 BM, confirming that development of the Tet-Zap70 T cells is dox dependent.

Repopulation of the peripheral lymphoid compartments by naïve, memory-like and T_{Reg} cells of WT CD45.1 and Tet-Zap70 CD45.2 origin in dox fed chimeras was assessed by FACS analysis. The T_{Reg} populations generated from the Tet-Zap70 BM, consistently displayed a lower level of CD44 surface staining compared to the WT cells, therefore the gate used to identify the Tet-Zap70 BM derived T_{Reg} population was moved, to include all the CD44^{lo} cells.

The peripheral TCR^{hi} $CD4^+$ compartment was successfully populated by the CD45.2 cells derived from the Tet-Zap70 BM progenitors **(Fig 4.2 A)**, although this was still below the frequency of CD45.1 positive cells generated from WT NM and was less than would be expected from the 10:1 ratio of Tet-Zap70:WT BM used to generate the chimeras. In contrast to the phenotype observed in the dox fed intact Tet-Zap70 mice, the replete chimeras did not show an elevated proportion of $CD4^+$ memory-like or T_{Reg} cells amongst the Tet-Zap70 population. However further examination of the composition of the chimeric mature $CD4^+$ compartment derived from the Tet-Zap70 and WT progenitors did reveal differences. In both the LN and spleen the overall frequency of Tet-Zap70 (CD45.2) $CD4^+$ memory-like and $CD4^+$ T_{Reg} populations were consistently reduced compared to the same subset of WT origin (CD45.1), and the frequency of Tet-Zap70 TCR^{hi} naïve lymphocytes was correspondingly increased in comparison to the naïve subset in the WT compartment.

The peripheral naïve TCR^{hi} $CD8^+$ population in the chimeric mice was largely derived from the WT CD45.1 BM progenitors. Consistent with the phenotype observed in Tet-Zap70 mice the frequency of naïve TCR^{hi} $CD8^+$ T cells generated from the Tet-Zap70 precursors was greatly reduced in comparison to the TCR^{hi} Tet-Zap70 $CD4^+$ T cell population **(Fig 4.2 B)**. However, in contrast to the Tet-Zap70 mice, there were virtually no mature memory-like $CD8^+$ T cells present within the LN or spleen of the chimeric mice.

4.2.2 Assessing the Competitive Fitness of Tet-Zap70 cells in Mixed BM Chimeras

The representation of the Tet-Zap70 CD45.2 cells in the peripheral T cell subsets was significantly lower than we would expect from the 10:1 ratio of Tet-Zap70:WT BM inoculum used to generate the mixed chimeras, suggesting expression of the transgene may affect the development and or maintenance of the thymocyte subsets. To address this in more detail we assessed the reconstitution of the chimeras by the WT and Tet-Zap70 progenitors at the different developmental checkpoints, specifically the pre-selection TCR^{lo} DP thymic compartment, the positively selecting TCR^{hi} DP thymocyte population, the TCR^{hi} CD4 and CD8 SP thymocytes subsets and the $CD44^{lo}$ naïve and $CD44^{hi}$ memory $CD4^{+}$ and $CD8^{+}$ T cell compartments **(Fig 4.3 B)** to determine the competitive fitness of the Tet-Zap70 cells through development. This was performed by calculating the ratio of Tet-Zap70 versus WT cells in each of the thymocyte and peripheral T cell compartments in individual chimeric mice. There was variation in the chimerism between individual mice, therefore we normalised these ratios against the starting ratio of Tet-Zap70:WT thymocytes in the most immature TCR^{lo} DP population.

The efficiency of thymic reconstitution in different thymocyte subsets of dox fed chimeras differed for the WT and Tet-Zap70 progenitors **(Fig 4.3 A)**. The ratio of Tet-Zap70:WT cells did not appear significantly different following differentiation of the TCR^{lo} DP compartment to the TCR^{hi} DP population suggesting Tre-Zap70 expression is sufficient for this developmental

checkpoint. However following differentiation of the TCR^{hi} DP thymocytes to the TCR^{hi} CD4 SP and TCR^{hi} CD8 SP lineages, the representation of Tet-Zap70 cells in both these populations decreased, suggesting the Tet-Zap70 cells were not as efficiently selected as WT cells **(Fig 4.3 B)**. Interestingly the cells derived from the Tet-Zap70 progenitors were least represented in the CD8 lineage compared to the CD4 lineage, suggesting the efficiency of thymic selection to the CD8 lineage is reduced in the Tet-Zap70 TCR^{hi} DP population. The ratio of Tet-Zap70:WT cells decreased further following differentiation to the naïve and memory-like compartments suggesting the expression of the transgene is unable to fully support the development and maintenance of the CD8 lineage cells. However it should be noted that this study is representative of one study and therefore needs to be repeated to confirm whether these observations are reproducible and biologically significant.

4.2.3 Survival of Peripheral T cell Subsets in the Absence of Continued Zap70 Expression

We next investigated whether Zap70 expression was required for the steady state survival of peripheral $CD4^+$ and $CD8^+$ T cells. Using mixed BM radiation chimeras discussed previously, we compared the survival of peripheral blood lymphocytes (PBL) in the presence and absence of continuous Zap70 expression. Chimeric mice were allowed to reconstitute for 9 weeks on dox (3mg/g). Dox was then removed from the diet of a cohort of chimeric mice (n = 5), to switch off Tre-Zap70 transgene expression, while a second group (n = 5) of chimeras were maintained ON dox as controls. Peripheral blood samples

were taken from these ON and OFF dox groups at the beginning of the experiment (day 0) and then 7, 12, 22, 30 and 44 d later. The frequency of naïve, memory-like and T_{Reg} cells were determined by FACS analysis.

Fig 4.4 shows the electronic gates used to determine the frequency of $CD4^+$ Tet-Zap70 (CD45.2) and WT (CD45.1) lymphocyte subsets within the mature TCR^{hi} $CD4^+$ population, in the peripheral blood of a representative chimera prior to switching off the Tre-Zap70 transgene at day 0, the same gates were used for all the time points analysed. Because the level of chimerism between the Tet-Zap70 and WT cells varied between the individual chimeras time points for individual chimeras were normalised to the frequency of cells in each lymphocyte subset at day 0. WT control frequencies did not change significantly over timecourse of the experiment and therefore they are only shown on the graphs displaying the mean percentages of cells recovered over time.

Following the removal of dox, the frequency of naïve TCR^{hi} Tet-Zap70 $CD4^+$ T cells declined steadily exhibiting a half life of ~ 44days **(Fig 4.5)**. In contrast the frequency of naïve $CD4^+$ T cells in the dox fed control chimeras remained relatively constant. Analysis of the frequency of Tet-Zap70 $CD4^+$ T_{Reg} cells **(Fig 4.6)** and $CD4^+$ memory-like cells **(Fig 4.7)** recovered from ON and OFF dox chimeras over the timecourse, failed to reveal any obvious difference in survival between the ON and OFF dox cohorts. However in the OFF dox mice there did appear to be a trend that suggested a subtle decline in the frequency of Tet-

Zap70 cells with time, but was unclear whether this was different from the dox fed controls. Further experiments are required to establish whether this apparent trend is reproducible.

Analysis of the reconstitution of the mature $CD8^+$ compartment by the Tet-Zap70 and WT cells in a representative chimera prior to switching off of the transgene (**Fig 4.8**) revealed that reconstitution of the $CD8^+$ T cell subsets by the Tet-Zap70 progenitors was significantly lower than the WT cells. The mature $CD8^+$ memory-like compartment showed only ~ 1% reconstitution by the Tet-Zap70 cells and was too low to be adequately followed over the 44d time period, therefore only the survival of the naïve $CD8^+$ Tet-Zap70 population is described here.

After the removal of dox, the naïve $CD8^+$ T cells generated from the Tet-Zap70 BM declined rapidly over the time course. The half life of the decay in naïve $CD8^+$ T cell survival was ~12d and much shorter than the time taken for an equivalent level of cell loss within the naive $CD4^+$ population **(Fig 4.9)**. Interestingly the naïve $CD8^+$ T cells recovered from the chimeras maintained on dox, also showed evidence of low level T cell loss over the timecourse compared to the WT control cells (ON and OFF dox), although it should be noted there was a much variation between the individual mice. This data implies that the loss of continuous Zap70 expression reduces the ability of the naïve $CD4^+$ and $CD8^+$ T cells to survive in the periphery. The data also suggests that the naïve $CD8^+$ T cell compartment has a greater dependence on

Zap70 for their survival given that this compartment showed a more rapid kinetic of cell loss than the naïve $CD4^+$ T cell population.

4.2.4 Peripheral Expression of IL-7R in Tet-Zap70 Mice

The data presented so far in this chapter demonstrates that a loss of peripheral Zap70 expression results in the gradual disappearance of the peripheral TCR^{hi} naïve $CD4^+$ and $CD8^+$ T cell populations. Since T cell survival also depends on IL-7 signalling we wanted to confirm that the only difference between the Tet-Zap70 and WT control T cells was the loss of Zap70 expression and that other survival signals were equivalent. Therefore we assessed whether the expression of IL-7R was comparable between WT and Tet-Zap70 T cells.

T cells from the LN and spleen of constitutively dox fed Tet-Zap70 and aged matched WT controls were analysed for the surface expression of the CD4 and CD8 coreceptors, CD5, CD44 and IL-7Rα chain (CD127). We compared IL-7R expression on the $CD5^{hi}$ naïve ($CD44^{lo}$) and $CD5^{hi}$ memory-like ($CD44^{hi}$) peripheral $CD4^+$ **(Fig 4.10 A and C)** and $CD8^+$ **(Fig 4.10 B and C)** T cell subsets generated in WT and dox fed (1mg/g) Tet-Zap70 mice. Unexpectedly, IL-7R expression was consistently reduced on the naïve $CD4^+$ and naïve $CD8^+$ T cells generated in Tet-Zap70 mice compared to the same subsets in the WT controls. Although IL-7R expression by the $CD4^+$ memory-like subset appeared similar on the Tet-Zap70 and WT cells, the IL-7R expression by the $CD8^+$ memory-like compartment was lower on the Tet-Zap70 cells compared to the WT.

Further comparison of IL-7R expression on the naïve $CD4^+$ and naïve $CD8^+$ T cells generated in the same mouse revealed that the MFI of IL-7R expression was comparable between the WT naïve $CD8^+$ and $CD4^+$ T cells. **(Fig 4.10 D)**. In contrast comparison of IL-7R expression between naïve $CD8^+$ and naïve $CD4^+$ T cells generated in the same Tet-Zap70 mouse revealed that IL-7R expression was reduced on the naïve $CD8^+$ T cells compared to the naïve $CD4^+$ T cells, suggesting that Zap70 signals may directly modulate IL-7R expression.

4.2.5 Thymic Expression of IL-7R in Tet-Zap70 Mice

We next examined whether the reduced peripheral expression of IL-7R in the naïve T cells generated in Tet-Zap70 mice was a result of impaired re-expression of IL-7R in the mature SP populations generated in Tet-Zap70 thymi. IL-7 signalling is essential for T cell development and IL-7R expression is tightly regulated during ontogeny (reviewed in (Mazzucchelli and Durum, 2007)). IL-7R signalling plays a critical role in the survival of the DN thymocyte subsets and aids TCR γ, δ and β gene rearrangements promoting thymic development (reviewed in (Candeias et al., 1997)). DN thymocytes thus express high levels of IL-7R, which is completely switched off following differentiation to the DP stage of thymopoiesis (Sudo et al., 1993). Following successful positive selection, the TCR^{hi} CD4 SP and TCR^{hi} CD8 SP populations then re-express IL-7R, although how IL-7R re-expression is controlled is not known.

Comparison of IL-7R expression between the TCR^{hi} CD4 SP and CD8 SP thymocytes generated in WT mice revealed that induction of IL-7R expression differed between these subsets of thymocytes **(Fig 4.11)**. WT CD4 SP thymocytes consistently displayed a higher level of IL-7R expression compared to the WT TCR^{hi} CD8 SP compartment **(Fig 4.11 B)**. Interestingly examination of IL-7R expression on the TCR^{hi} CD4 SP and TCR^{hi} CD8 SP thymocytes generated in Tet-Zap70 mice showed that, IL-7R expression was consistently reduced on Tet-Zap70 SP thymocytes in comparison to the WT SP controls **(Fig 4.11 A)**. As was the case in WT mice, IL-7R was also found to be lower on the CD8 SP thymocytes compared to the CD4 SP thymocytes in Tet-Zap70 mice **(Fig 4.11 B)**. Furthermore the existing difference in IL-7R expression between the CD4 SP and CD8 SP thymocytes appeared exacerbated by expression of the Tre-Zap70 transgene **(Fig 4.11 B)**. This suggests a role for Zap70 in the thymic induction of IL-7R expression post selection.

3.3 Discussion

Previous studies performed by several groups examining peripheral T cell survival in the absence of TCR (Polic et al., 2001), MHC (Martin et al., 2006; Witherden et al., 2000) or Lck and Fyn (Seddon and Zamoyska, 2002b) expression have shown that loss of homeostatic TCR mediated survival signals, results in the disappearance of the naïve $CD8^+$ and $CD4^+$ T cell subsets in replete mice. To dissect further how homeostatic TCR signals are transmitted intracellularly we assessed the role of Zap70 in the propagation of homeostatic TCR signals, since its activation and signalling potential is

dependent upon recruitment to the TCR/CD3 complex. In the present study we found that ablation of Zap70 expression resulted in the dramatic decline in both peripheral naïve $CD4^+$ and $CD8^+$ T cell populations. Notably, the naïve $CD8^+$ T cell compartment appeared most affected by a loss of Zap70 expression, suggesting that the extent to which Zap70 signalling is required for survival may differ between the naïve $CD4^+$ and $CD8^+$ T cell compartments. Surprisingly, our data also suggests, that Zap70 signalling may also influence T cell homeostasis through the regulation of IL-7R re-expression in the thymus and peripheral naïve T cell compartments.

The data reported in this chapter suggests that Zap70 is important for the transmission of homeostatic TCR survival signals. Abrogation of Zap70 expression resulted in a steady decline in the frequencies of peripheral Tet-Zap70 naïve $CD4^+$ and $CD8^+$ T cells in mixed BM chimeras **(Fig 4.5 and 4.9).** Additionally, the Tet-Zap70 BM seemed less efficient at reconstituting the peripheral T cell compartments of the mixed BM chimeras compared with control BM. Notably the cells derived from Tet-Zap70 BM, were progressively outcompeted by cells of WT origin at each stage of development in the thymus and the periphery **(Fig 4.2 and 4.3)**. This suggests that the expression of the Tre-Zap70 transgene is insufficient for the transmission of homeostatic TCR survival signals and as a consequence reduces the competitive fitness of the cells, in that they are less successful at competing for limited survival factors such as IL-7. Overall this data suggests that the peripheral lymphopenia observed in intact Tet-Zap70 mice, may result from reduced thymic output due

to less efficient thymopoiesis, but may also result from the failure of Tre-Zap70 transgene to fully support the survival of the naïve $CD4^+$ and $CD8^+$ T cell compartments. It is likely the combination of these defects in the development and homeostasis of Tet-Zap70 cells ultimately renders the intact Tet-Zap70 mice partially lymphopenic. Interestingly, the naïve Tet-Zap70 $CD8^+$ T cell compartment appeared more sensitive to a loss of Zap70 expression than the naïve $CD4^+$ T cell compartment, since the naïve $CD8^+$ T cells disappeared at a faster rate than their $CD4^+$ counterparts following ablation of Zap70 expression **(Fig 4.5 and 4.9)**. Similar survival differences have also been reported by (Polic et al., 2001) who show that following ablation of TCR expression, peripheral naïve $CD4^+$ T cells decline with a half life of ~46 days, whereas naïve $CD8^+$ T cells decay at a faster rate with a half life of ~16 days. Another study which also conditionally ablated TCR expression, also showed that naïve $CD8^+$ T cells declined much faster following a loss of TCR expression with a half life of 19 days, compared to naïve $CD4^+$ T cells which decayed with a half life of ~ 26.8 days (Labrecque et al., 2001). Consistent with this study (Seddon and Zamoyska, 2002b) reported that peripheral naïve $CD4^+$ T cells deficient for the expression of Lck and Fyn decayed with a half of ~26 days, whereas the naïve $CD8^+$ T cell compartment declined faster exhibiting a half life of ~18 days. These data imply that naïve $CD8^+$ T cells have a greater dependence on homeostatic TCR signals for their survival, and in our study this difference was particularly pronounced.

Our attempts to understand the survival defects we observed in the Tet-Zap70 mice revealed unexpected defects in other survival pathways. Surprisingly, IL-7R expression was much reduced on both naïve Tet-Zap70 $CD4^+$ **(Fig 4.10 A and C)** and $CD8^+$ T cells **(Fig 4.10 B and C)** compared to WT controls. Importantly, while comparison of IL-7R expression on naïve $CD8^+$ and naïve $CD4^+$ T cells in the same WT control mouse, showed no difference in the levels of IL-7R expression, naïve $CD8^+$ T cells generated in Tet-Zap70 mice showed lower levels of IL-7R compared to naïve $CD4^+$ T cells in the same mouse **(Fig 4.10. D)**. This expression defect appeared to extend throughout the mature T cell compartment as IL-7R expression was also lower on the peripheral memory-like $CD8^+$ T cells compared to WT cells expressing endogenous Zap70. Such altered expression of IL-7R has not been examined in the large majority of studies assessing the role of homeostatic TCR/MHC signals in naïve T cell survival. While it has been reported that TCR signals can downregulate IL-7R on activated human $CD4^+$ T cells *in* vitro (Franchimont et al., 2002), this does not appear to be the case for naïve $CD4^+$ T cells. A study by (Seddon et al., 2003) reported that naïve $CD4^+$ T cells lacking Lck and Fyn expression show only slightly reduced levels of surface IL-7R expression. This may suggest that the regulation of IL-7R expression is unique to Zap70, and perhaps represents an alternative pathway of Zap70 signalling.

The functional consequences of this decreased IL-7R expression in the Tet-Zap70 naïve $CD8^+$ and memory-like compartments is not known. However given that IL-7 is an essential homeostatic cytokine, it is possible that the

reduced levels of IL-7R observed on the naïve $CD8^+$ T cells in intact Tet-Zap70 mice, combined with the suboptimal expression of the Tre-Zap70 transgene, may provide insufficient homeostatic TCR and IL-7 survival signals. This may account for the dramatic depletion of the peripheral naïve $CD8^+$ but not naïve $CD4^+$ T cell compartment in intact Tet-Zap70 mice. Similarly the reduced levels of IL-7R expression on the memory-like $CD8^+$ T cell compartment may also impair their survival. Analysis of the forward scatter of T lymphocyte gated peripheral naïve $CD4^+$ and naïve and memory-like $CD8^+$ T cells generated in Tet-Zap70 and WT mice, did not reveal any differences in cell size (data not shown) implying that the trophic effects of IL-7 are not impaired in the Tet-Zap70 cells despite the reduced levels of IL-7R expression. Thus it still remains unclear what effects the reduced expression of IL-7R may be having on the function of the Tet-Zap70 T cells. In the survival experiments described in this chapter, IL-7R levels were not analysed, therefore it remains to be determined whether the decline in the survival of the naïve Tet-Zap70 naïve $CD4^+$ and $CD8^+$ T cells following ablation of Zap70 is purely attributed to the loss of homeostatic TCR signalling. In further studies it will be interesting to determine whether IL-7R levels decline on naïve $CD4^+$ and $CD8^+$ T cells following ablation of Zap70 expression. Given that IL-7R signals play an important role in the protection of cells from apoptosis we will also investigate whether IL-7R signalling is impaired in Tet-Zap70 T cells by measuring the intracellular levels of Bcl-2 and by comparing whether Tet-Zap70 (on and off dox) and WT naive $CD4^+$ and $CD8^+$ T cells exhibit different sensitivities to apoptosis *in vitro*.

Interestingly, the data presented here also suggests that IL-7R re-expression in the mature SP thymocyte compartment is not a passive process of maturation but is dependent on TCR differentiation signals. Under normal conditions IL-7R expression is silenced at the DP stage of thymopoiesis and re-expressed on the mature SP thymocytes (Fry and Mackall, 2002). While pre-TCR signalling has been reported to regulate IL-7Rα expression during early thymocyte development in the DN thymocyte subsets (Trigueros et al., 2003), how IL-7R re-expression is regulated within the mature SP thymocyte compartments in not known. Although IL-7R expression was switched back on in the Tet-Zap70 CD4 SP and CD8 SP thymocytes, the levels of IL-7R expression were reduced compared to WT controls **(Fig 4.11 A)**. Careful examination of WT thymocytes revealed a consistent difference in IL-7R expression between the mature CD4 SP and CD8 SP thymocytes and it may be this difference that is exacerbated in Tet-Zap70 mice **(Fig 4.11 B)**. The specific reason for this remains to be determined but suggests that Zap70 signals may play a role in the regulation of IL-7R re-expression in the thymus. One possibility is that IL-7R re-expression is influenced by positive selection signals, such that IL-7R levels increase as a function of signal strength through the TCR. Such a view would be consistent with the observation that Tet-Zap70 thymocytes were less efficiently selected during thymopoiesis than WT cells in the same hosts. To investigate this further we are assessing the expression of IL-7R in TCR transgenic thymocytes that either express TCRs that exhibit low or high avidity for self antigens. Preliminary data does indeed suggest that the strength of TCR ligation by

selecting ligands influences the re-expression of IL-7R during late T cell development.

The data presented in this chapter also demonstrates that increased frequency of peripheral T_{Reg} cells, and memory-like $CD4^+$ and $CD8^+$ T cells observed in intact dox fed Tet-Zap70 mice, is not a result of T cell intrinsic defects associated with the suboptimal expression Tre-Zap70 transgene. In mixed BM chimeras, reconstituted by Tet-Zap70 and WT BM progenitors, Tet-Zap70 derived T_{Reg} population and the memory-like $CD4^+$ and $CD8^+$ T cell populations, showed no evidence of peripheral expansion in these replete hosts. These data suggest that the peripheral expansion of T_{Reg} cells and memory-like T cells in Tet-Zap70 mice may be a result of the partial lymphopenia observed in these mice. Competition with WT cells for space and environmental stimuli such self peptide ligands and IL-7 in the mixed chimeras but not the intact Tet-Zap70 mice, may also account for the peripheral expansion of T_{Reg} and memory-like T cells observed in the intact Tet-Zap70 mice but not in the mixed BM chimeras.

Figure 4.1: Abrogated Tet-Zap70 thymocyte development in mixed BM chimeras reconstituted in the absence of dox treatment.

Sub-lethally irradiated C57BL/6J *Rag1*$^{-/-}$ hosts (n = 3) were injected intravenously with a 10:1 ratio of T cell depleted Tet-Zap70 (CD45.2) and WT BM (CD45.1) and maintained off dox treatment for the entire course of the experiment. 18wks later the thymi were analysed for the expression of the coreceptors CD4 and CD8, TCR, huCD2 and CD45.1 by FACS analysis. Figure shows FACS plots of the CD4 and CD8 coreceptor staining on T lymphocyte gated total thymocytes from a representative control chimera. Right hand side FACS plots shows the frequency of CD45.2^{+} huCD2^{+} thymocytes (induced Tet-Zap70 BM derived thymocytes), CD45.2^{+} huCD2^{-} thymocytes (uninduced Tet-Zap70 BM derived thymocytes) and CD45.1^{+} huCD2^{-} positive thymocytes (WT BM derived thymocytes) in the DP, CD4 SP and CD8 SP thymocyte subsets.

Control Chimera:

Off dox during reconstitution

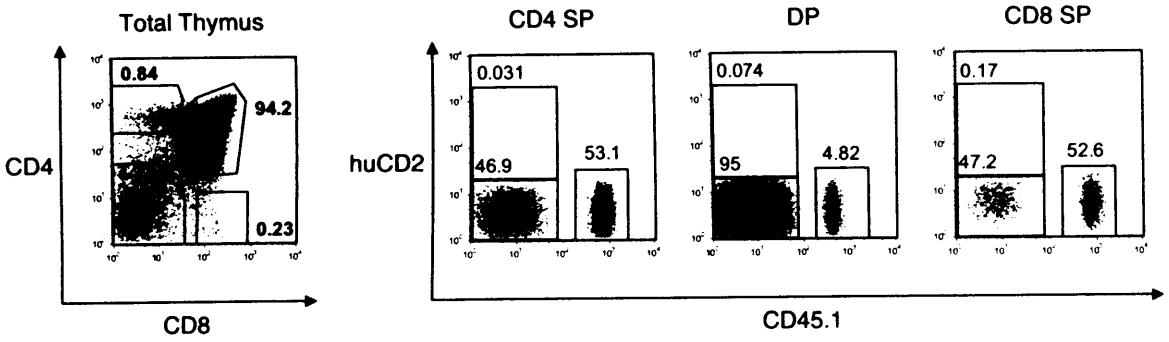
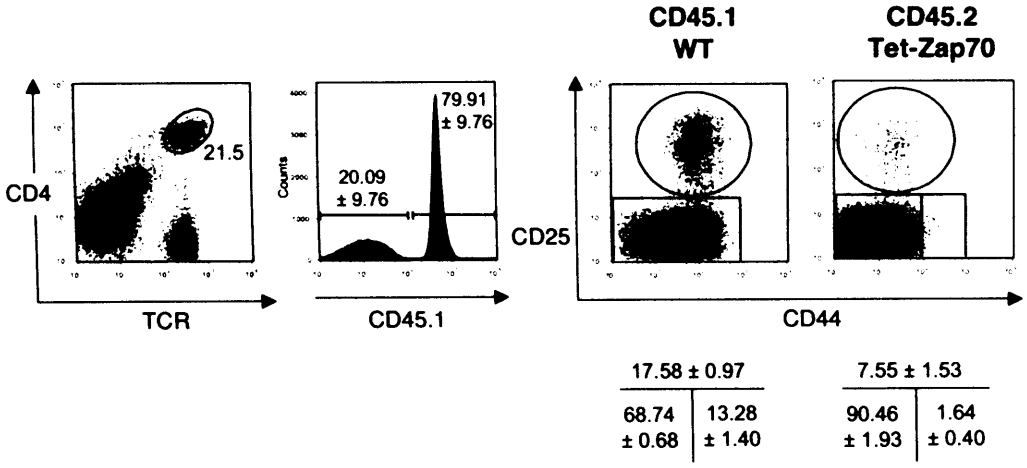


Figure 4.2: Peripheral phenotype of WT and Tet-Zap70 cells generated in replete chimeric mice.

Sub-lethally irradiated C57BL/6J *Rag1*$^{-/-}$ hosts (n = 3) were injected intravenously with a 10:1 ratio of T cell depleted Tet-Zap70 (CD45.2) and WT BM (CD45.1) and maintained on dox treatment (3mg/g) for the entire course of the experiment. 18wks later the thymi and LN were analysed for the expression of the coreceptors CD4 and CD8, TCR, huCD2, CD44, CD122 and CD45.1 by FACS analysis. **A)** Dot plots show the CD4 and TCR staining on T lymphocyte gated LN T cells from a representative dox fed chimera and indicates the TCR^{hi} CD4^{+} gate and the frequency of cells within the population. Histogram shows the CD45.1 expression on the TCR^{hi} CD4^{+} gated T cells. Gates indicate the CD45.2^{+} cells derived from the Tet-Zap70 BM, and the CD45.1 cells derived from the WT BM. Right hand side dots plots shows the frequency of CD44^{lo} CD25^{lo} (naïve), CD44^{hi} CD25^{lo} (memory-like) and CD44^{+} CD25^{hi} (T_{Reg}) T cells, within the CD45.1^{+} and CD45.2^{+} populations, using the gates shown in the histogram of CD45.1 expression. **B)** Left hand side dot plot shows the CD8 and TCR staining on T lymphocyte gated LN T cells from the same chimera. Gate denotes the TCR^{hi} CD8^{+} population of T cells. Histogram shows the CD45.1 expression on the TCR^{hi} CD8^{+} gated T cells. Right hand side dot plots shows the frequency of CD44^{lo} CD122^{lo} (naïve) and CD44^{hi} CD122^{hi} (memory-like) T cells, within the CD45.1^{+} and CD45.2^{+} populations, using the gates shown in the histogram of CD45.1 expression. Numbers reflect the mean percentage of cells present within each T cell population ± standard deviation (SD).

A. LN CD4+ T cells



B. LN CD8+ T cells

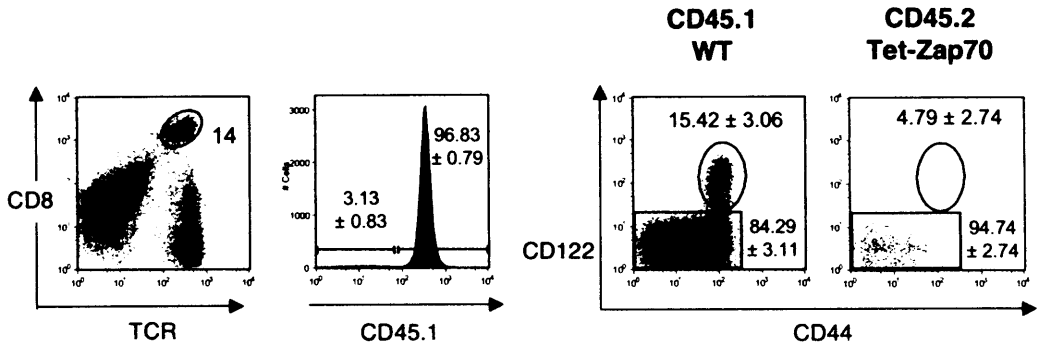
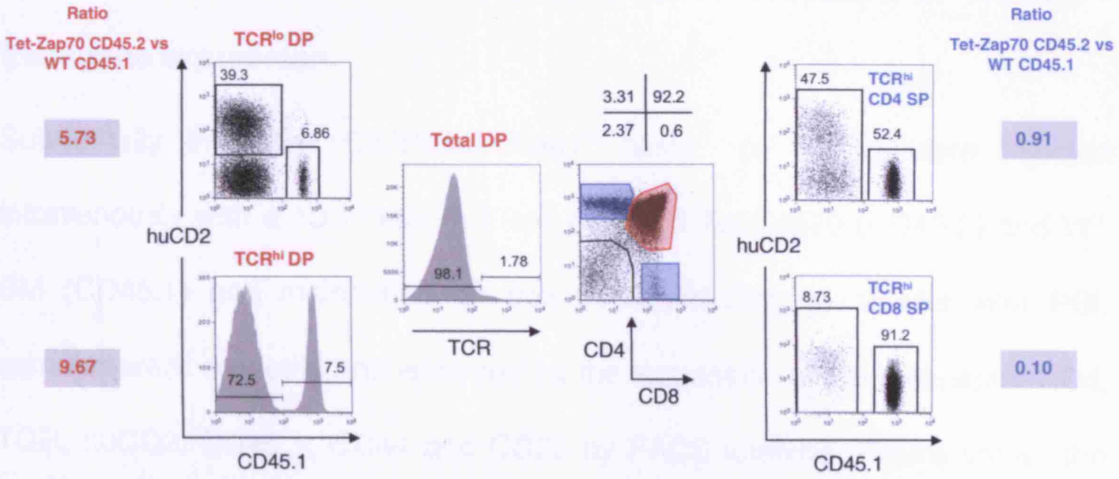


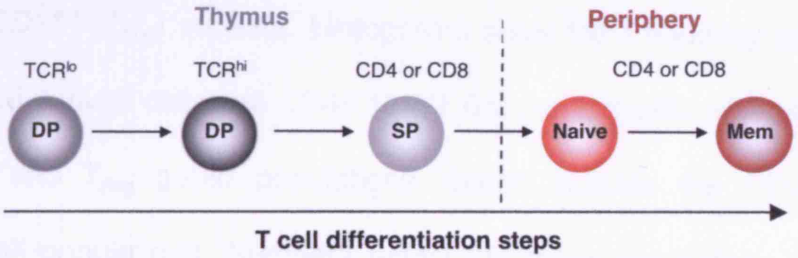
Figure 4.3: Competitive fitness of Tet-Zap70 cells is impaired in replete chimeras.

Sub-lethally irradiated C57BL/6J *Rag1*$^{-/-}$ hosts were injected intravenously with a 10:1 ratio of T cell depleted Tet-Zap70 (CD45.2) and WT BM (CD45.1). 18wks later the thymi, LN and spleen were analysed for the expression of the coreceptors CD4 and CD8, TCR, CD5, huCD2, CD45.1, CD122, and the CD44 and CD25 by FACS analysis **A)** The centre FACS profiles show the CD4 and CD8 coreceptor staining on the T lymphocyte gated thymocytes (gates denote the DP, CD4 SP, CD8 SP and DN thymocyte populations) and the profile of TCR expression on the DP gated thymocytes (population shaded in red). The upper left hand side FACS plot shows the huCD2 and CD45.1 expression on the TCR^{lo} gated DP population shown in the TCR histogram. The lower left hand side FACS plot shows the CD45.1 expression of the TCR^{hi} gated DP thymocytes. Right hand side FACS plots show the huCD2 versus CD45.1 staining on the TCR^{hi} SP subsets (TCR gating not shown). Numbers represent the percentage of cells in each gated region. Grey boxed numbers indicate the ratio of huCD2^{+} CD45.2^{+} (Tet-Zap70) thymocytes versus huCD2^{-} CD45.1^{+} (WT) thymocytes in each thymocyte subset. **Bi)** shows the T cell differentiation steps **Bii)** shows the ratio of huCD2^{+} CD45.2^{+} versus huCD2^{-} CD45.1^{+} thymocytes in all the thymocyte and T cell subsets illustrated in the schematic shown in **Bi).** Ratios were normalised against the starting ratio of Tet-Zap70:WT thymocytes in the most immature TCR^{lo} DP population. Ratios for individual mice are shown (n = 3) as well as the mean ratio amongst CD4 and CD8 lineages ± SD.

A.



B.



Tet-Zap70 vs WT Chimerism during T cell development

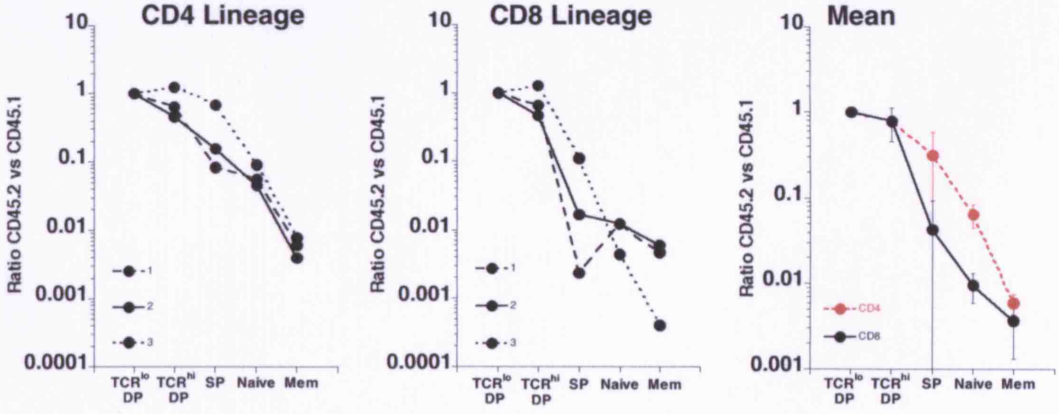


Figure 4.4: Phenotype and frequency of WT and Tet-Zap70 BM derived peripheral TCR^{hi} $CD4^+$ T cell subsets prior to cessation of Tre-Zap70 transgene expression.

Sub-lethally irradiated C57BL/6J *Rag1*$^{-/-}$ hosts (n = 10) were injected intravenously with a 10:1 ratio of T cell depleted Tet-Zap70 (CD45.2) and WT BM (CD45.1) and maintained on dox treatment (3mg/g). 9 wks later PBL samples were collected and analysed for the expression of the coreceptor CD4, TCR, huCD2, CD45.1, CD44 and CD25 by FACS analysis. Figure shows the CD4 versus TCR expression on the PBLs collected from a representative chimera. Gate denotes the TCR^{hi} $CD4^+$ T cell population. Center FACS plot shows the CD25 versus CD44 expression on the TCR^{hi} $CD4^+$ gated T cells. Gates denote the $CD44^{lo}$ $CD25^{lo}$ (naïve), $CD44^{hi}$ $CD25^{lo}$ (memory-like) and $CD44^{intermed}$ $CD25^{hi}$ (T_{Reg}) subsets. Histograms show the frequency of $CD45.2^+$ Tet-Zap70 BM derived cells and $CD45.1^+$ WT BM derived cells within the naïve, memory-like and T_{Reg} gated populations. Gates indicate the $CD45.2^+$ and $CD45.1^+$ T cell populations. Numbers reflect the mean percentage of T cells within each population ± SD.

Phenotype of chimeric peripheral blood CD4+ lineage T cells

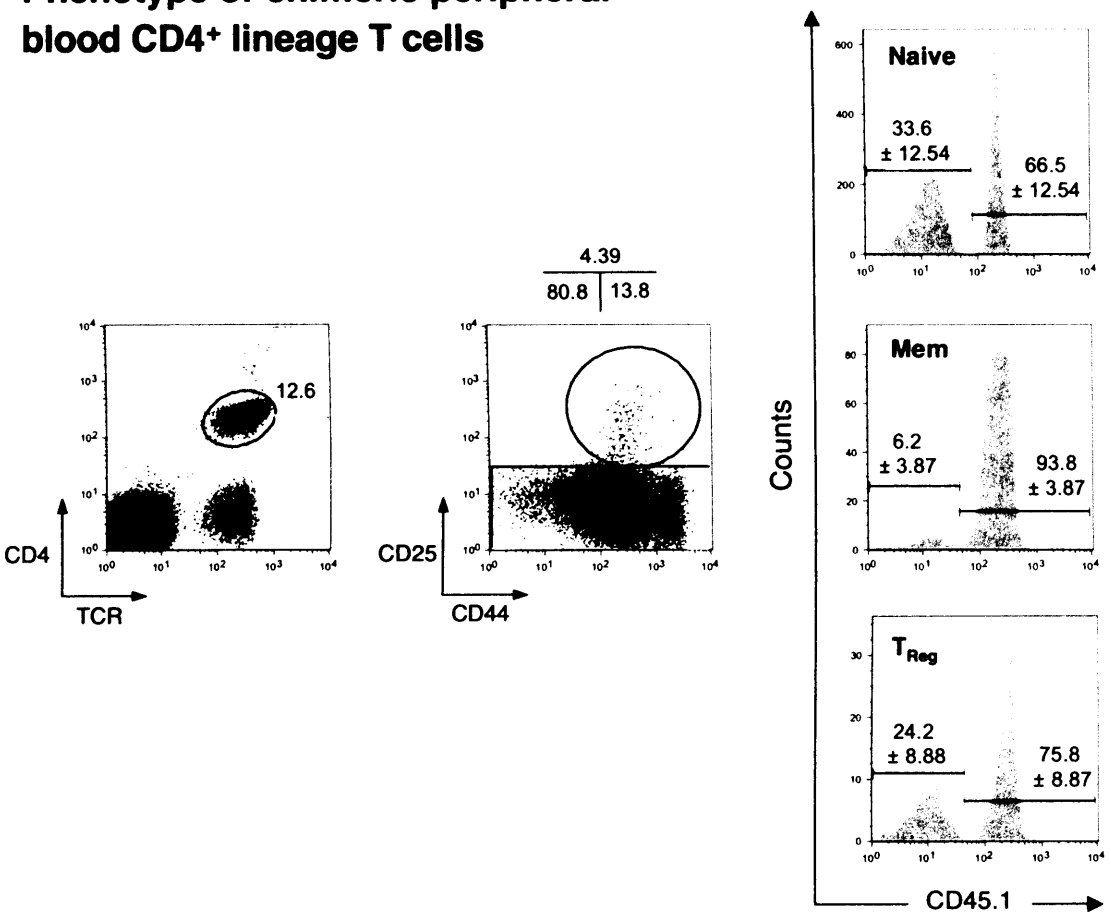
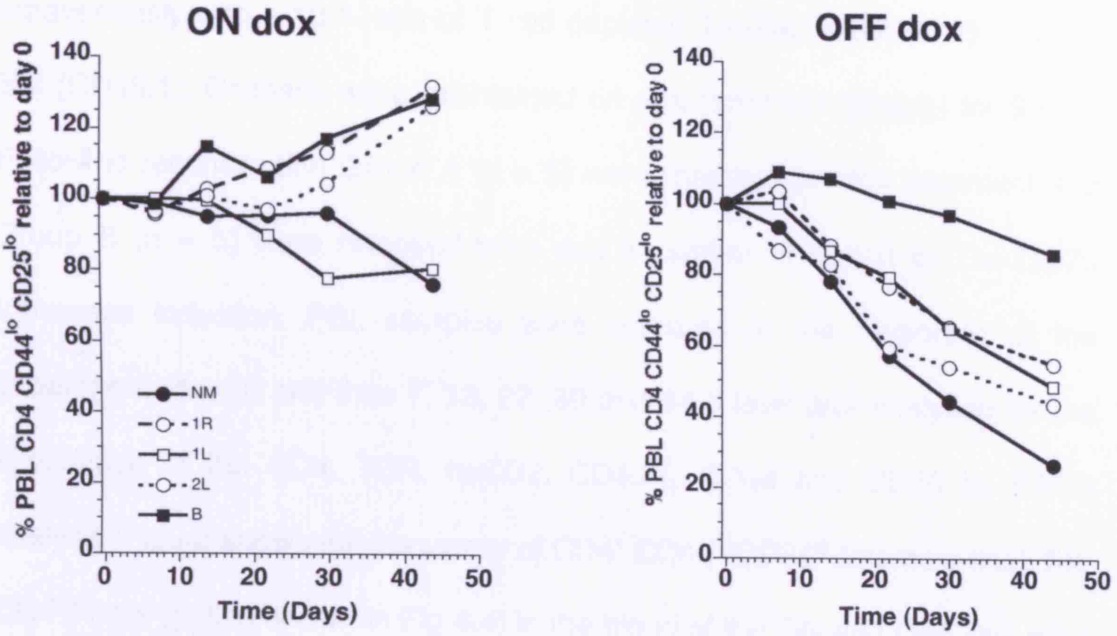


Figure 4.5: Peripheral naïve CD4$^+$ T cell survival is abrogated following ablation of Zap70 expression.

Sub-lethally irradiated C57BL/6J *Rag1*$^{-/-}$ hosts (n = 10) were injected intravenously with a 10:1 ratio of T cell depleted Tet-Zap70 (CD45.2) and WT BM (CD45.1). Chimeric were maintained on dox treatment (3mg/g) for 9 wks. Following reconstitution Group A (n = 5) were maintain on dox treatment and Group B (n = 5) were removed from dox treatment to switch of Tre-Zap70 transgene induction. PBL samples were collected at the beginning of the experiment (day 0) and then 7, 12, 22, 30 and 44 d later and analysed for the expression of the CD4, TCR, huCD2, CD45.1, CD44 and CD25 by FACS analysis. Figure shows the frequency of CD4$^+$ CD44^{lo} CD25^{lo} (naïve) Tet-Zap70 cells (gating shown in Fig 4.4) in the blood of the ON and OFF dox mice during the timecourse. Upper graphs show the frequency of naive Tet-Zap70 CD4$^+$ T cells from individual mice. Lower graph shows the mean frequencies of Tet-Zap70 and WT naïve CD4$^+$ T cells from the ON and OFF dox chimeras ± SD. Data is representative of two independent experiments. Statistical significance between WT control OFF dox and Tet-Zap70 cells OFF dox was calculated using the Mann Whitney test ($P< 0.05$ = * $P< 0.01$ = **)

Tet-Zap70 CD4+ CD44lo CD25lo



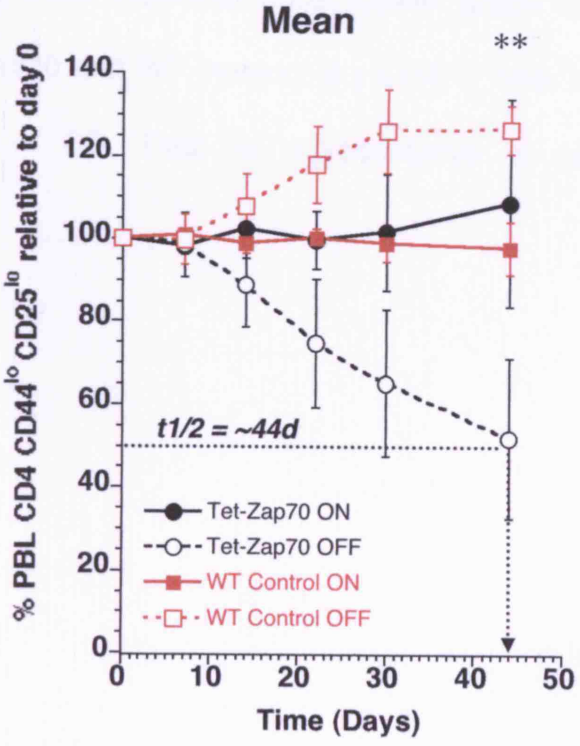
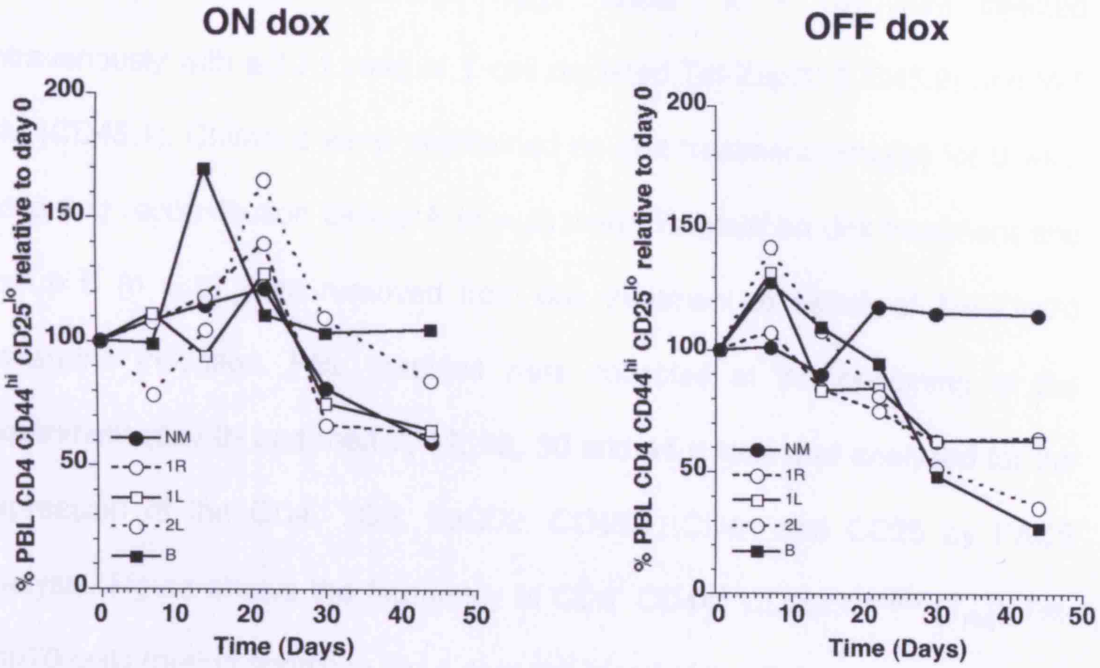


Figure 4.6: Survival of peripheral CD4$^+$ memory-like T cells following cessation of Zap70 expression.

Sub-lethally irradiated C57BL/6J *Rag1*$^{-/-}$ hosts (n = 10) were injected intravenously with a 10:1 ratio of T cell depleted Tet-Zap70 (CD45.2) and WT BM (CD45.1). Chimeric were maintained on dox treatment (3mg/g) for 9 wks. Following reconstitution Group A (n = 5) were maintain on dox treatment and Group B (n = 5) were removed from dox treatment to switch of Tre-Zap70 transgene induction. PBL samples were collected at the beginning of the experiment (day 0) and then 7, 12, 22, 30 and 44 d later and analysed for the expression of the CD4, TCR, huCD2, CD45.1, CD44 and CD25 by FACS analysis. Figure shows the frequency of CD4$^+$ CD44^{hi} CD25^{lo} (memory-like) Tet-Zap70 cells (gating shown in Fig 4.4) in the blood of the ON and OFF dox mice during the timecourse. Upper graphs show the frequency of memory-like Tet-Zap70 CD4$^+$ T cells from individual mice. Lower graph shows the mean frequencies of Tet-Zap70 and WT memory-like CD4$^+$ T cells from the ON and OFF dox chimeras ± SD. Data is representative of two independent experiments.

Tet-Zap70 CD4 CD44^{hi} CD25^{lo}



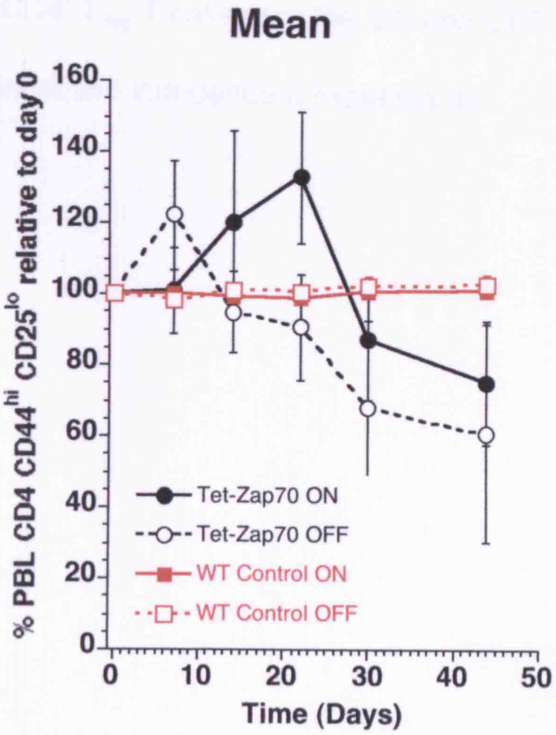


Figure 4.7: Survival of peripheral CD4$^+$ T_{Reg} cells following cessation of Zap70 expression.

Sub-lethally irradiated C57BL/6J *Rag1*$^{-/-}$ hosts (n = 10) were injected intravenously with a 10:1 ratio of T cell depleted Tet-Zap70 (CD45.2) and WT BM (CD45.1). Chimeric were maintained on dox treatment (3mg/g) for 9 wks. Following reconstitution Group A (n = 5) were maintain on dox treatment and Group B (n = 5) were removed from dox treatment to switch of Tre-Zap70 transgene induction. PBL samples were collected at the beginning of the experiment (day 0) and then 7, 12, 22, 30 and 44 d later and analysed for the expression of the CD4, TCR, huCD2, CD45.1, CD44 and CD25 by FACS analysis. Figure shows the frequency of CD4$^+$ CD44^{hi} CD25^{intermediate} (T_{Reg}) Tet-Zap70 cells (gating shown in Fig 4.4) in the blood of the ON and OFF dox mice during the timecourse. Upper graphs show the frequency of T_{Reg} Tet-Zap70 CD4$^+$ T cells from individual mice. Lower graph shows the mean frequencies of Tet-Zap70 and WT CD4$^+$ T_{Reg} T cells from the ON and OFF dox chimeras ± SD. Data is representative of two independent experiments.

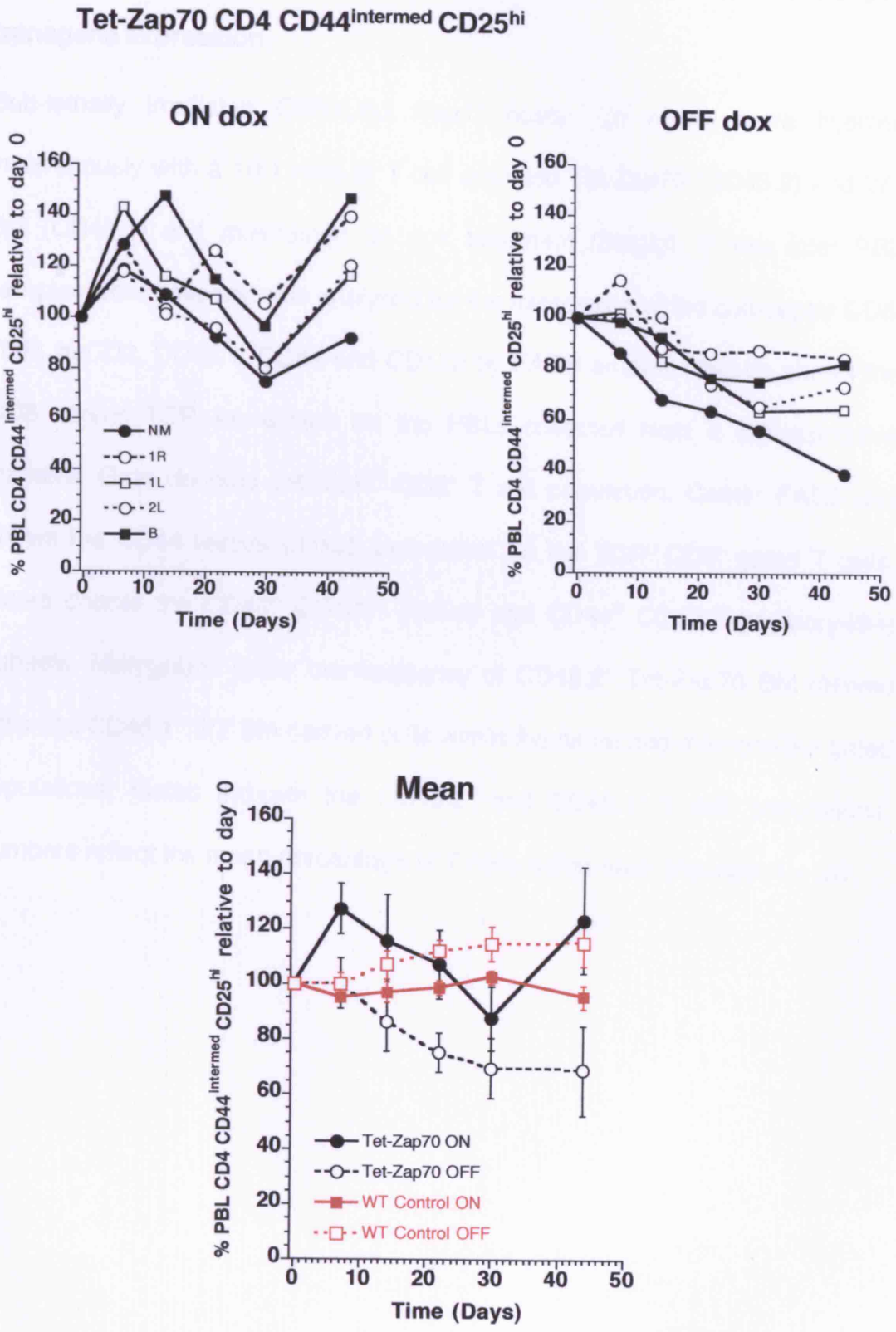

Tet-Zap70 CD4 CD44intermed CD25hi
ON dox
OFF dox
% PBL CD4 CD44intermed CD25hi relative to day 0
Time (Days)
NM
1R
1L
2L
B
Mean
Tet-Zap70 ON
Tet-Zap70 OFF
WT Control ON
WT Control OFF

Figure 4.8: Phenotype and frequency of WT and Tet-Zap70 BM derived peripheral TCR^{hi} $CD8^+$ T cell subsets prior to cessation of Tre-Zap70 transgene expression.

Sub-lethally irradiated C57BL/6J *Rag1*$^{-/-}$ hosts (n = 10) were injected intravenously with a 10:1 ratio of T cell depleted Tet-Zap70 (CD45.2) and WT BM (CD45.1) and maintained on dox treatment (3mg/g). 9 wks later PBL samples were collected and analysed for the expression of the coreceptor CD8, TCR, huCD2, CD45.1, CD44 and CD122 by FACS analysis. Figure shows the CD8 versus TCR expression on the PBLs collected from a representative chimera. Gate denotes the TCR^{hi} $CD8^+$ T cell population. Center FACS plot shows the CD44 versus CD122 expression on the TCR^{hi} $CD8^+$ gated T cells. Gates denote the $CD44^{lo}$ $CD122^{lo}$ (naïve) and $CD44^{hi}$ $CD122^{hi}$ (memory-like) subsets. Histograms show the frequency of $CD45.2^+$ Tet-Zap70 BM derived cells and $CD45.1^+$ WT BM derived cells within the naïve and memory-like gated populations. Gates indicate the $CD45.2^+$ and $CD45.1^+$ T cell populations. Numbers reflect the mean percentage of T cells within each population ± SD.

Phenotype of chimeric peripheral blood CD8 lineage T cells

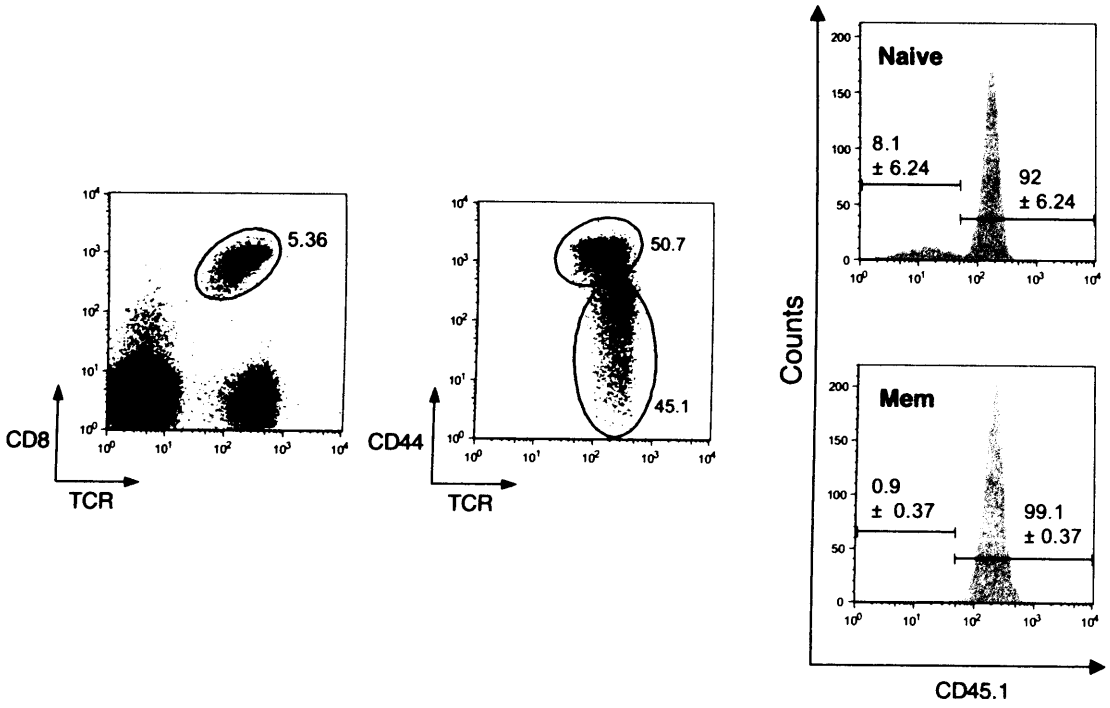
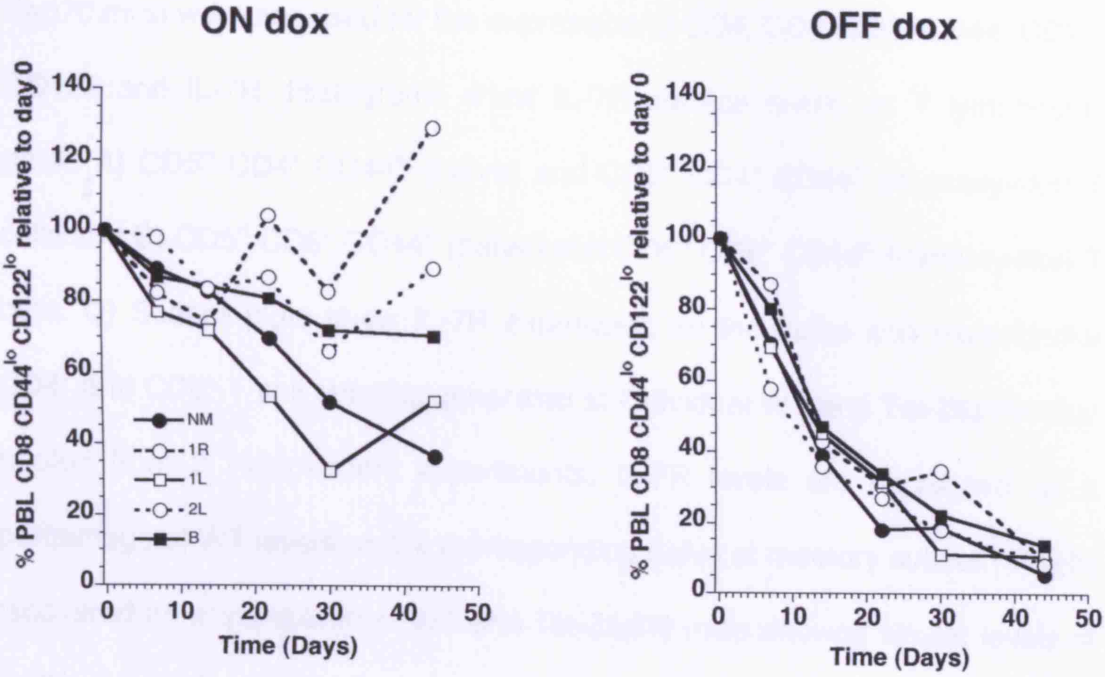


Figure 4.9: Peripheral Naïve CD8$^+$ T cell survival is abrogated following ablation of Zap70 expression.

Sub-lethally irradiated C57BL/6J *Rag1*$^{-/-}$ hosts (n = 10) were injected intravenously with a 10:1 ratio of T cell depleted Tet-Zap70 (CD45.2) and WT BM (CD45.1). Chimeras were maintained on dox treatment (3mg/g) for 9 wks. Following reconstitution Group A (n = 5) were maintain on dox treatment and Group B (n = 5) were removed from dox treatment to switch of Tre-Zap70 transgene induction. PBL samples were collected at the beginning of the experiment (day 0) and then 7, 12, 22, 30 and 44 d later and analysed for the expression of the CD8, TCR, huCD2, CD45.1, CD44 and CD122 by FACS analysis. Figure shows the frequency of CD8$^+$ CD44^{lo} CD122^{lo} (naïve) Tet-Zap70 cells (gating shown in Fig 4.8) in the blood of the ON and OFF dox mice during the timecourse. Upper graphs show the frequency of naive Tet-Zap70 CD8$^+$ T cells from individual mice. Lower graph shows the mean frequencies of Tet-Zap70 and WT naïve CD8$^+$ T cells from the ON and OFF dox chimeras ± SD. Data is representative of two independent experiments. Statistical significance between meaned WT control OFF dox and Tet-Zap70 OFF dox cells was calculated using the Mann Whitney test ($P< 0.05$ = * $P< 0.01$ = **).

Tet-Zap70 CD8+ CD44lo CD122lo



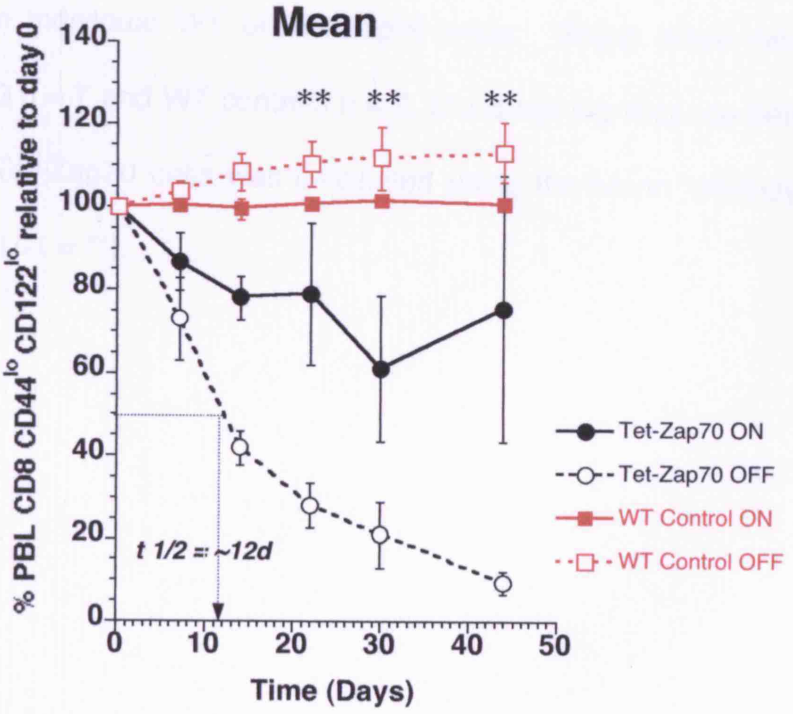
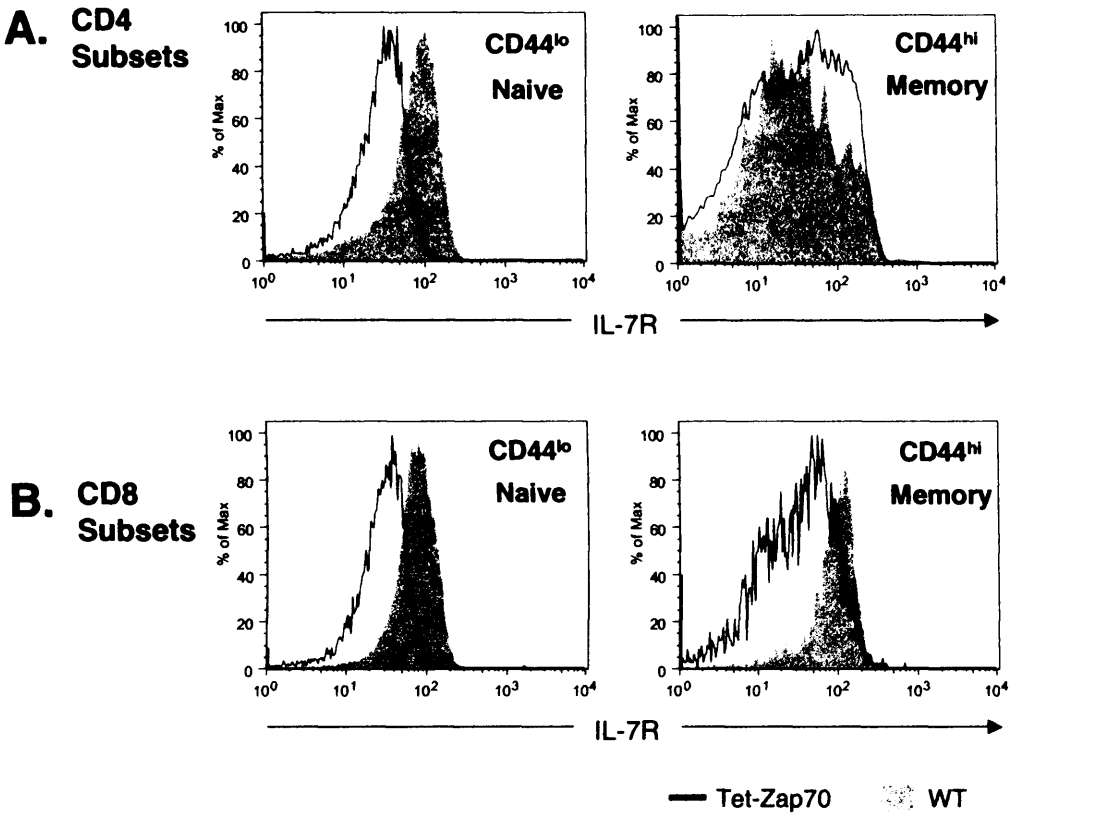


Figure 4.10: Decreased IL-7R surface expression in peripheral mature CD4$^+$ and CD8$^+$ T cells in Tet-Zap70 mice.

T cells from the LN of 5-12 wk old WT and constitutively dox fed (1mg/g) Tet-Zap70 mice were analysed for the expression of CD4, CD8, CD5, CD44, CD25, CD122 and IL-7R. Histograms show IL-7R surface levels on T lymphocyte gated **A)** CD5^{hi} CD4$^+$ CD44^{lo} (naïve) and CD5^{hi} CD4$^+$ CD44^{hi} (memory-like) T cells and **B)** CD5^{hi} CD8$^+$ CD44^{lo} (naïve) and CD5^{hi} CD8$^+$ CD44^{hi} (memory-like) T cells. **C)** Scatter plots show IL-7R expression on the naïve and memory-like CD4$^+$ and CD8$^+$ T cell subsets generated in individual WT and Tet-Zap70 mice pooled from 3 independent experiments. IL-7R levels are expressed as a percentage of WT levels on the corrosponding naïve or memory subset. T cells recovered from the spleen of WT and Tet-Zap70 mice showed similar levels of IL-7R expression therefore only LN data is shown. **D)** shows the IL-7R expression on the naïve CD4$^+$ and CD8$^+$ T cells generated in the same mouse. Naïve CD8$^+$ IL-7R expression is expressed as a percentage of naïve CD4$^+$ IL-7R expression in individual WT or Tet-Zap70 mice. Group sizes were as follows Tet-Zap70 n = 7 and WT controls n = 7. Statistical significance between WT control and Tet-Zap70 cells was calculated using the Mann Whitney test ($P< 0.05$ = * $P< 0.01$ = **).


A.
CD4 Subsets
CD44lo Naive
CD44hi Memory
% of Max
IL-7R
B.
CD8 Subsets
CD44lo Naive
CD44hi Memory
% of Max
IL-7R
Tet-Zap70
WT

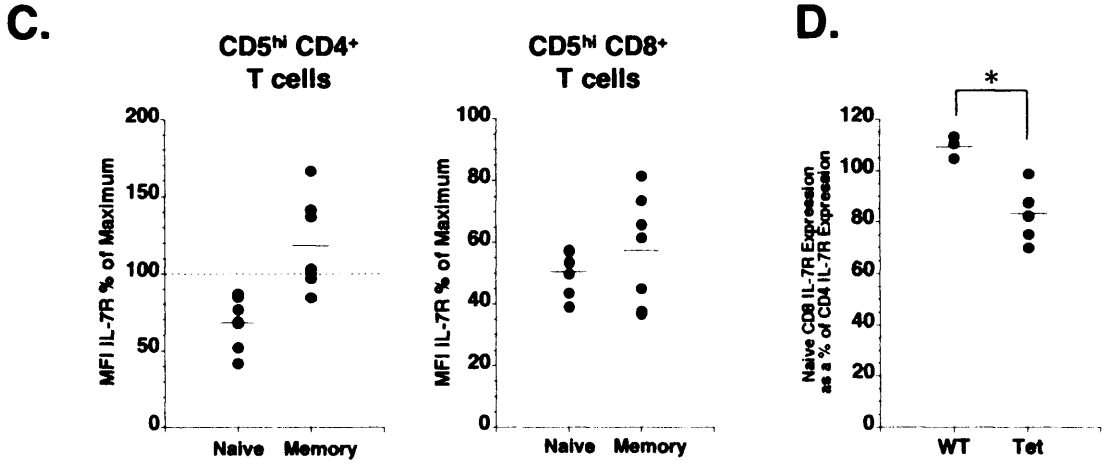
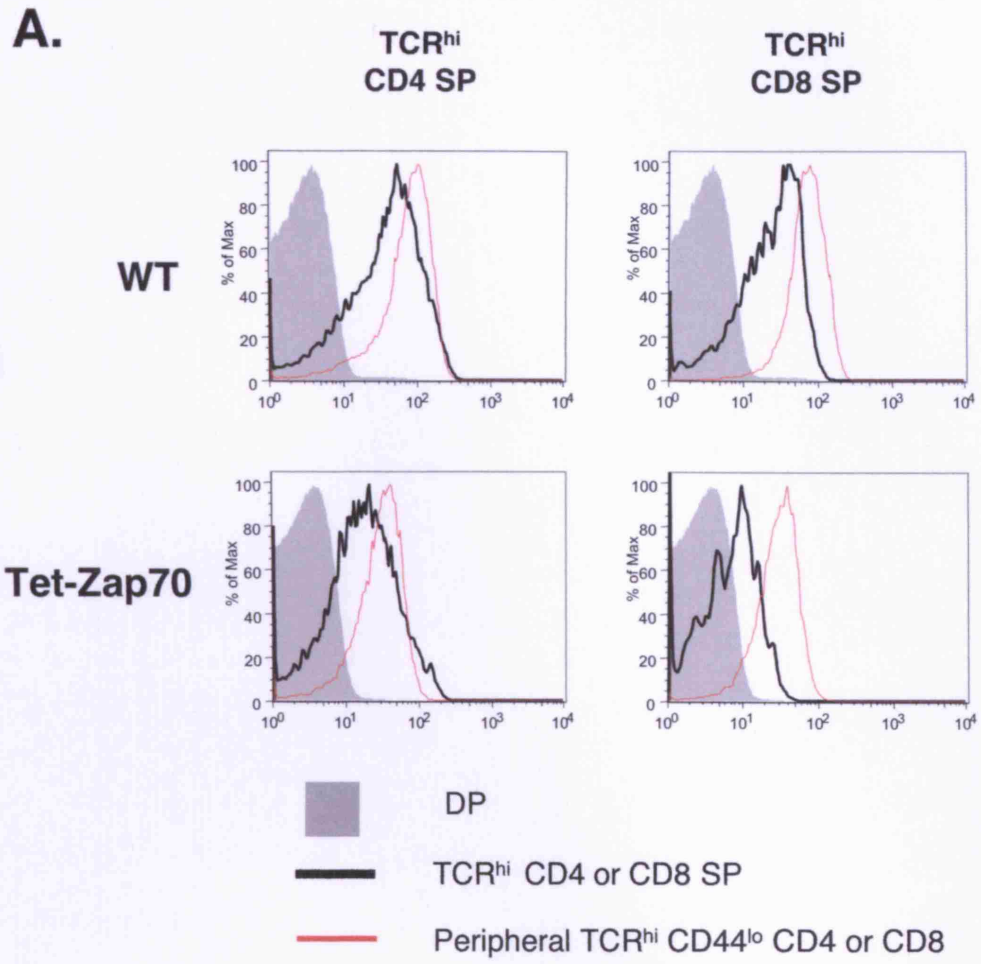
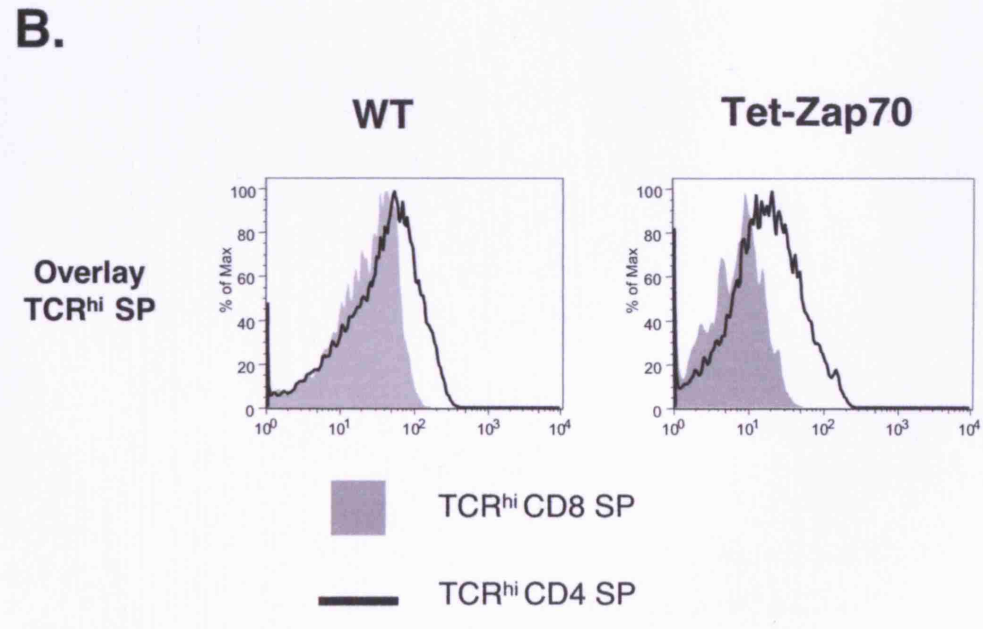

C.
CD5hi CD4+ T cells
CD5hi CD8+ T cells
MFI IL-7R % of Maximum
Naive
Memory
D.
Naive CD8 IL-7R Expression as a % of CD4 IL-7R Expression
WT
Tet
*

Figure 4.11: Impaired IL-7R re-expression in mature Tet-Zap70 CD4 SP and CD8 SP thymocytes.

Thymocytes and LN T cells from 5-12 wk old WT and constitutively dox fed (1mg/g) Tet-Zap70 mice were analysed for the expression of CD4, CD8, TCR and IL-7R. Histograms show the overlays IL-7R surface levels on T lymphocyte gated **A)** DP thymocytes, TCR^{hi} CD4 SP thymocytes, TCR^{hi} CD8 SP thymocytes and LN TCR^{hi} $CD4^{+}$ $CD44^{lo}$ and TCR^{hi} $CD8^{+}$ $CD44^{lo}$ (naïve) T cells generated in the same WT or Tet-Zap70 mouse. **B)** shows overlays of IL-7R expression TCR^{hi} CD4 SP thymocytes and TCR^{hi} CD8 SP thymocytes from either WT or Tet-Zap70 mice as indicated. Data is representative of three independent experiments.


A.
TCRhi CD4 SP
TCRhi CD8 SP
WT
Tet-Zap70
% of Max
DP
TCRhi CD4 or CD8 SP
Peripheral TCRhi CD44lo CD4 or CD8


B.
WT
Tet-Zap70
Overlay TCRhi SP
% of Max
TCRhi CD8 SP
TCRhi CD4 SP

Chapter 5

TCR and Cytokine Signals in Naïve T cell Homeostasis

5.1 Introduction

The Immune system has to respond to a diverse array of pathogens, it is therefore essential that the peripheral T cell compartment is subject to control by homeostatic mechanisms, which maintain the T cell pool at a constant size and composition. This homeostatic control is largely mediated by a balancing T cell survival, T cell proliferation as well as the thymic production of newly generated T cells. Conversely T cell death as a result of natural processes such as viral or bacterial infections or as a consequence of medical intervention also has a role in defining the nature of the peripheral lymphoid compartment.

The precise homeostatic balance within the T cell compartment is achieved because T cells are sensitive to their environment, responding to environmental cues that fine tune both cell survival and proliferation. The nature of the environmental cues that regulate these responses vary depending on the T cell subset and whether the cell is of naïve or memory phenotype. It is known that transmission of signals through the TCR and IL-7R on the T cell surface are involved in regulating the size of the peripheral T cell compartment, however the precise manner in which these signals cooperate to maintain naïve T cell

homeostasis is unclear. Over recent years several studies have established that the prolonged steady state survival of the naïve $CD4^+$ and $CD8^+$ compartments requires interaction of the TCR with self peptide MHC ligands (Brocker, 1997; Kirberg et al., 1997; Polic et al., 2001; Tanchot et al., 1997; Viret et al., 1999; Witherden et al., 2000) similar to those that mediate positive selection in the thymus (Ernst et al., 1999; Goldrath and Bevan, 1999; Viret et al., 1999). More recent reports have also implicated the TCR associated Src Kinases Lck and Fyn in the propagation of these homeostatic TCR signals that maintain T cell survival (Seddon and Zamoyska, 2002b). Furthermore, the cytokine IL-7, has been shown to play an essential role in the survival of mature naïve T cells *in vivo* (Tan et al., 2001; Vivien et al., 2001) at least in part by inhibiting the mitochondrial pathway of apoptosis, upregulating the expression of anti apoptotic proteins Bcl_{XL} (Amos et al., 1998) and Bcl-2 (Akashi et al., 1997; Maraskovsky et al., 1997) or by suppressing the activity of pro-apoptotic proteins including Bad (Li et al., 2004) Bax (Khaled et al., 2002) and Bim (Pellegrini et al., 2004).

Under conditions of T cell lymphopenia, naïve T cells have the capacity to undergo LIP (Ernst et al., 1999; Goldrath and Bevan, 1999; Kieper and Jameson, 1999). This is a homeostatic mechanism of cell division that occurs in response to the T cell deficiency within the naïve T cell compartment. Interestingly, the signals that support naïve T cell survival also play a critical role in influencing LIP. Interaction of the TCR with spMHC ligands under lymphopenic conditions can trigger naïve T cells to divide despite an absence of foreign antigenic stimulation (Ernst et al., 1999; Goldrath and Bevan, 1999;

Kieper and Jameson, 1999; Viret et al., 1999). Similarly homeostatic cell division of donor T cell in lymphopenic hosts is abrogated in the absence IL-7 signalling (Goldrath et al., 2002; Schluns et al., 2000; Tan et al., 2001). While these studies suggest that TCR and IL-7 signals are essential for naïve T cell survival and induction of LIP, the full relationship between these two homeostatic signalling pathways remain unclear. Does naïve T cell survival and LIP have different TCR and IL-7 signalling requirements? How do naïve T cells integrate IL-7 and TCR spMHC signals for survival and LIP?

A key shortcoming of previous studies assessing the signals that drive naive T cell survival and LIP is that they only investigate the requirement of TCR and IL-7 signals to a purely qualitative level and fail to address the quantitative role of these different signals in T cell homeostasis, largely as a result of difficulties in discriminating between survival, LIP and expansion under lymphopenic conditions. The objective of this study was to develop a system in which the dynamics of these homeostatic responses could be precisely quantified. This would allow us to quantify the effects of specific defects in TCR and IL-7R signalling and assess the differential contributions of TCR and IL-7R signals to the survival and the homeostatic expansion of the naive T cell compartment in *vivo*. To achieve this we aimed to establish a mouse model in which we could distinguish and measure and the survival and LIP of naïve MHC Class I H-2D^{b} restricted F5$^{+/+}$ CD8^{+} TCR transgenic T cells (F5 T cells) (Mamalaki et al., 1993) in T cell deficient hosts. F5 T cells transferred to lymphopenic *Rag1*$^{-/-}$ hosts undergo slow homeostatic cell divisions whilst maintaining a naïve (CD44^{lo}) phenotype. By using the cell dye CFSE to precisely measure cell division

together with kinetic analysis of cell recoveries from lymphopenic hosts, this mouse model would allow us to measure the number of divisions a specific cohort of cells underwent over time and calculate the extent to which the population expanded as a result of the measured cell divisions (expansion factor **EF**). By determining the expansion factor, we could then follow the survival of the original precursor population excluding the proliferative expansive effects induced by the lymphopenia. Using cytokine and MHC Class I deficient mice as hosts would allow us to dissect the role of TCR and IL-7 signals in these homeostatic responses.

5.2 Results

5.2.1 Measuring Naïve CD8$^+$ T cell Survival and LIP *In vivo*

Before we could dissect the roles of TCR and IL-7 signals in naïve T cell homeostasis, we firstly established the baseline homeostatic responses of naïve control F5 T cells in lymphopenic *Rag1*$^{-/-}$ hosts. F5 T cells were labelled with CFSE an intracellular cell dye, whose dilution in daughter cells is directly proportional to cell division, allowing the measurement of the proportion of cells in each cell division.

Following intravenous injection into *Rag1*$^{-/-}$ recipients, the cell recovery and phenotype of the F5 T cells in the LN and spleen of cohorts of mice at d 2, 7, 13 and 21 post transfer was determined, in order to determine the precise kinetics of the LIP response. The T cells recovered from the *Rag1*$^{-/-}$ recipients underwent several divisions over the time course with the majority of cells

undergoing at least one round of homeostatic cell division, evident in the CFSE profile of the cells **(Fig 5.1 A)**. Although the cells went underwent slow but sustained division they failed to upregulate CD44 and maintained a naïve phenotype.

The manner in which the size of the original precursor population, used to seed the T cell deficient hosts was calculated is detailed in **Fig 5.1 B**. The expansion factor was used to determine the fold expansion of a specific population of T cells calculated from the pattern of cell division revealed by the CFSE profile of the cells **(Fig 5.1 A)**. For example a population of cells will double with each round of cell division. The increase in the size of the population (predicted by the CFSE profile) compared to the starting input of cells is the expansion factor. By dividing the absolute cell recoveries in each division by the expansion factor, the size of the precursor population can be calculated (see also ***2.11 Materials and Methods***). By following the kinetics of the precursor population over time, cell death can detected by a reduction in the size of the precursor population. This allows the dissection of T cell survival from the expansive effects of LIP, and therefore allows us to more accurately follow the survival of the precursor population independently of expansion. This is shown in **Fig 5.1 C**. The data shows that the absolute number of donor F5 T cells recovered from the *Rag1*$^{-/-}$ hosts increased substantially over the timecourse. Calculation of the precursor population size (recovery of the cells excluding the effects of expansion) revealed no detectable cell death over the 21 d time period **(Fig 5.1 C)**. The expansion of the population with time was also reflected in the profiles of mean division (the average number of divisions that a cell has undergone)

(Fig 5.1 D) and predicted expansion (the fold expansion of the population calculated from the pattern of division predicted by the CFSE profile of the cells) **(Fig 5.1 E)**, which both increased over time.

5.2.2 The Degree of Homeostatic Proliferation in Lymphopenic Hosts is Dependent on Cell Dose

We next wished to determine whether the LIP response was proportional to size of the inoculum used to seed the lymphopenic hosts. To investigate this we transferred different numbers of F5 T cells into *Rag1*$^{-/-}$ hosts and observed the LIP response. $1.5x10^6$ or $20x10^6$ CFSE labelled naïve LN F5 T cells were intravenously injected into the tail vein of *Rag1*$^{-/-}$ recipients. The CFSE profile of the donor F5 T cells were assessed 14 d post transfer in the LN and spleen by FACS analysis.

Interestingly *Rag1*$^{-/-}$ hosts that received the high cell dose showed fewest cell divisions, with a large frequency of cells remaining undivided (division 0) **(Fig 5.2 A)**. In contrast the *Rag1*$^{-/-}$ hosts injected with the small cell dose showed the greatest number of cell divisions, reflected in the CFSE profile and the calculated number of mean divisions **(Fig 5.2 B)**. This suggests that cell dose does influence the degree of LIP within a given cohort of naïve T cells, implying that increased competition for host factors involved in LIP can restrict the expansion of the naïve T cell compartment. This is consistent with findings reported by (Moses et al., 2003; Troy and Shen, 2003) and (Ge et al., 2004)

that homeostatic expansion of peripheral naïve $CD4^+$ and $CD8^+$ T cells is regulated by the clonal competition for limited environmental factors.

5.2.3 TCR Signals are Essential for the LIP of Naïve T cells but not Their Survival in Lymphopenic Hosts

In order to examine the role of homeostatic TCR signals in naïve $CD8^+$ T cell survival and LIP, we transferred naïve F5 T cells into *F5*$^{+/+}$ *β2m*$^{-/-}$ *Rag1*$^{-/-}$ recipients that lack MHC Class I ligands. $1.5x10^6$ APC depleted naïve CFSE labelled LN F5 T cells were transferred by tail vein injection into groups of *Rag1*$^{-/-}$ and *F5*$^{+/+}$ *β2m*$^{-/-}$ *Rag1*$^{-/-}$ mice. *F5*$^{+/+}$ *β2m*$^{-/-}$ *Rag1*$^{-/-}$ mice (hereon referred to as *β2m*$^{-/-}$ *Rag1*$^{-/-}$) are a F5 $CD8^+$ TCR transgenic line lacking the invariant β2 microglobulin subunit of the MHC Class I complex, rendering the mice MHC Class I deficient, and unable to provide TCR ligands. Although the mice bear the F5 TCR transgene, in the absence of MHC Class I ligands, they fail to select any T cells and are consequently T cell deficient. Therefore we took advantage of this available strain as a *Rag1*$^{-/-}$ MHC Class I deficient host. The kinetics of F5 T cell LIP and survival in the presence or absence of MHC Class I ligands is shown in **Fig 5.3**. The F5 T cells recovered from the LN and spleen of *Rag1*$^{-/-}$ and *β2m*$^{-/-}$ *Rag1*$^{-/-}$ recipients were comparable at d 1 of the timecourse **(Fig 5.3 C)**, indicating that the initial seeding of the host mice with the donor cells was consistent between the two groups of mice. F5 T cells recovered from the *Rag1*$^{-/-}$ and *β2m*$^{-/-}$ *Rag1*$^{-/-}$ recipients over the timecourse did not upregulate the activation marker CD44 and retained their naïve phenotype (data not shown). As expected, in *Rag1*$^{-/-}$ hosts F5 T cells showed a slow but steady

pattern of cell division **(Fig 5.3 A and B)** also evident in the gradual expansion of the donor T cell population over the 21 days timecourse **(Fig 5.3 C).** In contrast the F5 T cells transferred to the *β2m*$^{-/-}$ *Rag1*$^{-/-}$ hosts showed both a substantially reduced level of cell division compared to the donor cells transferred into the MHC Class I sufficient hosts **(Fig 5.3 A and B)** and a failure to expand over time **(Fig 5.3 C)**. The precursor cell numbers (that exclude the predicted expansive effects of proliferation), showed a subtle decay in donor T cell numbers in *β2m*$^{-/-}$ *Rag1*$^{-/-}$ recipients, (most notable between d 1 and d 14). In contrast F5 T cells transferred to *Rag1*$^{-/-}$ hosts did not show this decline in cell number. However, this was not always evident in replicate experiments. Given the small number of cells recovered from the *β2m*$^{-/-}$ *Rag1*$^{-/-}$ hosts, it is unclear whether the subtle decay in the recovery of the F5 T cells from MHC Class I deficient recipients over the timecourse reflects a true survival difference or that the small number of T cells used to seed the recipients was too low to be accurately followed over the timecourse.

To more accurately address whether homeostatic TCR signals influence naïve T cell survival and LIP we performed the same experiment but used a larger number of donor F5 T cells to seed the *Rag1*$^{-/-}$ and *β2m*$^{-/-}$ *Rag1*$^{-/-}$ recipients. The use of a larger cell dose as reported earlier **(Fig 5.2)** is likely to increase the competition for limited survival factors, which may allow subtle survival defects to be detected. During CFSE labelling ~20-30% of the T cells die, therefore to maximise the number of T cells that could be transferred, we only CFSE labelled a small tracer population of the donor F5 T cells.

$2x10^6$ CFSE labelled and $17x10^6$ unlabelled APC depleted naïve LN F5 T cells were transferred into groups of *Rag1*$^{-/-}$ and *β2m*$^{-/-}$ *Rag1*$^{-/-}$ mice. Mice were sacrificed at 1, 7, 14 and 21 d post transfer and the absolute number and precursor population size in the LN and spleen were calculated. The F5 T cells appeared to seed both groups of mice with similar efficiency given the cell recoveries obtained from the *Rag1*$^{-/-}$ and *β2m*$^{-/-}$ *Rag1*$^{-/-}$ mice were similar at d 1 of the study. The F5 T cells transferred to *Rag1*$^{-/-}$ hosts displayed a progressive pattern of cell division **(Fig 5.4 A and B)** and showed a substantial degree of expansion over the study **(Fig 5.4 C)** as illustrated by the increase in the absolute number of T cells recovered over the timecourse. In contrast, as seen with earlier experiments the F5 T cells recovered from the *β2m*$^{-/-}$ *Rag1*$^{-/-}$ hosts failed to show a similar degree of cell division and expansion. However, a low level of cell division was consistently observed in the *β2m*$^{-/-}$ *Rag1*$^{-/-}$ hosts **(Fig 5.4 A and B)** suggesting this proliferation maybe independent of homeostatic TCR signalling. Analysis of the precursor size **(Fig 5.4 C)** could not reveal any differences in the survival of the F5 T cells transferred to the *Rag1*$^{-/-}$ and *β2m*$^{-/-}$ *Rag1*$^{-/-}$ hosts, suggesting homeostatic TCR signals are not essential for the short-term survival of naïve $CD8^+$ T cells under these conditions. Analysis of the intracellular levels of Bcl-2 in the F5 donor T cells transferred to the *Rag1*$^{-/-}$ and *β2m*$^{-/-}$ *Rag1*$^{-/-}$ and an *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ control revealed that as expected, Bcl-2 levels were reduced in the F5 T cells transferred to the *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ control showing that intact IL-7 signalling is required for optimal Bcl-2 expression **(Fig 5.5 A)**. Bcl-2 levels were similar between F5 T cells recovered from the *Rag1*$^{-/-}$

and *β2m*$^{-/-}$ *Rag1*$^{-/-}$ hosts, suggesting that homeostatic TCR signals do not influence IL-7R signalling **(Fig 5.5 A and B)**.

5.2.4 IL-7 Signalling is Essential for the Survival and LIP of Naïve CD8^{+} T cells

We next assessed the kinetics of F5 T cell survival, LIP and expansion in the presence and absence of IL-7 signalling. 1.5x10^{6} APC depleted naïve LN F5 T cells were CFSE labelled and adoptively transferred into groups of *Il-7*$^{+/+}$ *Rag1*$^{-/-}$, *Il-7*$^{+/-}$ *Rag1*$^{-/-}$ and *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ lymphopenic recipients. Mice were sacrificed at 2, 7, 13 and 21 d post transfer and absolute cell recoveries determined. **Fig 5.6 A** shows the profile of F5 T cell division in the spleen of injected hosts at d 13 post injection. The F5 T cells transferred to the *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ hosts proliferated more than T cells transferred into either *Il-7*$^{+/-}$ *Rag1*$^{-/-}$ or *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ recipients. This was also reflected in the graphs of mean division number **(Fig 5.6 B)**.

Interestingly, we observed a consistent IL-7 gene dose effect in the T cell response. Cells transferred to the *Il-7*$^{+/-}$ *Rag1*$^{-/-}$ hosts proliferated and expanded significantly less than in the *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ recipients. In the complete absence of IL-7 the cells failed to divide. This data suggests that in the presence of reduced levels of IL-7 as in the *Il-7*$^{+/-}$ hosts, IL-7 and TCR signals can maintain limited LIP, however in the complete absence of IL-7, TCR signals alone cannot support the LIP of the naïve T cell compartment.

Calculation of the absolute number of F5 T cells recovered from the experimental mice, revealed that the initial cell recoveries were different at d 2

post transfer suggesting that the initial seeding of the donor cells was not consistent between the recipient strains. However, cell recoveries were still far greater at later timepoints from the *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ hosts compared to the *Il-7*$^{+/-}$ *Rag1*$^{-/-}$ and *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ hosts **(Fig 5.6 D)**. Although variability within groups of mice was observed, the adjusted cell numbers **(Fig 5.6 D)** do highlight interesting differences in the survival of the naive T cell population in the presence or absence of IL-7. The decay in cell numbers is most notable in the donor cells from the *Il-7*$^{+/-}$ hosts. At d 2 and d 7 post transfer the cells persist, however at d 14 and d 21 there is a decline in the survival of the cells, exhibiting similar cell recoveries from the *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ mice, possibly suggesting that IL-7 may have become limiting **(Fig 5.6 D)**. However, given that a small dose of cells was used to seed the recipients in this experiment, it is unclear whether the small decline in F5 T cell survival observed in *Il-7*$^{+/-}$ *Rag1*$^{-/-}$ represents a significant survival defect. The donor cells transferred to *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ hosts survived, and showed no discernable decay in cell number. Surprisingly, F5 T cells transferred to the *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ hosts also persisted and were consistent between three replicate experiments. However, it is possible that the sensitivity of this assay was not sufficient for the small cell dose used to seed the various hosts.

To more accurately examine the requirement for IL-7 in naïve T cell survival we seeded *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ and *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ recipient mice with a larger cohort of donor cells so that any changes in the size of this precursor population could be followed more easily over time.

$2x10^6$ naïve CFSE labelled and $14x10^6$ unlabelled APC depleted LN F5 T cells were transferred into groups of *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ and *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ mice. Mice were sacrificed at 1, 7, 14 and 21 d post transfer and the absolute T cell number and precursor population size calculated. The T cell recoveries at d 1 post transfer suggested the initial seeding of the donor cells differed for the *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ and *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ recipient mice, therefore the T cell recoveries at each time point were normalised against the absolute number of T cells recovered at the start of the experiment, to aid the comparison of T cell survival and expansion over time (**Fig 5.7 B**). Consistent with the previous study T cells recovered from *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ recipients showed little if no proliferation (**Fig 5.7 A and B**), whereas T cells from the IL-7 sufficient hosts underwent significant cell division over the 21 d study confirming that IL-7 is crucial for the proliferative response of the naïve compartment to lymphopenia. Importantly, this supports earlier findings suggesting TCR signals alone are insufficient for the homeostatic expansion of the naïve T cell compartment.

Interestingly, the precursor population of naïve T cells from *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ and *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ recipients show different patterns of survival. F5 T cells recovered from the *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ recipients showed a detectable decay in the size of the precursor population during the first 7 d of the study to a level, which was largely maintained at the later time points (**Fig 5.7 C**). In contrast, the number of naive F5 T cells recovered from the *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ hosts declined exponentially throughout the entire duration of the timecourse exhibiting a half life of ~7 d. This data suggests that while there maybe a degree of functional redundancy between TCR and IL-7 signals supporting the survival of the naïve T cell

compartment, TCR signals cannot fully support naïve T cell survival in the absence of IL-7 signalling, inferring that IL-7 is crucial of the maintenance of the peripheral naïve T cell compartment.

5.2.5 Competition for Homeostatic TCR signals Restricts LIP

The data shown so far confirms the importance of homeostatic TCR signals in LIP, since F5 T cell proliferation was markedly reduced in the absence of MHC Class I ligands. However, we also observed a gene dose effect of IL-7 on LIP of the naïve T cell compartment, suggesting that the amount of IL-7 available also influences whether T cells divide. It is therefore unclear whether the requirement for homeostatic TCR signals to support LIP is only qualitative or whether quantitative differences in these signals can also influence the magnitude of the LIP response as is the case for IL-7 (Kieper et al., 2004). To address this we transferred F5 T cells into hosts lacking competition for IL-7, but had a varying degree of competition for MHC Class I ligands.

To exclude the role of competition for IL-7 in the regulation of LIP, we took advantage of mice that conditionally express IL-7Rα on the surface of the $CD8^+$ T cells (Buentke et al., 2006). For this study $F5^{+/-}$ Tre-IL-7R rtTA.C^{huCD2} *Il-7R*$^{-/-}$ (F5 Tet-IL-7R hereon) host mice were removed from dox treatment for a period of 3 d or 6 wk (F5 Tre-IL-7R^{OFF}) prior to the beginning of the study to ablate IL-7Rα expression. In the absence of IL-7R expression there is no competition for IL-7, regardless of the size of the host T cell compartment. Since F5 T cells die in the absence of continued IL-7R expression (Tan et al., 2001) using F5 Tre-IL-7R^{OFF} mice off dox for varied times created a variety of host conditions with

respect to the size of the host (IL-7R deficient) T cell population. This also meant that while the transferred F5 T cells had no competition for IL-7 from the host *Il-7R*$^{-/-}$ T cells, both populations were still however competing for available MHC Class I ligands.

CFSE labelled F5 CD8^{+} T cells were adoptively transferred into WT F5$^{+/+}$ *Rag1*$^{-/-}$ control replete hosts and F5 Tet-IL-7R^{OFF} hosts exhibiting varying degrees of lymphopenia, as a result of ablating IL-7R expression for 3 days or 6 wks. The extent of lymphopenia in these different hosts was directly assessed by measuring the frequency of host (CD45.2) F5 T cells in the periphery of these mice **(Fig 5.8)**. The extent of cell division within the F5 donor (CD45.1) T cell population recovered from the LN and spleen was determined by FACS analysis. The CFSE profile of the donor F5 T cells recovered from the various recipients 10 d post transfer is shown in **Fig 5.8**. Very few donor F5 T cells were recovered from the F5 replete hosts, suggesting the donor T cells were outcompeted by the host cells for the limited MHC Class I ligands and IL-7. In contrast the donor F5 T cells showed significant T cell proliferation in all the F5 Tet-IL-7R^{OFF} hosts that lacked IL-7R expression. The extent of the donor F5 T cell LIP depended on the size of the host F5 T cell population and thus competition for the available spMHC ligands. F5 donor T cells proliferated more in Tet-IL-7R^{OFF} recipients that had the most reduced host population of T cells suggesting that in the absence of competition for IL-7 signalling LIP of naïve T cells requires enhanced homeostatic TCR signalling that is limited by competition for spMHC ligands.

5.2.6 IL-15 is Required for the LIP of the Naïve CD8$^+$ T cell Compartment

Several groups have shown that IL-7 and IL-15 are essential for memory T cell homeostasis. While IL-7 has been shown to play a significant role in naïve T cell survival and homeostatic cell division, the analysis of naïve T cell homeostasis in irradiated *Il-15*$^{-/-}$ hosts (Goldrath et al., 2002) have not been able to reveal any role for IL-15 signalling in naïve CD8$^+$ T cell LIP. To investigate this is more detail, we examined the influence of IL-15 on LIP in our F5 TCR transgenic system.

F5 Tet-IL-7R CD8$^+$ T cells were removed from dox treatment for a period of 3 days prior to the beginning of the experiment to cease IL-7R expression. CD8$^+$ T cells from F5 Tet-IL-7R^{OFF} and (F5 *Rag1*$^{-/-}$ x CD45.1) F1 control mice were CFSE labelled and co-transferred by intravenous injection into *Il-15ra*$^{+/+}$ *Rag1*$^{-/-}$ and *Il-15ra*$^{-/-}$ *Rag1*$^{-/-}$ lymphopenic hosts. Although not IL-15 deficient, *Il-15ra*$^{-/-}$ *Rag1*$^{-/-}$ hosts cannot transpresent IL-15 without IL-15Rα expression, and so are functionally deficient for IL-15 (Sandau et al., 2004) The extent of cell division as determined from the CFSE profile of the cells recovered from the LN and spleen was performed by FACS analysis 7 d post transfer.

As expected control F5 T cells from the *Il-15ra*$^{+/+}$ *Rag1*$^{-/-}$ hosts showed extensive homeostatic proliferation **(Fig 5.9 A and B)**. In contrast control F5 T cells recovered from IL-15Rα deficient hosts did show evidence of LIP although it was much reduced compared to the *Il-15ra*$^{+/+}$ control. Interestingly this low level of LIP was absent from the F5 Tet-IL-7R^{OFF} T cells transferred to the Il-

15Rα deficient hosts, suggesting that IL-7 signalling is responsible for this cell division. Although the reduction in LIP was greatest in the absence of IL-7R expression, this data suggests IL-7 is more important than IL-15 for LIP but that IL-15 signalling does contribute.

5.2.7 Strength of Homeostatic TCR Signalling in LIP is Reduced in IL-7 Deficient Hosts

In line with the current literature, the experiments described in this chapter identify roles for TCR and IL-7 signals in naïve $CD8^+$ T cell survival and LIP. However, how these signals influence one another is unknown. To dissect the relationship between the two signalling pathways we assessed whether cytokine signalling can effect the reception of homeostatic TCR signals.

CD5 expression levels on T cells vary depending on the strength of TCR ligation with self ligands in the thymus (Azzam et al., 1998) and the peripheral lymphoid compartment (Smith et al., 2001a). We therefore examined homeostatic TCR signalling in the context of cytokine signalling (presence or absence of IL-7) by examining CD5 expression on the donor naïve F5 $CD8^+$ T cells in the presence or absence of IL-7 expression.

APC depleted naïve F5 $CD8^+$ T cells were CFSE labelled and adoptively transferred by tail vein injection into lymphopenic *Il-7*$^{+/+}$ *Rag1*$^{-/-}$, *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ *and* *β2m*$^{-/-}$ *Rag1*$^{-/-}$ hosts. The expression of CD5 was analysed on the CD8 TCR^+ or $CD5^+$ T cells recovered from the spleen of the different hosts by FACS analysis at different time points.

CD5 expression was highest on the F5 T cells transferred to *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ hosts at d 11 post transfer. In the absence of MHC contact F5 T cells from *β2m*$^{-/-}$ *Rag1*$^{-/-}$ hosts showed the lowest expression of CD5, indicative of reduced homeostatic TCR signalling (**Fig 5.10 A**). Unexpectedly, the level of CD5 expression detected on F5 T cells recovered from *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ hosts was consistently lower than observed in F5 T cells from *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ controls, eventhough these mice were lymphopenic and had no competition for MHC Class I ligands. This suggests that homeostatic TCR signalling is decreased in the absence of IL-7.

We also examined TCR expression levels on the F5 T cells transferred to the lymphopenic hosts since TCR down modulation is indicative of TCR engagement. As expected TCR expression was consistently increased on F5 T cells recovered from the MHC Class I deficient *β2m*$^{-/-}$ *Rag1*$^{-/-}$ recipients, providing further evidence of reduced TCR engagement in these hosts. Surprisingly, F5 T cells from *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ hosts and *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ did not show the same level of TCR expression. While low levels of TCR expression were detected on the F5 T cells from the *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ hosts, TCR downmodulation was not as great in F5 T cells transferred to *Il-7*$^{-/-}$ hosts suggesting TCR engagement is reduced in the absence of cytokine signalling.

To gain a better understanding of the role of IL-7 in the regulation of homeostatic TCR signalling, we analysed the kinetics of TCR **(Fig 5.10 B)** and CD5 expression **(Fig 5.10 C)** on the F5 T cells transferred to *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ and *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ recipients. The MFI of TCR and CD5 expression on F5 T cells from

the spleen of recipient mice was normalised against the MFI of *ex vivo* naïve F5 control splenic T cells at each timepoint. TCR levels steadily declined on F5 T cells transferred to *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ hosts. In contrast TCR downmodulation was less apparent in the F5 T cells transferred to the *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ recipients. CD5 expression appeared similar on the F5 T cells from *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ and *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ at d1 of the experiment, however at d 3 and d 7 CD5 expression by F5 T cells was upregulated in *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ hosts, indicating these cells were receiving strong homeostatic TCR signals in the lymphopenic recipients. In contrast F5 T cells recovered from the IL-7 deficient hosts failed to show a similar level of CD5 upregulation at the early timepoints. Interestingly, at later timepoints CD5 levels did reach levels of expression comparable to the same F5 T cells in *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ hosts, suggesting that homeostatic TCR signalling was increased in *Il-7*$^{-/-}$ hosts but that this increase was greatly delayed in the absence of IL-7 expression. This data suggests that IL-7 signals can influence homeostatic TCR signalling, such that in the absence of IL-7 the strength of homeostatic TCR signals is reduced.

5.2.8 Interaction of T cells with MHC Expressing APCs is Reduced in IL-7 Deficient Hosts

Given that the strength of homeostatic TCR signalling appeared to be effected by IL-7 signalling, we wished to determine the manner in which IL-7 could modulate TCR signalling. We proposed that IL-7 might affect the delivery of homeostatic TCR signals via T cell-APC interaction, such that in the absence of IL-7, contact of the T cells with APCs delivering homeostatic TCR signals

maybe impaired. To address this in more detail we assessed the influence of IL-7 on T cell-APC interactions. We examined this by measuring the passive acquisition of MHC Class II ligands on the surface of the F5 T cells themselves to quantitate the contact between the T cell and the MHC Class II expressing APC. Mouse T cells do not express MHC Class II molecules (Seddon and Mason, 1996) by de novo synthesis, but can acquire it following interaction with dcs. This is illustrated in **Fig 5.11 A** which shows MHC Class II acquistion by naïve CD8 T cells in WT polyclonal and MHC Class II deficient mice. MHC Class II could not be detected on the naïve $CD8^+$ T cells from the MHC Class II deficient mice confirming the specificity of FACS staining.

APC depleted naïve F5 $CD8^+$ T cells were CFSE labelled and adoptively transferred by tail vein injection into lymphopenic *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ *and Il-7*$^{-/-}$ *Rag1*$^{-/-}$ hosts. The acquisition of MHC Class II ($I\text{-}A^b$) complexes on the surface of the $CD8^+$ T cells from the spleen of recipients was assessed by FACS at 1, 3, 7, 10 and 14 d post transfer. The MFI of MHC Class II expression on the donor F5 T cells from the various hosts was normalised against control Class II staining on *ex vivo* splenic F5 T cells from replete F5 controls.

At early timepoints F5 T cells in the *Il-7*$^{+/+}$ hosts but not *Il-7*$^{-/-}$ hosts had a greater acquisition of Class II on their cell surface compared to F5 T cells from replete controls. The increase in MHC Class II acquisition on F5 T cells transferred to *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ hosts was observed as early as d 1 post transfer **(Fig 5.11 B)**. Consistent with the pattern of CD5 expression observed on F5 T cells transferred to IL-7 deficient hosts, MHC Class II acquisition did at later

timepoints increase to comparable levels as the same cells in *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ control hosts. This data suggests that the failure of T cells to receive homeostatic TCR signals from the APCs in *Il-7*$^{-/-}$ hosts, is due to a failure of T cells to make enhanced contacts with the APCs despite an absence of host T cell competition. This suggests that in the absence of IL-7 signalling, F5 T cells are delayed in sensing and responding to lymphopenia within the naïve T cell pool.

5.3 Discussion

Previous studies have shown that TCR and IL-7 cytokine signals are essential for the survival and induction of LIP in naïve T cells under conditions of T cell deficiency (Ernst et al., 1999; Goldrath and Bevan, 1999; Goldrath et al., 2002; Kieper and Jameson, 1999; Schluns et al., 2000; Tan et al., 2001; Viret et al., 1999). However, these studies have failed to address how TCR and IL-7 signalling pathways in naive T cell homeostasis influence one another and whether the requirement for IL-7 and TCR signalling differ for naïve T cell survival versus LIP. In the present study, we found that IL-7 but not homeostatic TCR signals are essential for naïve T cell survival in conditions of T cell lymphopenia. Importantly our data also suggests that the induction of LIP responses requires enhanced TCR and IL-7 signalling and surprisingly that homeostatic TCR signals can only function in the context of enhanced IL-7 cytokine signalling, implying that IL-7 signalling may directly modulate the reception of homeostatic TCR signals in LIP.

To assess whether there existed cooperative interactions between homeostatic TCR signals and IL-7 signals in naïve T cell survival, we assessed the manner in which these signals could support the survival of F5 T cells in T cell deficient hosts. Interestingly, homeostatic TCR signals appeared not to be essential for short-term naïve T cell survival, since F5 T cells transferred to *β2m*$^{-/-}$ *Rag1*$^{-/-}$ hosts appeared to persist despite an absence of typical MHC Class I ligands **(Fig 5.4 C)**. Our results differ from those reported in a recent study examining the survival and LIP of different Class I restricted TCR transgenic T cells under lymphopenic conditions and in the absence of appropriate MHC Class I expression (Hao et al., 2006). This study reported that HY and P14 CD8^{+} T cells expressing H-2D^{b} restricted TCRs could not survive in the absence of H-2D^{b} MHC complexes. Furthermore, the cells completely disappeared 24 h post transfer suggesting an essential role for homeostatic TCR signals in peripheral P14 and HY T cell maintenance. A second study performed by (Murali-Krishna and Ahmed, 2000a) also reported that P14 TCR transgenic T cells required homeostatic TCR signals for their survival, although, the decline in naïve T cell survival was not observed until between 8 and 20 d post transfer into H-2D^{b} x H-2K^{d} x *β2m*$^{-/-}$ MHC Class I deficient hosts, with the greatest decline in P14 T cell survival occurring between 20 and 50 d post transfer. One explanation for the differences between our study and the findings reported by (Hao et al., 2006) and (Murali-Krishna and Ahmed, 2000a) could be that CD8^{+} T cells expressed different transgenic TCRs and therefore it is possible that these different CD8^{+} clones possess intrinsic survival differences. Indeed it has been shown that different CD8^{+} TCR transgenic clones exhibit different LIP

responses depending on the avidity of the TCR for self peptide ligands (Kieper et al., 2004), hence it is possible that the differing sensitivity of these $CD8^+$ T cell clones towards self ligands may also influence their survival responses. The disparity between the requirement of MHC Class I expression in naïve $CD8^+$ T cell survival between the two studies and our study may also reflect intrinsic differences in the experimental systems used. In contrast to our study, the MHC Class I deficient hosts used to assess the survival of the different TCR transgenic populations in the Hao *et al* and the Murali-Krishna *et al* study, were sublethally irradiated. Irradiation has been shown to induce the expression of cytokines and growth factors changing the cytokine milieu of the host and thus creating an altered environment to that of normal un-irradiated mice (Hong et al., 1999; Limanni et al., 1995; Peterson et al., 1994). This is consistent with other studies have reported augmented LIP responses in irradiated hosts compared with un-irradiated hosts (Schluns et al., 2000). It is also important to note that our study only addressed the survival of the F5 T cells in the absence of MHC Class I ligands over a relatively a short timecourse of 21 d. In contrast the Murali-Krishna *et al* study assessed naïve T cell survival over a longer 50 d time period, it is therefore possible that homeostatic TCR signals are not required for the short-term survival but do play a role in long-term naïve $CD8^+$ T cell maintenance. Future studies will involve assessing the long-term survival of the F5 T cells over an extended timecourse.

While the $\beta2m^{-/-}$ $Rag1^{-/-}$ hosts used in our study are functionally MHC Class I deficient, in the absence of the β2m subunit, they have been reported to

express low levels of misfolded H-2D^b Class I MHC complexes at the cell surface (Allen et al., 1986; Zijlstra et al., 1990). This low level of MHC expression is unable to support the selection of endogenous T cells during thymic selection (Reynolds et al., 2004; Smyth et al., 1998) and as expected F5 T cells transferred to these hosts show evidence of reduced homeostatic TCR signalling such that CD5 levels are significantly lower than MHC Class I sufficient hosts **(Fig 5.10 A)**. Although the homeostatic TCR signals in these β2m$^{-/-}$ Rag1$^{-/-}$ hosts are unable to support the LIP of the F5 naive T cells **(Fig 5.3 A and B and Fig 5.4 A and B)**, we cannot exclude that this low level of H-2D^b MHC Class I expression may be sufficient in providing a basal survival signal, that has the capacity to support the survival of the F5 T cell population in the absence of normal MHC ligands **(Fig 5.4 C)**.

Surprisingly, transfer of a large F5 T cell number into the *β2m$^{-/-}$ Rag1$^{-/-}$* mice also failed to reveal a survival defect. Under these conditions, we would anticipate that the transfer of a large cell dose would increase the competition for limited survival factors. The persistence of a large cohort of F5 T cells in the *β2m$^{-/-}$ Rag1$^{-/-}$* mice **(Fig 5.4 C)** may be explained by the inadvertent co-transfer of MHC Class I expressing APCs into these recipients. However, this seems unlikely given that the F5 T cells appeared to persist at later timepoints, at which time the terminally differentiated APCs would have died (Garcia et al., 1999). It is also possible that the MHC Class I proteins present on the surface of the F5 CD8$^+$ T cells themselves may provide a survival signal in the absence of professional APC derived MHC ligands. We investigated this possibility

further by generating naïve MHC Class I deficient F5 $CD8^+$ T cells in a BM chimera model, and followed their survival in a periphery devoid of T cell MHC Class I ligands. Preliminary experiments failed to show any evidence that T cell derived MHC Class I ligands could support naive T cell survival in the absence of APC derived ligands, however further replicates of this study are required to fully determine their role if any in naïve $CD8^+$ T cell survival.

Although our model does address the requirement for TCR signals in naïve T cell survival it does so in a lymphopenic environment in which other survival signals are available in excess. The pro-survival cytokines IL-7 and IL-15 may accumulate in the periphery of the *Rag1*$^{-/-}$ recipients since these hosts lack a preformed resident T cell population that would consume these cytokines. This view is consistent with the reports that T cell deficiency in humans is associated with an increase in circulating IL-7 levels (Fry et al., 2001; Napolitano et al., 2001). It is therefore possible that these cytokines maintain the survival of the F5 donor T cell population in the absence of homeostatic TCR signals, masking a more subtle requirement for homeostatic TCR signalling in naïve $CD8^+$ T cell survival. To address this question more accurately, the survival of a cohort of $CD8^+$ T cells could be assessed in replete hosts that specifically lack MHC Class I ligands, to determine whether increased competition and an absence of homeostatic TCR signalling affects the survival of the donor T cell population. Critically what this data does suggest is that in lymphopenia there does not appear to be an acute

requirement for homeostatic TCR signalling in short-term naïve $CD8^+$ T cell survival.

Consistent with the literature (Tan et al., 2001; Vivien et al., 2001) we found that even in conditions of T cell lymphopenia, IL-7 signalling was essential for the survival of a large T cell cohort, since F5 donor T cell precursor population decayed significantly following transfer into IL-7 deficient hosts **(Fig 5.7 C)**. Interestingly, however the survival of a small T cell cohort, did not appear to require IL-7 signalling **(Fig 5.6 C)**. Furthermore, the transferred F5 T cells were consistently detected in the IL-7 deficient mice, even with as little a $1x10^6$ cells used as the seeding inoculum. This data suggests that in the absence of IL-7, other survival signals perhaps including homeostatic TCR signals, IL-15 cytokine signals (Berard et al., 2003), T cell-dendritic cell contact (Feuillet et al., 2005) or other as of yet unknown survival signals can maintain the survival of a small T cell population. Although we cannot identify which of these non-IL-7 signals is more crucial. However, our data shows that these non-IL-7 survival signals are easily saturated in the absence of IL-7, given that the survival of a larger T cell cohort in the absence of IL-7 signalling was greatly impaired **(Fig 5.7)**. This suggests that enhanced competition for these survival resources within a larger T cell population limits the extent to which these non-IL-7 signals can compensate for IL-7 in survival. The use of lymphopenic recipients thus reveals the activity of those non-IL-7 survival signals that are not easily revealed in replete hosts.

The disparity between the requirement of homeostatic TCR survival signals in lymphopenic versus replete conditions may be a consequence of the nature in which IL-7 is used by T cells. In replete hosts it has been suggested that resting T cells utilise IL-7 in an altruistic fashion, such that once a T cell has received an IL-7 signal, IL-7Rα expression is downregulated (Park et al., 2004). It has been reported that this occurs through the suppression of IL-7Rα transcription by IL-7 itself as well as IL-6 and the γc cytokines IL-2, IL-4 and IL15 (Park et al., 2004; Xue et al., 2002). IL-15 cytokine signals also influence naïve (Berard et al., 2003; Kennedy et al., 2000a) and memory T cell survival (Kennedy et al., 2000a) therefore it is interesting that it also plays a role in the regulation of IL-7 signalling by suppressing IL-7Rα expression. (Mazzucchelli and Durum, 2007) suggest that this receptor regulation may help the T cells integrate survival signals effectively such that if a T cell is provided with a survival signal other than IL-7, it downregulates IL-7Rα expression as a means to limit IL-7 consumption, although as of yet, this has not been formally shown.

Interestingly, (Franchimont et al., 2002) have also reported that TCR signals may also influence the expression of IL-7Rα, since *in vitro* activation of peripheral human $CD4^+$ T cells resulted in the downregulation of IL-7Rα expression. Similarly, (Trigueros et al., 2003) have also reported that pre-TCR signals can regulate IL-7Rα. This is however in contrast to a study performed by (Seddon et al., 2003) in which the ablation of the TCR proximal Src Kinases Lck and Fyn essential for the transmission of homeostatic TCR signals, did not impair IL-7Rα expression. Similarly our study showed that in lymphopenia, IL-7

signalling can maintain T cell survival sufficiently in the absence of homeostatic TCR signals. In addition Bcl-2 levels were comparable in the F5 T cells transferred to the MHC Class I deficient and sufficient hosts, whereas Bcl-2 levels were reduced in F5 T cells transferred to *Il-7*$^{-/-}$ hosts. This suggests that TCR signals are not required for the activity of IL-7 signalling, and infer these pathways function independently of one another in the context of naïve T cell survival. The discrepancy between these studies is difficult to reconcile, however it possible that TCR signals may be coupled to IL-7 signalling by an as of yet unidentified pathway independently of the Lck and Fyn pathway.

Interestingly, while homeostatic TCR signals were dispensible for naïve T cell survival in the presence of excess IL-7, the same was not true for the induction of LIP within the naïve F5 T cell compartment. Both IL-7 **(Fig 5.6 and Fig 5.7)** and homeostatic TCR signalling **(Fig 5.3 and 5.4)** were essential for the efficient induction of LIP, since the absence of one or the other of these signals were sufficient to almost completely block LIP. Importantly, we also found that LIP required an increase in signalling from both receptors, suggesting both signals function quantitatively rather than simply at a qualitative level to regulate LIP. The finding that homeostatic TCR signalling could support the survival of a small naïve T cell population in the absence of IL-7 expression **(Fig 5.6 C and D)** but not the LIP **(Fig 5.6 A and B)**, suggests that enhanced homeostatic TCR signalling above the threshold required for survival, is necessary for the induction and/or maintenance of LIP responses. This view is consistent with the literature which suggests that naïve T cells need to receive

a certain amount of homeostatic signalling to undergo LIP (Kassiotis et al., 2003; Kieper et al., 2004). We found that LIP was reduced in hosts in which IL-7 levels were fixed, but where competition for MHC Class I ligands was increased **(Fig 5.8)**, suggesting that LIP requires enhanced homeostatic TCR signalling that can be restricted by competition for spMHC ligands. While we like many other studies, found an absolute requirement for IL-7 in LIP, we in agreement with (Kieper et al., 2004) identified a quanitative role for IL-7, since the magnitude of the F5 LIP response was sensitive to gene dose effects in *Il-7*$^{+/-}$ versus *Il-7*$^{+/+}$ *hosts* **(Fig 5.5 6)**. In agreement with a recent study by (Sandau et al., 2007) we also found that IL-15 signals contribute to LIP, albeit at a lesser extent than IL-7 signals. It would be interesting to examine how IL-7 and IL-15 signals cooperate to induce LIP. Given that IL-7 has been shown to exert its proliferative effects through the regulation of proteins involved in cell cycle progression (Khaled et al., 2005; Li et al., 2006), it would be interesting to determine whether IL-15 activates similar intracellular signalling pathways to induce homeostatic cell division and whether there exists a functional synergy between these two signals.

While there is much evidence to suggest that IL-7 signals and homeostatic TCR signals both play crucial roles in LIP, it is not clear how if at all these signals cooperate to induce homeostatic cell division versus cell survival under conditions of T cell deficiency. Recent studies have shown that mice over expressing IL-7, exhibit increased numbers of $CD4^+$ and $CD8^+$ $CD44^{lo}$ T cells as well as a significantly elevated proportion of $CD44^{hi}$ T cells compared to non-IL-

7 transgenic controls (Kieper et al., 2002; Mertsching et al., 1995; Samaridis et al., 1991). Further analysis of the LIP of different TCR transgenic $CD8^+$ T cells expressing high (OT-1 TCR transgenic) or low affinity TCRs (HY TCR transgenic) and expressing an IL-7 transgene revealed that only $CD8^+$ T cell clones that exhibited high affinity TCR spMHC interactions could utilise the excess IL-7 to homeostatically divide (Kieper et al., 2002). Given that interaction of the TCR with self ligands is essential for LIP, one explanation for this observation proposed by (Sprent and Surh, 2003), is that IL-7 may directly amplify or potentiate homeostatic TCR signals, such that this signalling overcomes the threshold required for the induction of LIP. Sprent *et al also* suggest that the full costimulatory effects of IL-7 are only apparent if TCR affinity for self is above a critical threshold (Ge et al., 2004; Kieper et al., 2004; Sprent and Surh, 2003). However, the manner in which IL-7 can modulate homeostatic TCR interactions is unclear although there is evidence that IL-7 can directly affect cell cycle progression via its inhibitory effects on the cyclin-dependent kinase $p27^{Kip1}$ (Li et al., 2006). A possible point where TCR and IL-7 signals converge has been suggested by a recent study by (Riou et al., 2007). Interestingly this study reported that the convergence of IL-7 and TCR signals was essential for the survival of human $CD4^+$ central memory T cells by inducing the phosphorylation of the fork head box O3a (FOXO3a) transcription factor. FOXO3a regulates the transcription of the proapoptotic genes including Bim (Essafi et al., 2005), and its phosphorylation leads to its transcriptional inactivation. Importantly this study showed that neither IL-7 signals nor TCR signalling alone was sufficient for FOXO3a phosphorylation, inferring a degree

of synergy between these signalling pathways in memory T cell survival. It would be interesting to determine whether FOXO3a plays a similar role in naïve T cell survival and whether TCR and IL-7 signals could also converge to regulate FOXO3a in naïve T cells.

In our study we have found another potential mechanism by which IL-7 and homeostatic TCR signals can converge to regulate T cell homeostasis. Surprisingly, we found evidence to suggest that IL-7 signals can directly modulate the reception of homeostatic TCR signalling to influence naïve T cell homeostasis. The strength of homeostatic TCR signalling was substantially reduced in F5 T cells transferred to lymphopenic hosts lacking IL-7 expression **(Fig 5.10)**. In the absence of IL-7 signalling, the enhancement of homeostatic TCR signals was greatly delayed, since F5 donor T cells in *Il-7*$^{-/-}$ hosts failed to both upregulate CD5 levels and downmodulate TCR levels as observed in IL-7 sufficient hosts **(Fig 5.10)**. The reduced capacity of the F5 T cells to receive homeostatic TCR signals in IL-7 deficient hosts, appeared to be a consequence of reduced T cell contact with APCs that deliver the spMHC ligands essential for homeostatic TCR signalling, since the acquisition of APC derived MHC Class II complexes on the surface of F5 T cells transferred to *Il-7*$^{-/-}$ hosts, was reduced compared to *Il-7*$^{+/+}$ controls and *ex vivo* F5 control T cells **(Fig 5.11)**. This suggests that IL-7 is in some way regulating the ability of T cells to interact with APCs that is likely to be essential for enhanced homeostatic TCR signalling. However, it is important to note that the *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ recipients used in this study fail to develop LN organs given that IL-7/IL-7R interactions are required for normal lymphogenesis (Coles et al., 2006), therefore it cannot be

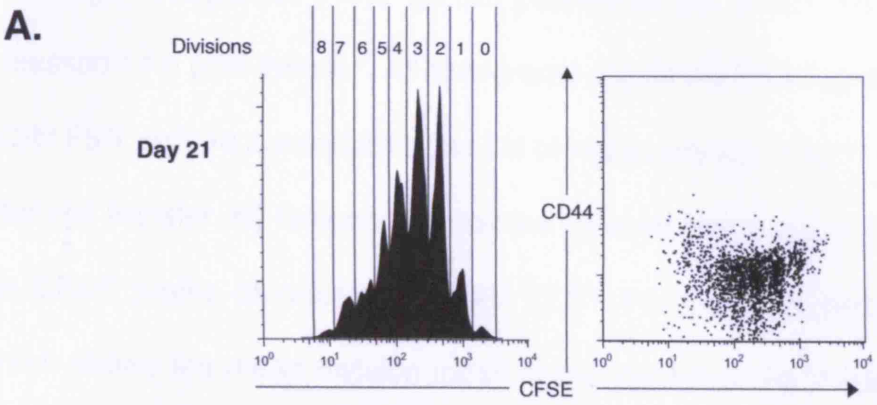
ruled out that the decreased reception of homeostatic TCR signals in these hosts may occur as a result of stromal defects or differences in how the cells traffic within the remaining lymphoid organs in these mice. To address this further, we are currently repeating these experiments by transferring F5 Tet-IL-7R^{OFF} T cells into Rag1$^{-/-}$ hosts to confirm whether IL-7 can regulate the magnitude of homeostatic TCR signalling in lymphopenia in a more physiological model.

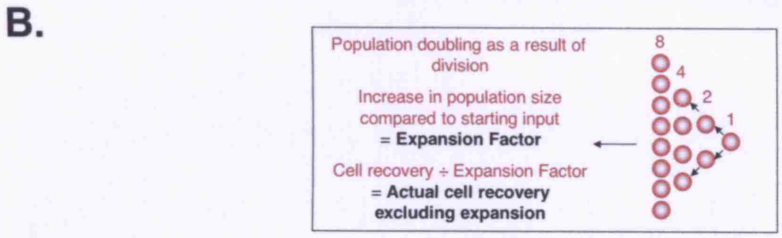
It has been known for sometime that cytokines and growth factors can regulate cell glucose uptake and metabolism in dependent cells. Early studies using IL-3 dependent cell lines revealed that the cytokine IL-3 could regulate cellular metabolism through its effects on glucose import by upregulating the expression of the GLUT1 glucose transporter (Rathmell et al., 2000). Recently a similar role has been suggested for IL-7 in light of its ability to maintain glucose metabolism in primary T cells cultured *in vitro* (Rathmell et al., 2001) and regulate GLUT1 expression and mitochondrial homeostasis in a human lymphoblastic leukaemic cell line in a PI3K dependent manner (Barata et al., 2004). In light of the literature, we propose that IL-7 signalling may influence T cell:APC interactions in lymphopenia as a consequence of its metabolic effects. We suggest that enhanced IL-7 signalling increases the mobility of the T cells within the LN organs, thus facilitating the enhanced T cell:APC interactions required for the induction of LIP. Employing stereo-microscopy and time-lapse imaging, we have examined the behaviour of fluorescently labelled (CFSE) T cells *in vivo*, in LN sections (data not shown). Preliminary data suggests that

the movement of F5 IL-7R^{OFF} T cells lacking IL-7R expression is indeed impaired compared with that of control F5 T cells in the same hosts. Remarkably, these data suggest that IL-7 may act as a sensor for lymphopenia through its influence on T cell metabolism and thereby indirectly influence homeostatic TCR signalling, by affecting a T cells ability to make sufficient APC contacts. Limitations of this microscopy assay have prevented us from fully assessing the conditions in which IL-7 may influence T cell movement, through it effects on glucose metabolism. To investigate this further we are currently employing Two-photon microscopy to more accurately follow the trafficking of the naïve T cells within intact LN organs, in varied host conditions ranging from replete hosts and lymphopenic *Rag1*$^{-/-}$ mice of varying IL-7, IL-15 and MHC class I genotypes.

Figure 5.1: Homeostatic proliferation of F5 TCR transgenic CD8$^+$ T cells in lymphopenic hosts.

CFSE labelled F5 *Rag1*$^{-/-}$ T cells (1.25 x10^6 donor cells per recipient) were transferred intravenously into *Rag1*$^{-/-}$ recipients (n = 3 per timepoint). The survival, proliferation and expansion of the CFSE labelled CD8$^+$ TCR^{hi} population was measured over time. **A)** histogram shows the CFSE profile of the CD8$^+$ TCR^{hi} F5 T cells recovered from the LN of a representative *Rag1*$^{-/-}$ recipient at d 21 of the timecouse. FACS plot shows the surface expression of CD44 on the dividing cells. **B)** shows a schematic of how the expansion factor (EF) calculated from the pattern of cell division is used to measure the size of the precursor T cell population. **C)** graph shows the absolute cell number of CD8$^+$ TCR^{hi} F5 T cells ± SD and adjusted precursor cell numbers (excluding the effects of proliferation) ± SD recovered from the LN and spleen of the mice over a 21 d timecourse. **D)** shows the mean division index of the cells ± SD. **E)** shows the EF predicted by the CFSE profile of the cells ± SD. Absolute and precursor cell numbers were calculated as described in ***Chapter 2 Materials and Methods***. Data is representative of five independent experiments.


A.
Divisions
8 7 6 5 4 3 2 1 0
Day 21
CD44
CFSE


B.
Population doubling as a result of division
Increase in population size compared to starting input
= Expansion Factor
Cell recovery ÷ Expansion Factor
= Actual cell recovery excluding expansion
8
4
2
1

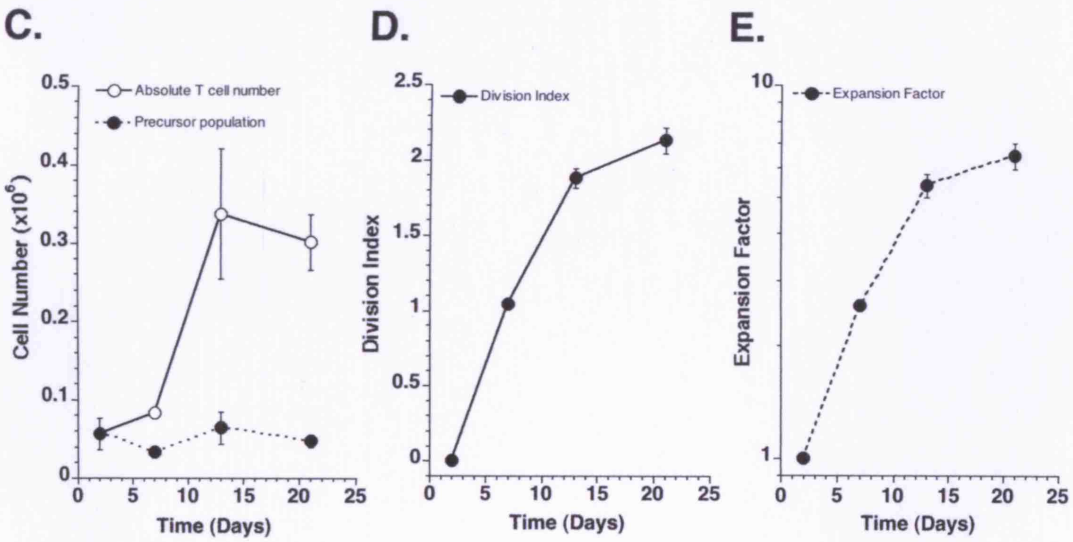
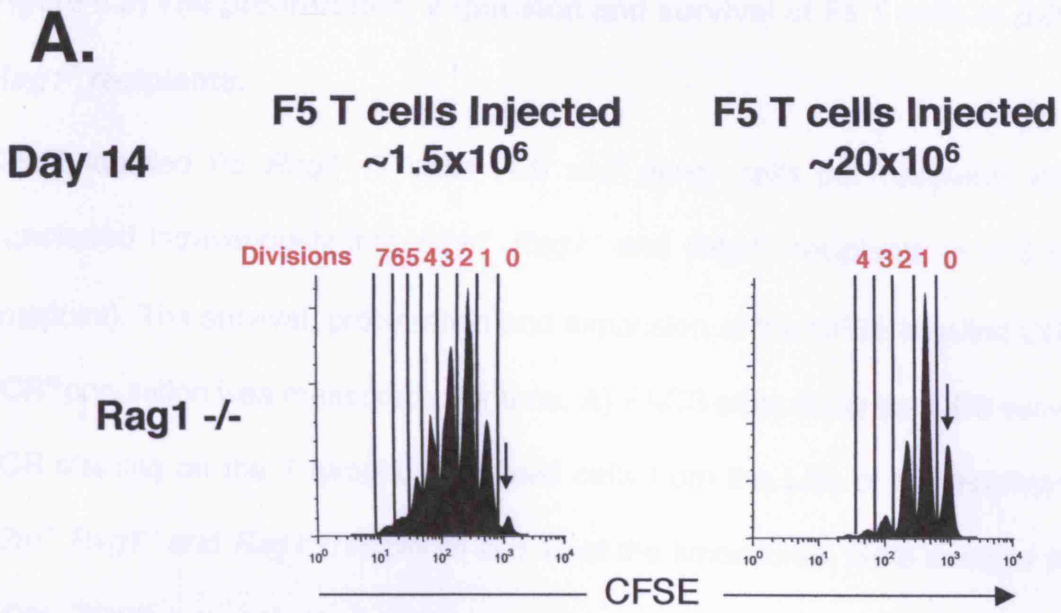

C.
Absolute T cell number
Precursor population
Cell Number (x10^6)
Time (Days)
D.
Division Index
Time (Days)
E.
Expansion Factor
Time (Days)

Figure 5.2: The extent of LIP in *Rag1*$^{-/-}$ is dependent on cell dose.

$1.5x10^6$ or $20x10^6$ CFSE labelled naïve F5 T cells were injected intravenously into *Rag1*$^{-/-}$ recipients. The CFSE profiles of the donor F5 T cells were assessed 14 d post transfer. **A)** histograms shows the CFSE profile of the $CD8^+$ TCR^{hi} F5 T cells recovered from the LN of representative *Rag1*$^{-/-}$ recipients 14 d after cell transfer. **B)** Groups of mice (n= 3) were taken at d 7, 14 and 21 and the CFSE profile of recovered $CD8^+$ TCR^{hi} F5 T cells assessed by FACS. Graph shows the mean division index of the recovered F5 T cells ± SD at the different time points. Data is representative of 5 independent experiments.


A.
Day 14
F5 T cells Injected ~1.5x10^6
F5 T cells Injected ~20x10^6
Divisions 7 6 5 4 3 2 1 0
4 3 2 1 0
Rag1 -/-
CFSE

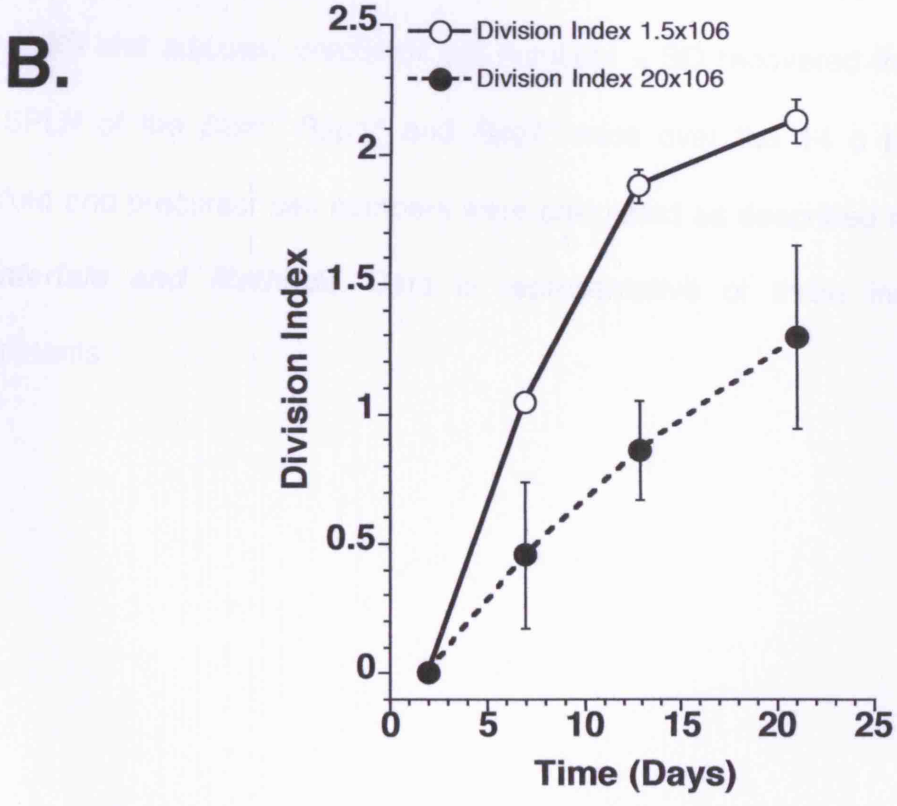
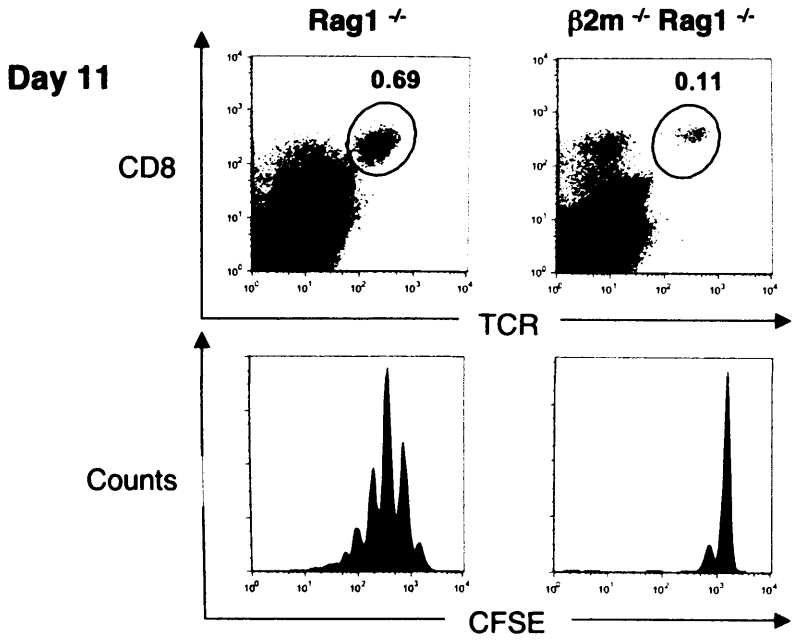

B.
Division Index 1.5x106
Division Index 20x106
Division Index
Time (Days)
0
0.5
1
1.5
2
2.5
0
5
10
15
20
25

Figure 5.3: The proliferation, expansion and survival of F5 T cells in *β2m*$^{-/-}$ *Rag1*$^{-/-}$ recipients.

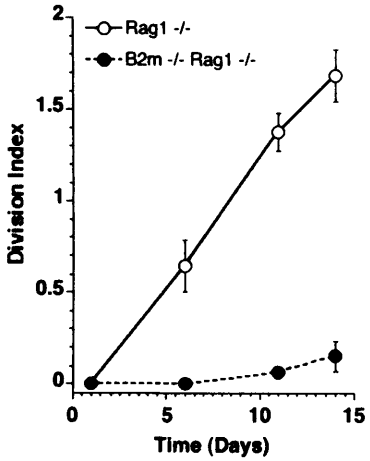
CFSE labelled F5 *Rag1*$^{-/-}$ T cells (1.5 x10^6 donor cells per recipient) were transferred intravenously into *β2m*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients (n = 3 per timepoint). The survival, proliferation and expansion of the CFSE labelled CD8$^+$ TCR^{hi} population was measured over time. **A)** FACS plots show the CD8 versus TCR staining on the T lymphocyte gated cells from the LNs of representative *β2m*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients at d 11 of the timecourse. Gate denotes the CD8$^+$ TCR^{hi} population of F5 donor T cells and numbers represent the percentage of cells within the CD8$^+$ TCR^{hi} population. Histograms show the CFSE profile of the CD8$^+$ TCR^{hi} gated F5 T cells. **B)** shows the mean division index of F5 T cells ± SD in groups of *β2m*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ hosts at d 1, 7, 11, 14 post transfer. **C)** shows the absolute cell number of CD8$^+$ TCR^{hi} F5 T cells ± SD and adjusted precursor cell numbers ± SD recovered from the LN and SPLN of the *β2m*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ mice over the 14 d timecourse. Absolute and precursor cell numbers were calculated as described in ***Chapter 2 Materials and Methods***. Data is representative of three independent experiments.

A.



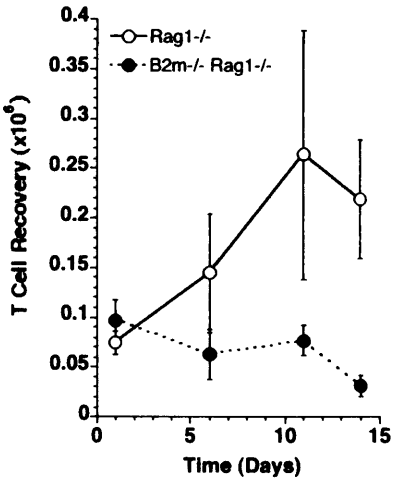
B.

Division Index



C.

Absolute Cell Number



Precursor Population

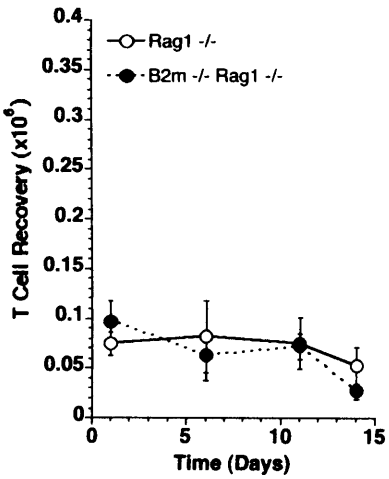
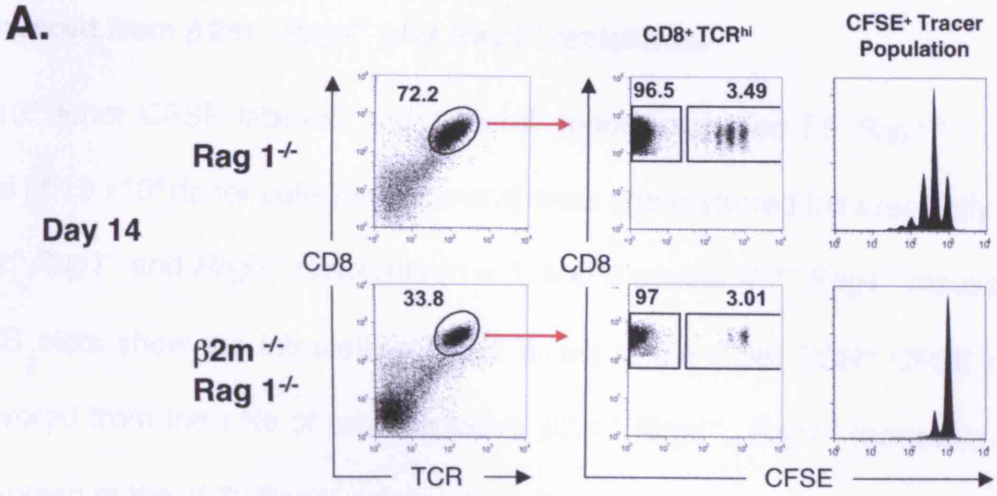
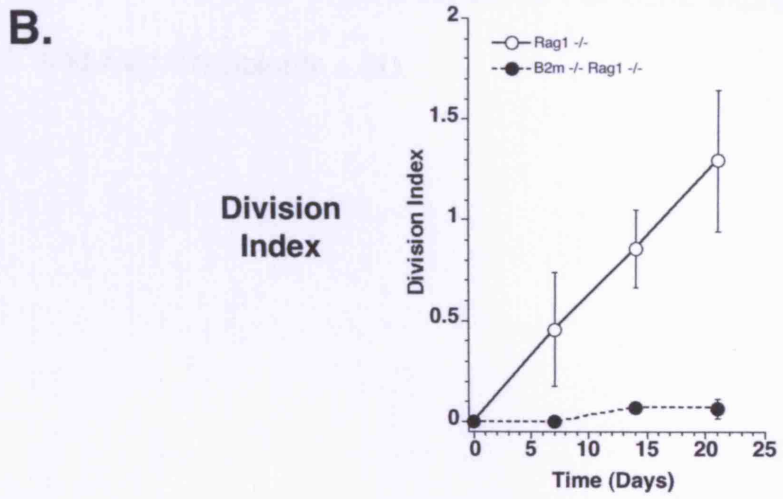


Figure 5.4: The proliferation and expansion but not the survival of a large cohort of F5 donor T cells is impaired in *β2m*$^{-/-}$ *Rag1*$^{-/-}$ recipients.

2 x10^6 donor CFSE labelled and 17 x10^6 donor unlabelled F5 *Rag1*$^{-/-}$ T cells (total of 19 x10^6 donor cells per recipient) were cotransferred intravenously into *β2m*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients. The survival, proliferation and expansion of the CD8$^+$ TCR^{hi} population was measured over time. **A)** FACS plots show the CD8 versus TCR staining on the T lymphocyte gated F5 T cells recovered from the LNs of representative *β2m*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients at d 14 of the timecourse. Gate denotes the CD8$^+$ TCR^{hi} population of donor T cells. Centre FACS plots show the CD8 coreceptor expression versus CFSE dilution on the CD8$^+$ TCR^{hi} gated cells. Gates denote the CD8$^+$ CFSE$^+$ tracer population and the CD8$^+$ CFSE$^-$ F5 T cells. Numbers represent the frequency of cells in each T cell population. Histograms shows the CFSE profile of the CFSE$^+$ tracer population recovered from the *β2m*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients. **B)** shows the mean division index of the F5 cells ± SD in groups (n = 5 per timepoint) of the indicated host at d 1, 7, 14 and 21 **C)** shows the absolute and adjusted precursor cell number of CD8$^+$ TCR^{hi} F5 T cells ± SD from the LN and SPLN of the *β2m*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients over a 21 d timecourse.


A.
CD8+ TCRhi
CFSE+ Tracer Population
Rag 1-/-
Day 14
72.2
96.5
3.49
CD8
CD8
β2m -/-
Rag 1-/-
33.8
97
3.01
TCR
CFSE


B.
Division Index
Rag1 -/-
B2m -/- Rag1 -/-
Division Index
Time (Days)

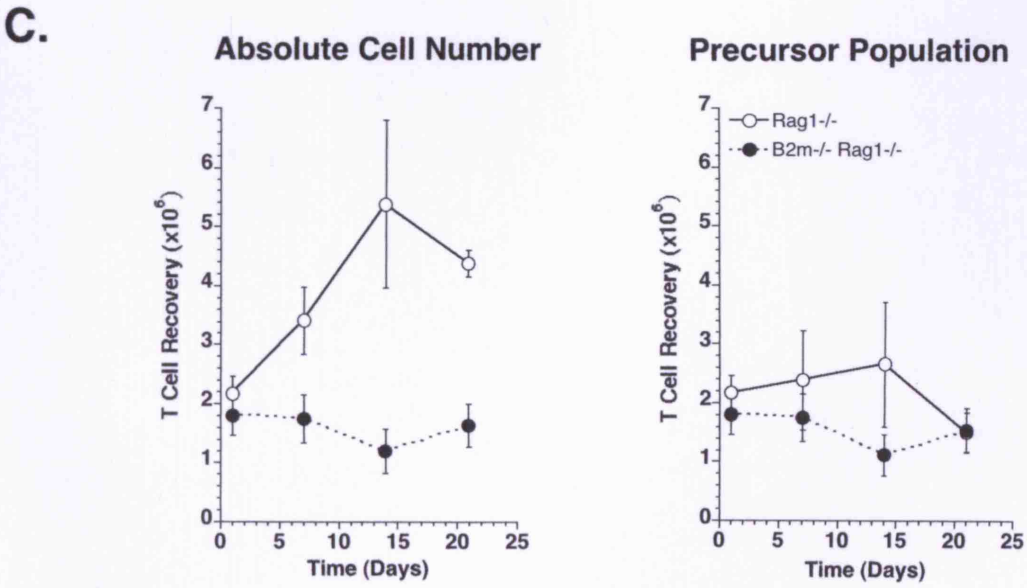
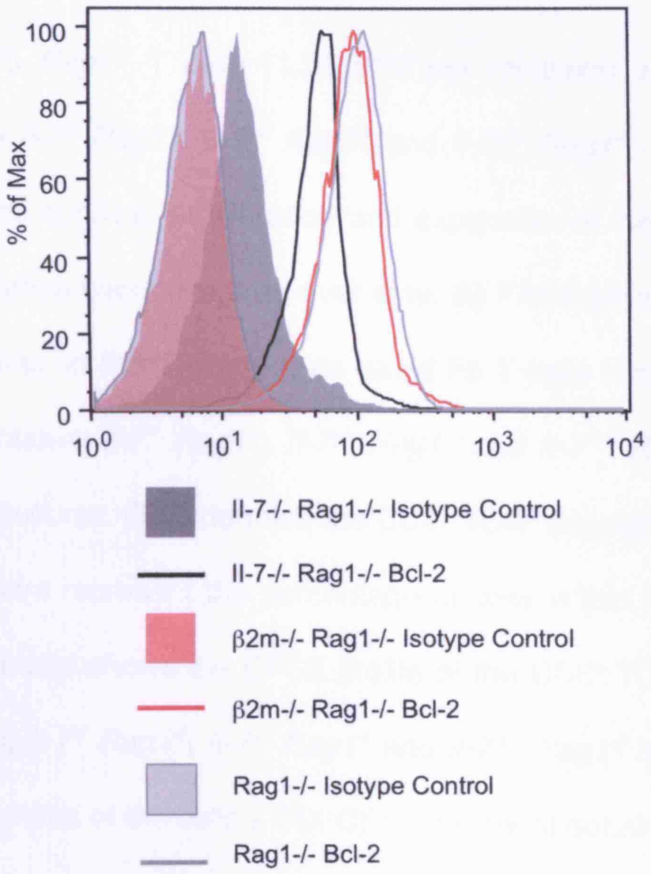

C.
Absolute Cell Number
Precursor Population
T Cell Recovery (x10^6)
Time (Days)
Rag1-/-
B2m-/- Rag1-/-

Figure 5.5: Intracellular Bcl-2 levels are similar between F5 T cells recovered from *β2m*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients.

2 x10^6 donor CFSE labelled and 17 x10^6 donor unlabelled F5 *Rag1*$^{-/-}$ T cells (total of 19 x10^6 donor cells per recipient) were cotransferred intravenously into *β2m*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients (n = 5) and a control *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ mouse. **A)** FACS plots show the intracellular Bcl-2 levels in the CD8$^+$ TCR^{hi} CFSE$^-$ cells recovered from the LNs of representative *β2m*$^{-/-}$ *Rag1*$^{-/-}$, *Rag1*$^{-/-}$ recipients and the spleen of the *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ control 7 d following transfer (gating shown in **Fig 5.4 A**). **B)** bar chart shows the meaned MFI of Bcl-2 expression in the *β2m*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$recipients ± SD.

A.


100
80
60
40
20
0
% of Max
10^0
10^1
10^2
10^3
10^4
Il-7-/- Rag1-/- Isotype Control
Il-7-/- Rag1-/- Bcl-2
β2m-/- Rag1-/- Isotype Control
β2m-/- Rag1-/- Bcl-2
Rag1-/- Isotype Control
Rag1-/- Bcl-2

B.

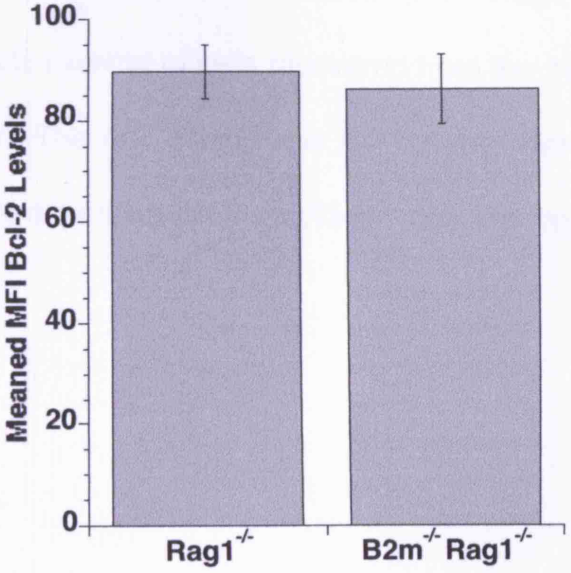
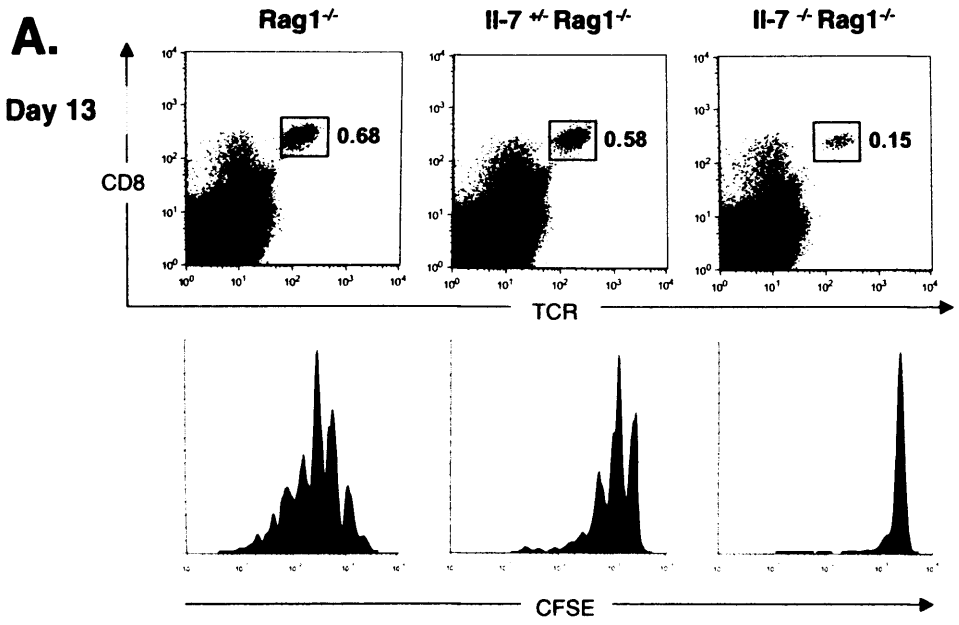
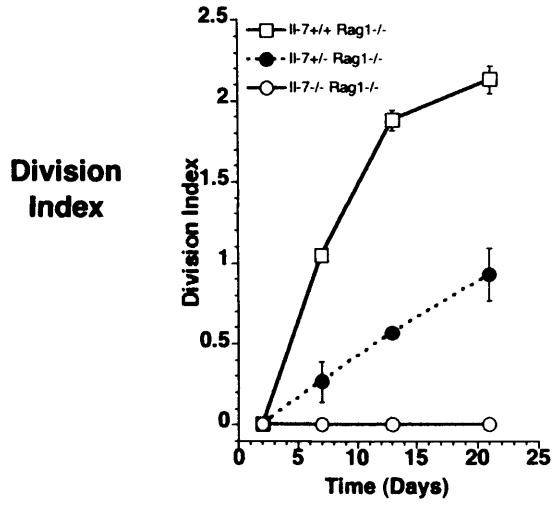

100
80
60
40
20
0
Meaned MFI Bcl-2 Levels
Rag1-/-
B2m-/- Rag1-/-

Figure 5.6: The proliferation, expansion and survival of F5 T cells in the absence of IL-7 signalling.

CFSE labelled F5 *Rag1*$^{-/-}$ T cells (1.25 x10^6 per recipient) were transferred intravenously into *Il-7*$^{-/-}$ *Rag1*$^{-/-}$, *Il-7*$^{+/-}$ *Rag1*$^{-/-}$ and *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ recipients (n = 3 per timepoint). The survival, proliferation and expansion of the CFSE labelled CD8$^+$ TCR^{hi} population was measured over time. **A)** FACS plots show the CD8 versus TCR staining on the T lymphocyte gated F5 T cells recovered from the SPLN of representative *Il-7*$^{-/-}$ *Rag1*$^{-/-}$, *Il-7*$^{+/-}$ *Rag1*$^{-/-}$ and *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ recipients at d 13 of the timecourse. Gate denotes the CD8$^+$ TCR^{hi} population of F5 donor T cells and numbers represent the percentage of cells within the CD8$^+$ TCR^{hi} population. Histograms shows the CFSE profile of the CD8$^+$ TCR^{hi} gated F5 T cells recovered the *Il-7*$^{-/-}$ *Rag1*$^{-/-}$, *Il-7*$^{+/-}$ *Rag1*$^{-/-}$ and *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ hosts. **B)** shows the mean division index of the cells ± SD. **C)** shows the absolute cell number of CD8$^+$ TCR^{hi} F5 T cells ± SD and calculated precursor cell numbers ± SD recovered from the LN and SPLN of the *Il-7*$^{-/-}$ *Rag1*$^{-/-}$, *Il-7*$^{+/-}$ *Rag1*$^{-/-}$ and *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ recipients mice over a 21 d timecourse. (*Il-7*$^{-/-}$ *Rag1*$^{-/-}$ recipients lacked LNs therefore absolute number of cells recovered from the mice was calculated using the SPLN only). The *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ and *Il-7*$^{+/+}$ *Rag1*$^{-/-}$ data is representative of three independent experiments. *Il-7*$^{+/-}$ *Rag1*$^{-/-}$ hosts were only used in this experiment.


A.
Day 13
Rag1-/-
Il-7 +/- Rag1-/-
Il-7 -/- Rag1-/-
0.68
0.58
0.15
CD8
TCR
CFSE


B.
Division Index
Il-7+/+ Rag1-/-
Il-7+/- Rag1-/-
Il-7-/- Rag1-/-
Division Index
Time (Days)

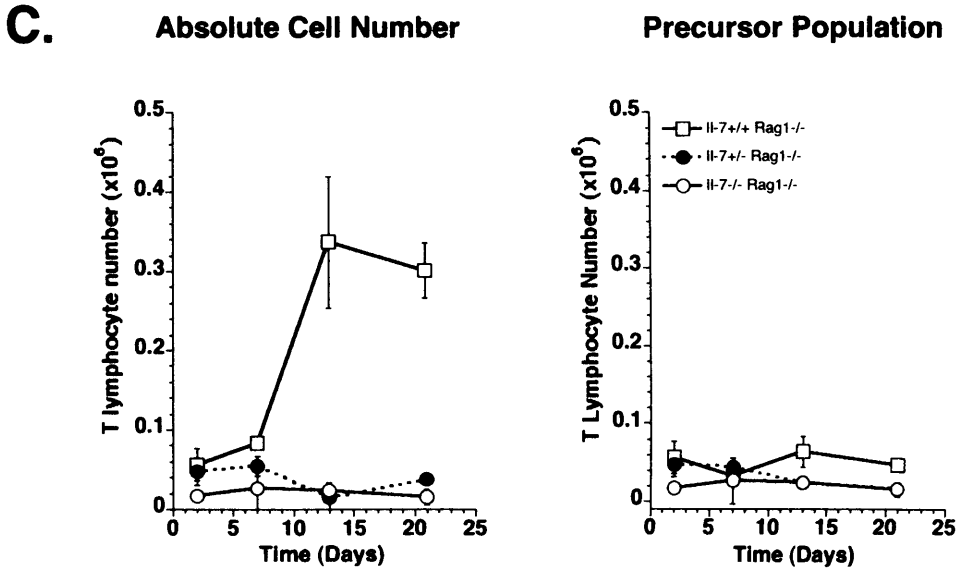
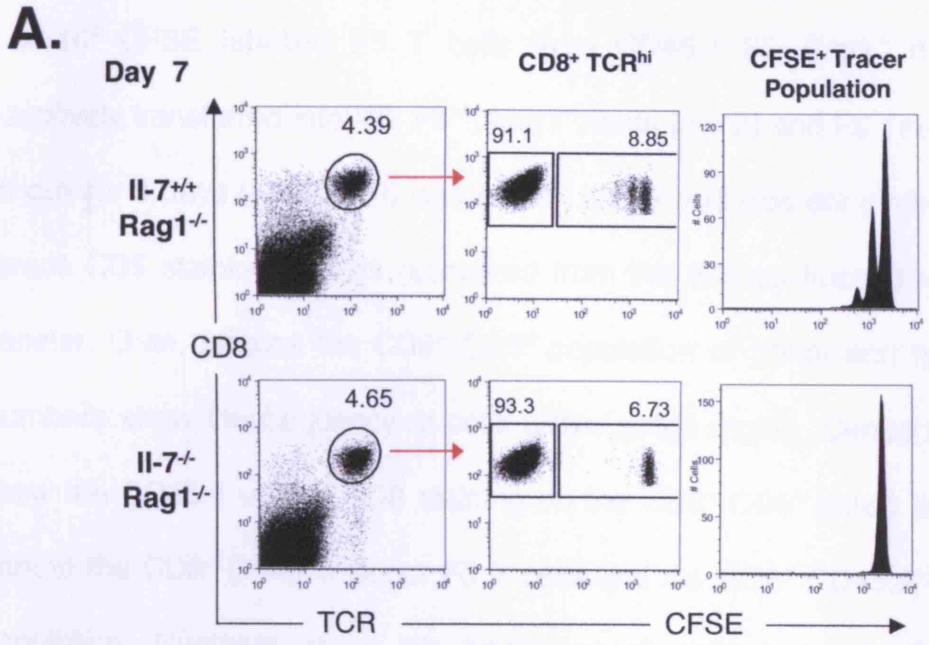

C.
Absolute Cell Number
Precursor Population
T lymphocyte number (x10^6)
T Lymphocyte Number (x10^6)
Time (Days)
Il-7+/+ Rag1-/-
Il-7+/- Rag1-/-
Il-7-/- Rag1-/-

Figure 5.7: IL-7 signalling is essential for the survival and LIP of a large cohort of F5 T cells *In vivo*.

2 x10^6 donor CFSE labelled and 14 x10^6 donor unlabelled F5 *Rag1*$^{-/-}$ T cells (total of 19 x10^6 donor cells per recipient) were cotransferred intravenously into *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients (n = 5 per timepoint). The survival, proliferation and expansion of the CD8$^+$ TCR^{hi} population was measured over time. **A)** dot plots show the CD8 versus TCR staining on the T lymphocyte gated F5 T cells recovered from the spleen of representative *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients at d 7 of the timecourse. Gate denotes the CD8$^+$ TCR^{hi} population of donor T cells. Centre dot plots show the CD8 coreceptor expression versus CFSE dilution on the CD8$^+$ TCR^{hi} gated cells. Gates denote the CD8$^+$ CFSE$^+$ tracer population and the CD8$^+$ CFSE$^-$ F5 T cells. Numbers represent the frequency of cells in each T cell population. Histograms shows the CFSE profile of the CFSE$^+$ tracer population recovered from the *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients. **B)** shows the absolute cell number of CD8$^+$ TCR^{hi} F5 T cells ± SD and adjusted precursor cell numbers ± SD recovered from the LN and SPLN of the *Rag1*$^{-/-}$ recipients and the only the SPLN of *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ hosts over the 21 d timecourse.


A.
Day 7
CD8+ TCRhi
CFSE+ Tracer Population
4.39
91.1
8.85
Il-7+/+ Rag1-/-
CD8
4.65
93.3
6.73
Il-7-/- Rag1-/-
TCR
CFSE

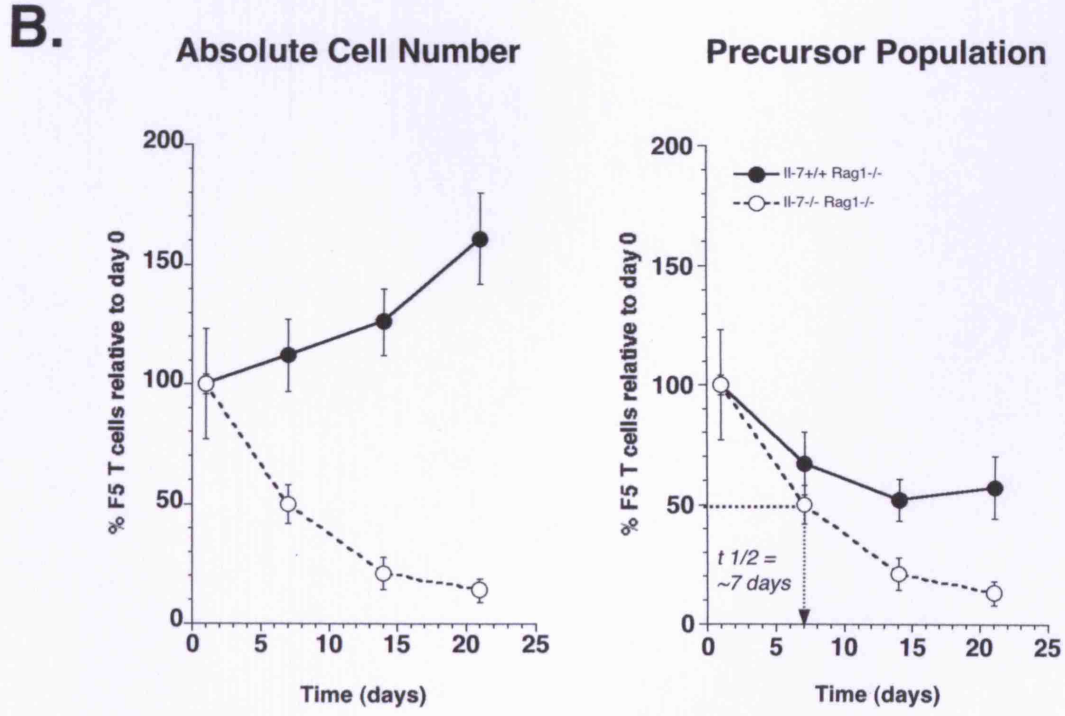

B.
Absolute Cell Number
Precursor Population
% F5 T cells relative to day 0
Time (days)
Il-7+/+ Rag1-/-
Il-7-/- Rag1-/-
t 1/2 =
~7 days

Figure 5.8: Competition for MHC ligands in the absence of competition for IL-7 restrains the LIP of the naïve T cell compartment.

2 x 10^6 CFSE labelled F5 T cells from CD45.1 F5 *Rag1*$^{-/-}$ donors were adoptively transferred into WT F5$^{+/+}$ *Rag1*$^{-/-}$ hosts (n = 2) and F5 Tet-IL-7R hosts off dox for 3 days (n = 3) or 6 wks (n = 3). Left hand side dot plots are of CD8 versus CD5 staining of cells recovered from the various hosts 10 d following transfer. Gate denotes the CD8$^+$ CD5^{hi} population of donor and host T cells. Numbers show the frequency of cells within gated region. Centre FACS plots show the CD45.1 versus CD8 staining on the CD8$^+$ CD5^{hi} gated cells. Gates denote the CD8$^+$ CD45.1$^+$ donor F5 T cells and the CD8$^+$ CD45.2$^+$ IL-7R$^-$ host population. Numbers show the frequency of cells in each T cell gate. Histograms show the CFSE dilution on the CD8$^+$ CD45.1$^+$ gated donor T cells. Data is representative of three independent experiments.

.

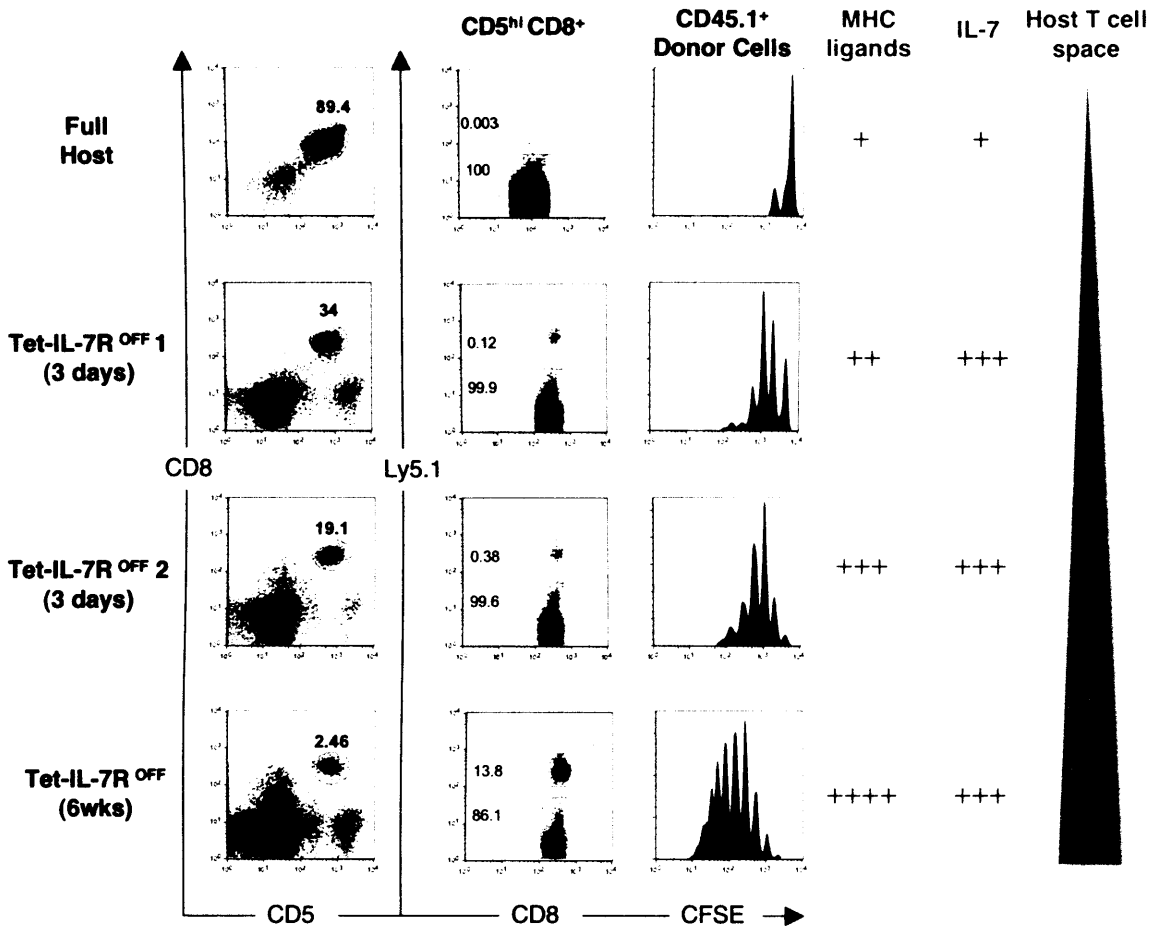
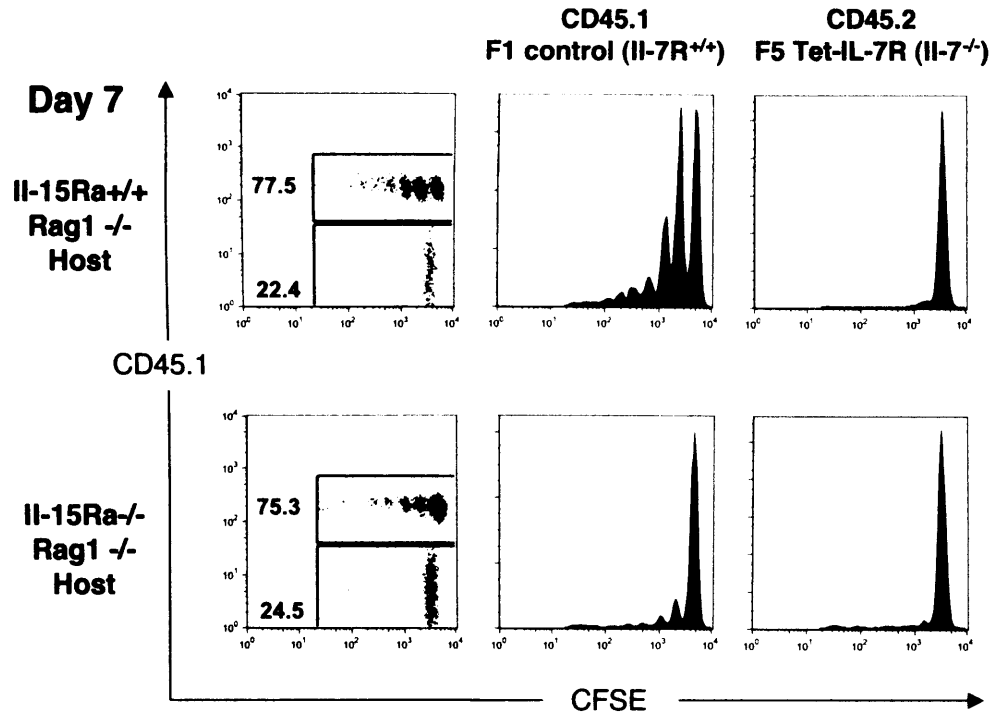

Day 10
CD5hi CD8+
CD45.1+ Donor Cells
MHC ligands
IL-7
Host T cell space
Full Host
89.4
0.003
100
+
+
Tet-IL-7R OFF 1 (3 days)
34
0.12
99.9
++
+++
CD8
Ly5.1
Tet-IL-7R OFF 2 (3 days)
19.1
0.38
99.6
+++
+++
Tet-IL-7R OFF (6wks)
2.46
13.8
86.1
++++
+++
CD5
CD8
CFSE

Figure 5.9: LIP is abrogated in the absence of IL-15 signalling.

1.4 x 10^6 CFSE labelled (F5 *Rag1*$^{-/-}$ x CD45.1) F1 control T cells and 1.4 x 10^6 CFSE labelled CD45.2$^+$ F5 Tet-IL-7R cells (off dox for 3 d prior to the experiment) were cotransferred intravenously into the same *Il-15ra*$^{+/+}$ *Rag1*$^{-/-}$ and *Il15ra*$^{-/-}$ *Rag1*$^{-/-}$ recipients (n = 2). The extent of cell division in the different hosts was analysed 7 d post transfer. **A)** FACS plots show the CD45.1 staining versus CFSE dilution on the T lymphocyte gated CD8$^+$ TCR^{hi} donor T cells recovered from the LNs of representative *Il-15ra*$^{+/+}$ *Rag1*$^{-/-}$ and *Il-15ra*$^{-/-}$ *Rag1*$^{-/-}$ recipients. Gates denotes the CFSE$^+$ CD45.1$^+$ F1 control T cell population and the CFSE$^+$ CD45.2$^+$ F5 Tet-IL-7R population. Numbers represent the percentage of cells within each gated region. Histograms shows the CFSE profile of the CFSE$^+$ CD45.1$^+$ F1 control population and the CFSE$^+$ CD45.2$^+$ F5 Tet-IL-7R population **B)** shows the mean division index of the cells ± SD. Data is representative of two independent experiments.

A.


CD45.1
F1 control (Il-7R+/+)
CD45.2
F5 Tet-IL-7R (Il-7-/-)
Day 7
Il-15Ra+/+
Rag1 -/-
Host
77.5
22.4
CD45.1
Il-15Ra-/-
Rag1 -/-
Host
75.3
24.5
CFSE

B.

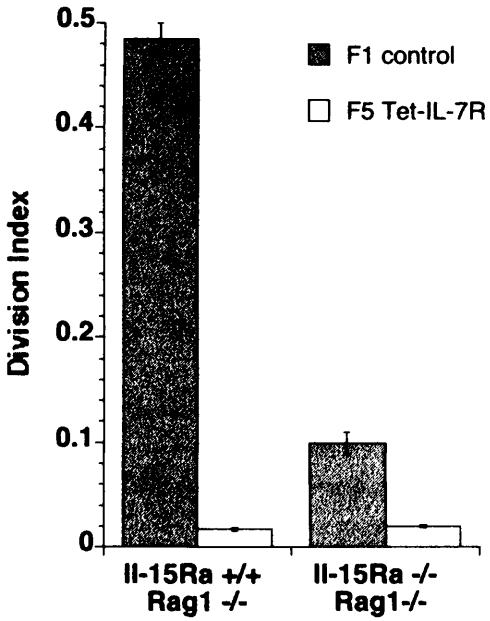

0.5
0.4
0.3
0.2
0.1
0
Division Index
F1 control
F5 Tet-IL-7R
Il-15Ra +/+
Rag1 -/-
Il-15Ra -/-
Rag1-/-

Figure 5.10: CD5 upregulation is impaired in the absence of IL-7 signalling.

A) CFSE labelled F5 *Rag1*$^{-/-}$ T cells (1.5 x10^6 per recipient) were transferred intravenously into *Il-7*$^{-/-}$ *Rag1*$^{-/-}$, *β2m*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients (n = 3). The surface expression of TCR and CD5 was measured 11 d post transfer. Histograms show the TCR and CD5 expression on T lymphocyte gated CD8$^+$ TCR^{hi} T cells recovered from the SPLN of the various hosts. **B)** shows the mean levels of TCR expression ± SD on CD8$^+$ TCR^{hi} CFSE$^-$ F5 T cells (gating shown in **Fig 5.7 A**) following transfer into *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients (n = 5 per timepoint). MFI of TCR staining on the F5 T cells recovered from the *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients was normalised against the MFI of TCR staining on *ex vivo* F5 control T cells. **C)** Unlabelled F5 *Rag1*$^{-/-}$ T cells (3.5 x10^6 donor cells per recipient) were transferred intravenously into *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients (n = 5 per timepoint). Histogram shows the CD5 expression on T lymphocyte gated CD8$^+$ CD5$^+$ F5 donor T cells recovered from the SPLN of representative *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients 3 d following transfer. Graph shows the mean levels of CD5 expression ± SD on the CD8$^+$ CD5^{hi} F5 donor T cells over the 15 d timecourse. MFI of CD5 staining on the F5 T cells recovered from the *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients was normalised against the MFI of staining on *ex vivo* F5 control T cells (plotted as a percentage of maximum). Data is representative of three independent experiments.

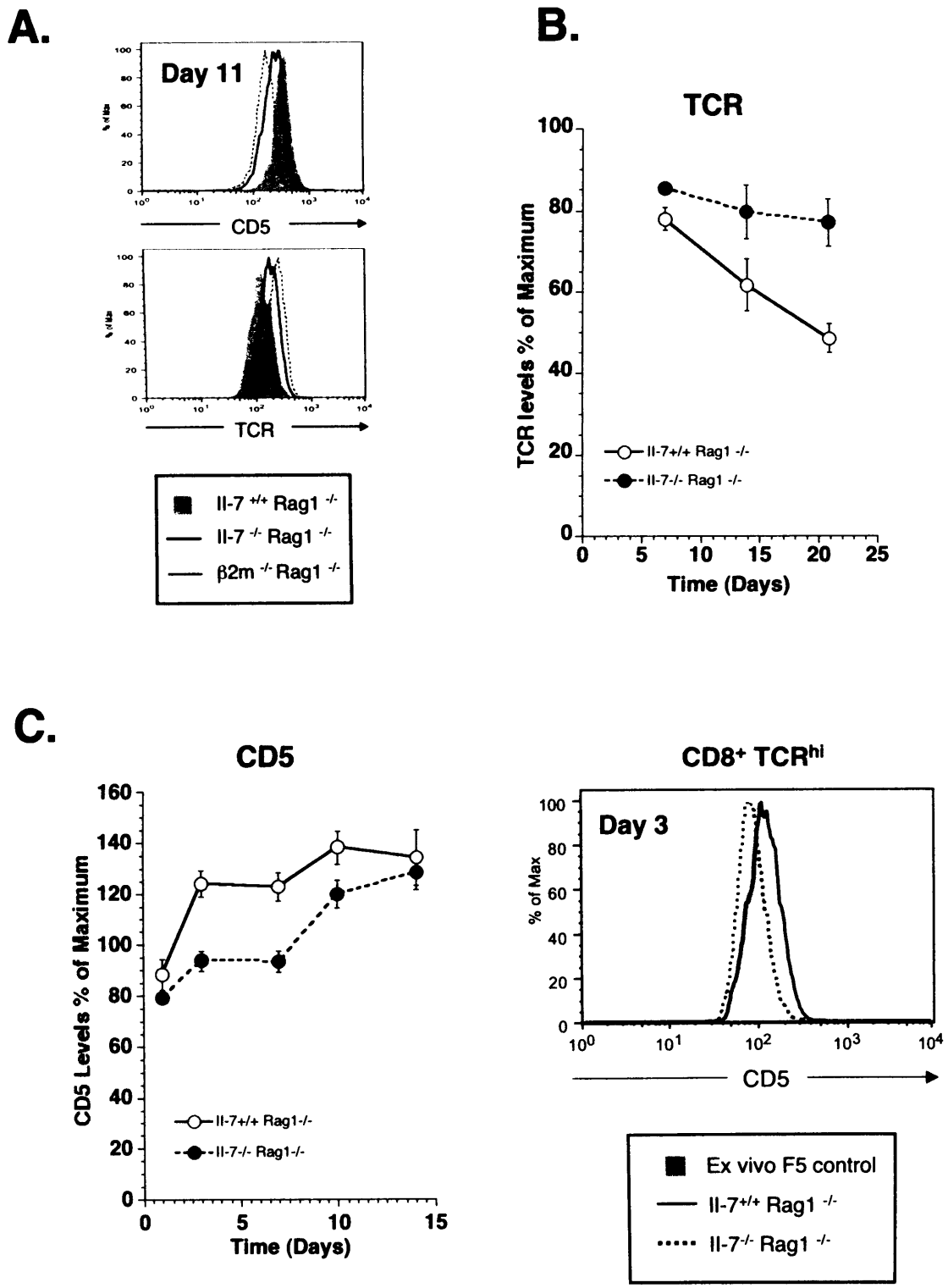
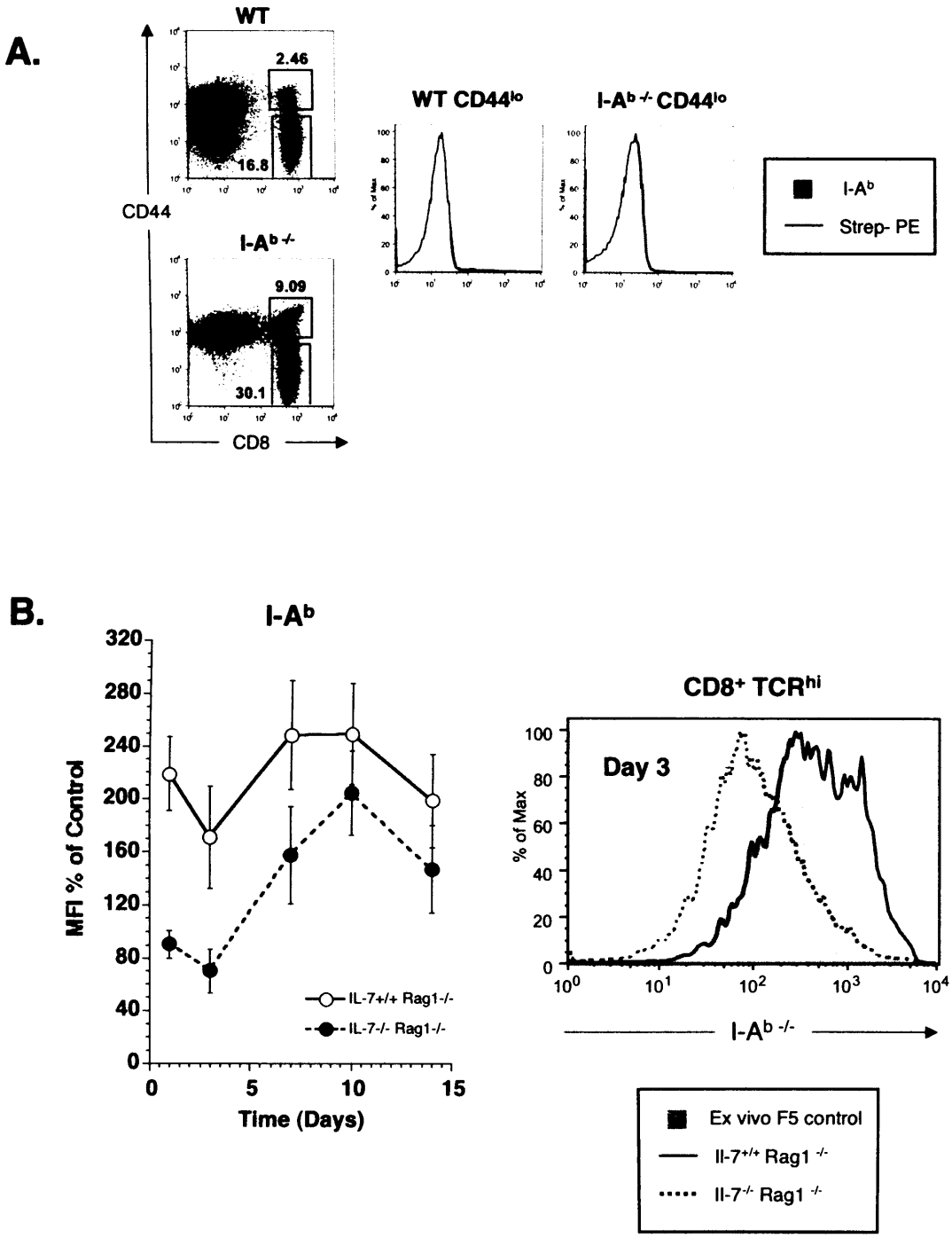

A.
Day 11
CD5
TCR
II-7 +/+ Rag1 -/-
II-7 -/- Rag1 -/-
β2m -/- Rag1 -/-
B.
TCR
TCR levels % of Maximum
Time (Days)
II-7+/+ Rag1 -/-
II-7-/- Rag1 -/-
C.
CD5
CD5 Levels % of Maximum
Time (Days)
II-7+/+ Rag1-/-
II-7-/- Rag1-/-
CD8+ TCRhi
Day 3
% of Max
CD5
Ex vivo F5 control
II-7+/+ Rag1 -/-
II-7-/- Rag1 -/-

Figure 5.11: MHC class II acquisition by F5 T cells is reduced in *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ hosts.

A) FACS plots show the CD44 versus CD8 expression on CD8^{+} TCR^{hi} cells from WT and *I-A*$^{b-/-}$ mice. Gates denote the CD8^{+} CD44^{lo} (naïve) T cells and the CD8^{+} CD44^{hi} (memory) T cells. Numbers show the frequency of cells in each gated region. Histograms show the I-A^{b} staining and negative control streptavidin-PE staining on the CD8^{+} CD44^{lo} T cell population. **B)** Unlabelled F5 *Rag1*$^{-/-}$ T cells (3.5 x10^{6} donor cells per recipient) were transferred intravenously into *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients (n = 5 per timepoint). Histogram shows the surface I-A^{b} levels on T lymphocyte gated CD8^{+} CD5^{+} F5 donor T cells recovered from the SPLN of representative *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients 3 d following transfer. Graph shows the mean levels of I-A^{b} expression ± SD on the CD8^{+} CD5^{hi} F5 donor T cells over the 15 d timecourse. MFI of I-A^{b} staining on the F5 T cells recovered from the *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ recipients was normalised against the MFI of staining on *ex vivo* F5 control T cells (plotted as a percentage of maximum). Data is representative of three independent experiments.


A.
WT
2.46
16.8
CD44
I-Ab -/-
9.09
30.1
CD8
WT CD44lo
I-Ab -/- CD44lo
I-Ab
Strep- PE
B.
I-Ab
MFI % of Control
320
280
240
200
160
120
80
40
0
0
5
10
15
Time (Days)
IL-7+/+ Rag1-/-
IL-7-/- Rag1-/-
CD8+ TCRhi
Day 3
% of Max
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
I-Ab -/-
Ex vivo F5 control
Il-7+/+ Rag1 -/-
Il-7-/- Rag1 -/-

Chapter 6

Final Discussion

The overall aim of this study was to investigate how naïve T cell homeostasis is regulated by homeostatic TCR signals and IL-7 cytokine signals in replete and lymphopenic settings. More specifically we wished to investigate whether the TCR proximal tyrosine kinase Zap70 is involved in the propagation of TCR survival signals and assess the functional overlap between TCR and IL-7R signalling in naïve T cell survival and lymphopenia induced proliferation. The data produced in this thesis supports the view that Zap70 expression is required for the transmission of homeostatic TCR survival signals *in vivo*. We have shown that naïve $CD8^+$ T cell survival in lymphopenic hosts requires different homeostatic signals than naïve T cells in replete hosts. We also demonstrate that naïve $CD4^+$ and $CD8^+$ T cells differ in their dependency on homeostatic TCR signals for their survival. Unexpectedly we found that homeostatic TCR and IL-7 signals may cooperate to maintain naïve T cell homeostasis, such that homeostatic TCR signals have the capacity to influence IL-7R expression, and that IL-7 signalling can indirectly affect the reception of homeostatic TCR signals by facilitating enhanced contacts with MHC expressing DCs. Together these data report that naïve T cell homeostasis requires dynamic regulation by TCR and IL-7 signals, and that efficient homeostasis is maintained by the convergence of these signals.

6.1 Is Zap70 Required for Naïve T cell Survival?

The initial experiments performed in this thesis aimed to generate and characterise mice bearing a tetracycline inducible Zap70 transgene. Initial characterisation of dox fed Tet-Zap70 mice revealed reduced cellularity of the peripheral naïve $CD4^+$ T cell pool and a more marked depletion of the naïve $CD8^+$ T cell compartment compared to WT mice **(Fig 3.11 and 3.13)**. Our data suggests that this occurs a result of combined defects in the efficiency of thymopoiesis **(Fig 3.6)** and peripheral T cell survival **(Fig 4.5 and 4.9)**. More specific analysis of the requirement of Zap70 in naïve T cell survival, revealed that cessation of Zap70 expression in chimeric mice, resulted in the gradual disappearance of the naive $CD4^+$ **(Fig 4.5)** and $CD8^+$ Tet-Zap70 populations **(Fig 4.9)**, inferring that Zap70 is involved in the transmission of homeostatic TCR signals.

Interestingly, we observed differences in the extent to which homeostatic TCR signals were required to maintain the survival of the naïve $CD4^+$ and $CD8^+$ Tet-Zap70 compartments. Notably, the naïve $CD8^+$ compartment decayed at a much faster rate than the naïve $CD4^+$ T cell pool **(Fig 4.5 and 4.9)**, suggesting that naïve $CD8^+$ T cells are more dependent on homeostatic TCR signals for their survival, this is consistent with findings reported by (Labrecque et al., 2001; Polic et al., 2001) and (Seddon and Zamoyska, 2002b). The reason for the faster kinetic of T cell loss within the naïve T cell $CD8^+$ compartment, compared to the naïve $CD4^+$ T cell population is unclear in our model system, given that the naïve $CD4^+$ and $CD8^+$ Tet-Zap70 T cells also exhibited reduced

IL-7R expression compared to WT cells **(Fig 4.10)**, suggesting that Zap70 signalling may also regulate IL-7R expression. WT polyclonal naïve $CD4^+$ and $CD8^+$ T cells express roughly equivalent levels of IL-7R **(Fig 4.10 E)**. However, in thymectomized mice treated with anti-IL-7R antibodies (Vivien et al., 2001), or transfer of C57Bl/6 naïve $CD4^+$ or naïve $CD8^+$ T cells into un-irradiated *Il-7*$^{-/-}$ hosts (Tan et al., 2001) has shown that the survival of the naïve $CD4^+$ T population is most abrogated by a loss of IL-7R signalling, suggesting this compartment is more sensitive to a loss of IL-7 survival signals than naïve $CD8^+$ T cells despite expressing similar levels of IL-7R. This is in contrast to our Tet-Zap70 mice, the levels of IL-7R expression differed between the naïve $CD4^+$ and naïve $CD8^+$ T cell populations, IL-7R expression appeared most reduced in naïve $CD8^+$ T cells and correlated **(Fig 4.10 E)** with the marked reduction in cellularity observed in the naïve $CD8^+$ compartment but not the naïve $CD4^+$ T cell pool **(Fig 3.11 and 3.13)**. It is clear that the expression of the Tre-Zap70 transgene in peripheral T cells is unable to fully rescue TCR signalling as demonstrated by the reduced level of CD5 expression in peripheral $CD4^+$ and $CD8^+$ T cells generated in Tet-Zap70 mice **(Fig 3.17)**. Therefore is unclear whether the lower levels of IL-7R expression on the naïve $CD8^+$ T cells reflects a more profound survival defect within this T cell compartment or whether the differences in survival between the naïve $CD4^+$ or $CD8^+$ T cell compartments is purely attributed to differing sensitivities to the suboptimal transmission of homeostatic TCR signals. Another possibility is that in the (Vivien et al., 2001) and (Tan et al., 2001) studies that signals transmitted by the cytokine IL-15 may augment the survival of the naïve $CD8^+$ T

cell compartment. Evidence to support this comes from the analysis of *Il-15*$^{-/-}$ mice which exhibit reduced numbers of peripheral naïve CD8$^+$ T cells but not naïve CD4$^+$ T cells (Kennedy et al., 2000b) suggesting a role for IL-15 in naïve CD8$^+$ T cell survival. The reduced surface levels of CD122 observed on the naïve CD8$^+$ T cells generated in our Tet-Zap70 mice **(Fig 3.14)** may abrogate this low level of IL-15 signalling, which combined with the suboptimal expression of the Tre-Zap70 transgene and reduced levels of IL-7R expression may further perturb the survival of the naïve CD8$^+$ T cell compartment.

It is also possible that the reduction in IL-7R expression on the surface of the Tet-Zap70 naive CD4$^+$ and CD8$^+$ T cells may not have an appreciable effect on the homeostasis of these compartments. Our studies of F5 TCR transgenic T cells **(Chapter 5),** have shown that these cells also express very low levels of IL-7R compared to polyclonal WT CD8$^+$ T cells (Seddon and Zamoyska, 2002a), but, appear to persist in lymphopenic hosts lacking MHC class I ligands despite the reduced levels of IL-7R **(Fig 5.4)**. However it should be noted that the survival of the F5 T cells was studied in T cell deficient hosts, and therefore the competition for limited IL-7 in such an environment would be low. This is in contrast to the Tet-Zap70 mice which, although partially lymphopenic, do possess a substantial population of T cells and B cells which would increase the competition for the limited IL-7. It is possible that under these competitive conditions the effects of reduced IL-7R expression is more profound. Future experiments will assess whether the low levels IL-7R expression observed in Tet-Zap70 mice combined with the limited levels of

circulating IL-7 have an impact on the survival of the peripheral naïve $CD8^+$ T cell population.

6.2 Survival Signals in Lymphopenic and Replete Hosts.

While there is a large body of evidence supporting a role for TCR/spMHC interactions in the maintenance of the naïve T cell compartment, there appears to be some differences in the signals required for the survival of naïve T cells in a lymphopenic environment versus a replete setting. Notably, (Martin et al., 2006) has shown that homeostatic TCR signals are not required for the long-term survival of naïve $CD4^+$ T cells in T cell deficient hosts, but are critical for the survival of the naïve $CD4^+$ T cell population in replete hosts, or $CD8^+$ T cell containing mice. Therefore, one of the first questions we addressed was whether TCR signals generated from the recognition of self ligands were required for naïve $CD8^+$ T cell survival in lymphopenic conditions and replete mice. In agreement with the Martin *et al*, 2006 study, our data demonstrates that homeostatic TCR signals are dispensable for the short-term survival of naïve $CD8^+$ T cells in conditions of lymphopenia **(Fig 5.4).** Alternatively this data infers that IL-7 (and possibly other unidentified survival factors) can support the survival of the naïve T cell population in the absence of TCR signals in T cell deficient hosts. The non-redundant role of IL-7 survival signals was also apparent given that large cohorts of naïve $CD8^+$ T cells transferred into *Il-7*$^{-/-}$ *Rag1*$^{-/-}$ hosts, declined significantly with time **(Fig 5.6)**, confirming that TCR signals alone cannot support the survival of large numbers of naïve $CD8^+$ T cells in lymphopenic settings. These data imply that IL-7 signals are more

essential than TCR signals for the homeostasis of naïve $CD8^+$ T cells in conditions of lymphopenia.

In agreement with studies by (Martin et al., 2006; Polic et al., 2001; Witherden et al., 2000) and (Seddon and Zamoyska, 2002b) assessment of naïve T cell survival in a lymphoreplete environment using the Tet-Zap70 and WT bone marrow chimeras, confirm that both the naïve $CD4^+$ and $CD8^+$ compartments require homeostatic TCR signals for their survival given that the frequency of peripheral naïve $CD4^+$ and $CD8^+$ Tet-Zap70 T cells declined following ablation of Zap70 expression **(Fig 4.5 and 4.9)**. The reason for the differential requirements of homeostatic TCR signalling between naïve T cell populations in T cell deficient versus replete hosts, is unclear, but is believed to involve competition for limited IL-7. IL-7 is believed to be produced in a constitutive fashion and at levels that remain constant despite changes to the size of the T cell pool (Mazzucchelli and Durum, 2007). In lymphoreplete conditions one possibility is that competition for and consumption of IL-7 is high, and such low levels of IL-7 maybe insufficient for the survival of the naïve T cell compartment and necessitates the requirement of a secondary homeostatic TCR signal to maintain survival. In contrast, in lymphopenic hosts the reduced size of the naïve T cell population, is likely to reduce competition for IL-7, therefore elevated levels of IL-7 through decreased consumption may be sufficient to maintain the survival of the naïve T cell population in the absence of a secondary TCR survival signal. Although many studies suggest that the levels of circulating IL-7 regulate T cell survival, this has not been shown directly. The effects of increasing IL-7 availability on naïve T cell survival *in vivo* are difficult

to determine given that IL-7 enhances T cell proliferation in hosts overexpressing IL-7 (Bosco et al., 2005). These studies fail to compensate for the effects of cell division, therefore is unclear whether the increased numbers of T cells found in such mice result purely from increased proliferation or is also due to augmented survival. In our experiments, naïve $CD8^+$ T cells transferred into *Il-7*$^{+/-}$ hosts appeared to survive better at early timepoints, compared to *Il-7*$^{-/-}$ hosts. However these experiments involved the transfer of small cohorts into the recipients and therefore it is unclear whether these differences in survival are significant. Future experiments will attempt to address this issue by transferring larger T cell cohorts into *Il-7*$^{+/-}$ and *Il-7*$^{-/-}$ mice.

6.3 The Relationship Between Homeostatic TCR and IL-7R Signals.

6.3.1 Can Homeostatic TCR Signals Influence IL-7 Signalling?

Our work has also uncovered the surprising finding that Zap70 signals can modulate IL-7R re-expression in the thymus. CD4 SP and CD8 SP thymocytes generated in Tet-Zap70 mice, showed reduced expression of IL-7R compared to control thymocytes **(Fig 4.11)**. Interestingly this difference mirrored an existing difference in IL-7R expression we also observed between CD4 SP and CD8 SP thymocytes in WT mice, but that appeared exacerbated in Tet-Zap70 mice. To explain these findings we propose that IL-7R re-expression in the thymus may be regulated by positive thymic selection signals transmitted by Zap70. We also suggest that the level of IL-7R expression on mature SP

thymocytes may be modulated by the strength of TCR ligation. This hypothesis would therefore suggest that thymocytes possessing high aviditiy TCRs would also express higher levels of IL-7R than thymocytes expressing low avidity TCRs. Indeed it has been recognised for some time that F5 TCR transgenic mice express a low avidity TCR and that F5 CD8 SP thymocytes are virtually negative for IL-7R expression (Seddon and Zamoyska, 2002a). We suggest that low avidity TCR signals in F5 mice are insufficient for full induction of IL-7R expression in the thymus.

Currently we are investigating this further, by assessing the regulation of IL-7R on thymocytes expressing high and low avidity TCRs. We are also crossing the Tet-Zap70 mice onto different MHC class I TCR transgenic backgrounds, to see how the suboptimal expression of Tre-Zap70 transgene affects IL-7R expression. Our current model **(Fig 6.1)** proposes that in polyclonal Tet-Zap70 mice, the heterogeneity of the polyclonal population may preferentially skew the repertoire toward cells that are better equipped at functioning with suboptimal levels of Zap70 expression. On low avidity TCR transgenic backgrounds where expression of the TCR is fixed, suboptimal Zap70 signalling cannot be compensated for by adjusting the repertoire of selecting T cells, toward cells with a higher avidity for spMHC and therefore we believe the Tre-Zap70 transgene will be unable to optimally transmit positive selection signals and thus fail to rescue IL-7R expression. Indeed preliminary experiments using Tet-Zap70 mice bred onto an class I restricted F5 TCR transgenic background suggest this.

The regulation of IL7R expression by positive selection signals may represent a mechanism by which weakly selecting cells (that may not be particularly useful) are purged from the peripheral repitoire. Reduced IL-7R expression and hence IL-7 signalling in weakly selecting cells that are already less competitive for spMHC ligands may increase the likely hood of their loss/removal from the peripheral repertoire, and serve to enrich the peripheral T cell repertoire with useful cells.

In peripheral T cells it is unclear whether homeostatic TCR signals influence IL-7R signalling. In Tet-Zap70 T cells, the sub-optimal expression of the Tre-Zap70 transgene and thus reduced homeostatic TCR signalling does appear to modulate peripheral IL-7R expression **(Fig 4.10)**. Therefore, we would expect IL-7R expression and/or signalling pathways to be more profoundly abrogated in cells unable to receive homeostatic TCR signals. However we did not find any evidence that homeostatic TCR signals could modulate IL-7R signalling under such conditions given that Bcl-2 levels appeared normal in F5 $CD8^+$ T cells recovered from $\beta 2m^{-/-}$ recipients lacking MHC class I ligands **(Fig 5.5)**. However, it is possible that the use of F5 TCR transgenic T cells, which express low levels of IL-7R may not be receptive to such regulation by homeostatic TCR signals. It would be interesting to investigate whether the absence of homeostatic TCR signals affects IL-7R expression or IL-7 signalling in TCR transgenic $CD8^+$ T cell clones expressing WT levels of IL-7R such as the OT-1 TCR transgenic. Bcl-2 is not the only pro-survival factor activated by IL-7 signalling. IL-7 signals influence the activity and/or expression of a wide

range of pro and anti-apoptotic proteins **(Fig 1.3)**, it therefore possible that homeostatic TCR signals may affect other survival factors downstream of the IL-7R. Interestingly, IL-7 and TCR signals have been reported to modulate the survival of $CD4^+$ central memory T cell in humans by phosphorylating the transcription factor FOXO3a (Riou et al., 2007), it would be interesting to investigate whether dual TCR and IL-7 signalling has the same effect on FOXO3a in naïve T cells.

Although the expression of IL-7R **(Fig 4.10)** and the transmission of homeostatic TCR signals **(Fig 3.17)** was reduced in the naïve peripheral $CD4^+$ and $CD8^+$ T cells generated in Tet-Zap70 mice, the cells still appeared capable of LIP, as demonstrated by the increased frequency of cells that had upregulated the activation maker CD44 **(Fig 3.11 and 3.13).** This suggests that these cells can still respond to lymphopenia, despite the reduced TCR and possibly IL-7 signalling. Given that both IL-7 and TCR signals are essential for LIP it is possible these signals converge to regulate the same intracellular proteins involved in the LIP response. Two such possible candidates are the cell cycle proteins p27kip1 (Li et al., 2006) and Cdc25A (Khaled et al., 2005), which mediate the proliferative effects IL-7. It would be interesting to examine whether the activity of these cell cycle proteins are also regulated by homeostatic TCR signals.

6.3.2 Can IL-7R Signals Influence Homeostatic TCR Signalling?

It is widely accepted that IL-7 signals are essential for LIP. Our data supports the findings by (Kieper et al., 2004) and (Bosco et al., 2005) that IL-7 functions in a quantitative fashion, such that increased availability of IL-7 augments the LIP of the naïve T cell compartment. However, it is not clear how IL-7 mediates such affects. (Sprent and Surh, 2003) suggest that IL-7 signals enhance TCR signalling to amplify LIP responses, although there is still no direct evidence to suggest this. Unexpectedly, our data revealed that the strength of homeostatic TCR signalling was reduced in F5 $CD8^+$ T cells transferred to *Il-7*$^{-/-}$ but not *Il-7*$^{+/+}$ hosts, suggesting that IL-7 has the capacity to influence homeostatic TCR signalling. Further analysis revealed that naïve T cells transferred to IL-7 deficient hosts, showed evidence of reduced contact with MHC expressing DCs, implying that IL-7 signals may modulate homeostatic TCR signalling indirectly, by influencing homeostatic T cell-APC interactions **(Fig 5.11)**. Given that recent reports suggest role for IL-7 in the regulation of glucose metabolism (Barata et al., 2004; Rathmell et al., 2001) we are currently assessing whether the loss of IL-7 signals impairs the movement of naïve T cells in the secondary lymphoid organs, and thus indirectly abrogates the reception of homeostatic TCR signals by decreasing the number of contacts T cells make with DCs. Preliminary experiments do indeed suggest this to be true although more experiments using more accurate microscopy equipment is needed to confirm this observation. However, If this is the case, this model would therefore suggest that increasing the availability of IL-7 not only acts as a sensor alerting T cells to the space within their environment in which they can expand, but may

also facilitate enhanced contacts with DCs delivering the self ligands required for the induction of LIP.

Our data is also in agreement with (Sandau et al., 2007) who report that IL-15 signals contribute to LIP *in vivo*. IL-15 has also been shown to upregulate GLUT4 mRNA expression and increase glucose metabolism in rat skeletal tissue (Busquets et al., 2006), therefore it would be interesting to investigate whether IL-15 signals can also influence glucose uptake in T cells and perhaps influence homeostatic TCR signalling by also influencing T cell metabolism and motility.

6.4 How do TCR Signals Induce Survival Versus LIP?

While there is evidence suggesting that IL-7 signals amplify LIP responses, it is not clear how homeostatic TCR signals function in the context of LIP. The current opinion is that homeostatic TCR signals function in a purely qualitative fashion. However, in our studies we conclude that, homeostatic TCR signals also function quantitatively. We demonstrated that even in conditions where the competition for IL-7 is low, the availability and competition for self ligands is sufficient to reduce the LIP of naïve donor T cells **(Fig 5.8)**. This suggests that homeostatic TCR signals play a non-redundant role in the LIP response, and that enhanced TCR signalling is required for LIP. The implications of this, is that TCR signals that influence naïve $CD8^+$ T cell survival may differ from the TCR signals that drive LIP quantitatively. Our data shows that competition for IL-7 is not the only factor, which restrains the LIP of the naïve T cell

compartment, but that competition for self ligands contributes significantly to the regulation of LIP. It appears the both IL-7 and homeostatic TCR signals are equally essential for the LIP of the naïve $CD8^+$ T cell compartment. We propose that scanning of APCs by T cells in replete hosts provides the minimal homeostatic TCR signal, that supports the survival of the naïve T cell compartment, but that anything that increases the rate of T cell scanning (for example: increased levels of IL7 or lymphopenia) allows accumulation of the homeostatic TCR to a level that triggers cell division. This would also be consistent with a recent study that suggests homeostatic proliferation involves discrete single divisions rather than a programmed response of many divisions, as occurs with antigen (Yates *et al* JI in press).

6.5 Clinical Perspectives

Understanding the precise signalling mechanisms involved in the survival and proliferation of naïve and memory T cells in lymphopenic and replete conditions is particularly important in trying to design therapeutics that enhance immune reconstitution in patients with profound lymphopenia. Many human diseases are associated with lymphopenia the most obvious example of this is acquired immuno deficiency syndrome (AIDS), in which naïve $CD4^+$ T cell numbers are substantially reduced (Hellerstein et al., 1999; Margolick and Donnenberg, 1997). Other viral diseases such as Measles (Okada et al., 2000) and highly virulent influenza A (H5N1) (Tumpey et al., 2000) also result in marked but transient depletion of the T cell pool. An obvious clinical application would be the use of IL-7 therapy in enhancing the proliferation of naïve and memory T

cells in patients exhibiting profound lymphopenia. Indeed *in vivo* non-human primate models are currently under development to evaluate the effects of IL-7 therapy on immune reconstitution. Preliminary results show that IL-7 can induce a substantial increase in total $CD4^+$ and $CD8^+$ T cell counts as a result of enhanced thymopoiesis and peripheral T cell proliferation (Beq et al., 2006; Fry et al., 2003). More recently IL-7 has been shown to reduce spontaneous apoptosis of *ex vivo* HIV-infected $CD4^+$ and $CD8^+$ T cells following culture *in vitro* (Vassena et al., 2007), further supporting the use of IL-7 as a tool to aid the immune reconstitution of HIV infected patients.

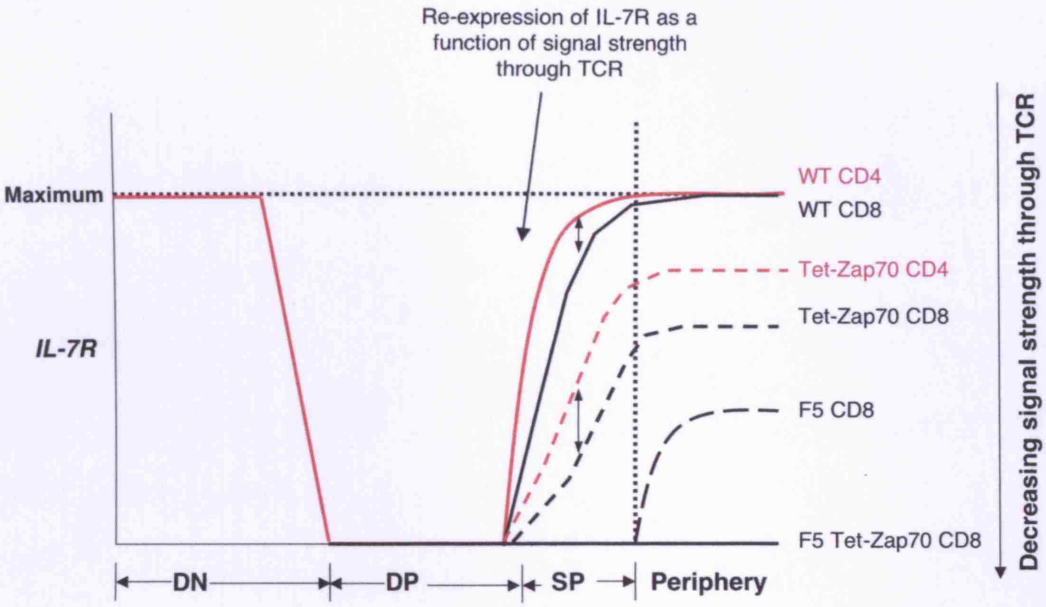
More recently it has been shown that IL-7 levels are increased in the serum of HIV-infected patients and correlates with the level of $CD4^+$ T-cell depletion (Fry et al., 2001; Llano et al., 2001; Napolitano et al., 2001). Interestingly IL-7Rα expression is also downregulated on HIV-infected $CD4^+$ and $CD8^+$ T cells appearing most pronounced within the memory T cell compartments (Koesters et al., 2006; MacPherson et al., 2001). Another recent study reports that the reduced IL-7R expression on infected memory $CD4^+$ T cells is associated with low Bcl-2 levels and with the reduced survival of the T cells following culture with IL-7 *in vitro* (Rethi et al., 2005). It is unclear why and how surface IL-7R levels decrease in HIV-infected cells. Understanding of how IL-7R expression is regulated in normal cells may help the development of therapeutics to enhance IL-7 responsiveness in HIV patients.

The use of memory-like cells generated under conditions of lymphopenia may also have potential clinical applications. It has been shown that LIP is skewed

towards weak antigens that have a low affinity for the TCR (Ernst et al., 1999; Goldrath and Bevan, 1999). Therefore, memory-like cells generated in lymphopenic conditions maybe particularly useful in diseases driven by weak low affinity antigens, such as tumour antigens. Indeed (Dummer et al., 2002) have reported that homeostatic T cell proliferation and tumor antigen presentation in lymph nodes can trigger a beneficial anti-tumor autoimmune response, and the generation of long-term memory. Thus patients undergoing tumour immunotherapy may benefit from adjuvants that stimulate LIP, such as IL-7.

Figure 6.1: Model of IL-7R expression during thymocyte development.

IL-7R expression is high within the DN compartment and is switched off following transition to the DP compartment and re-expressed on the mature SP thymocytes. How IL-7R re-expression is regulated is unknown. Our model suggests that positive selection signals regulate the level of IL-7R expressed on the peripheral T cells, such that IL-7R expression increases as a function of TCR signal strength. Arrows denote the difference in IL-7R expression between CD4 SP and CD8 SP thymocytes in WT and dox fed Tet-Zap70 mice.


Re-expression of IL-7R as a function of signal strength through TCR
Maximum
IL-7R
WT CD4
WT CD8
Tet-Zap70 CD4
Tet-Zap70 CD8
F5 CD8
F5 Tet-Zap70 CD8
DN
DP
SP
Periphery
Decreasing signal strength through TCR

Appendix

The Zap70 protein detected in the dox fed Tet-Zap70 mice was ~5.5kb heavier than endogenous Zap70 protein, resulting with the addition of an amino terminal peptide tag to the transgenic Zap70 protein.

Sequencing of the Tre-Zap70 DNA construct 1 used to generate the Tet-Zap70 mice identified an alternative in frame ATG start codon upstream Zap70 ORF **(Fig A)**. This second start sequence was found between the SnaBI and EcoRI restriction sites in the remnant of the MCS (polylinker primers) originally introduced into the tetracycline inducible plasmids to aid the subsequent subcloning steps. Subcloning of the huβg intronic sequence into the second Tre-huβg-Zap70 construct, was performed using the SnaBI and EcoRI restriction sites and therefore the alternative start codon was not present in Tre-huβg-Zap70 construct **(Fig B)**.

Analysis of the protein sequence of the peptide tag using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST), which finds regions of local similarity between sequences, could find no sequence homology between the peptide tag and all other proteins in the database.

Figure A: Nucleotide sequence of Tre-Zap70 Construct 1.

Nucleotide sequence of the MCS and region upstream of the Zap70 ORF. Blue boxes highlight the ATG start sequence.

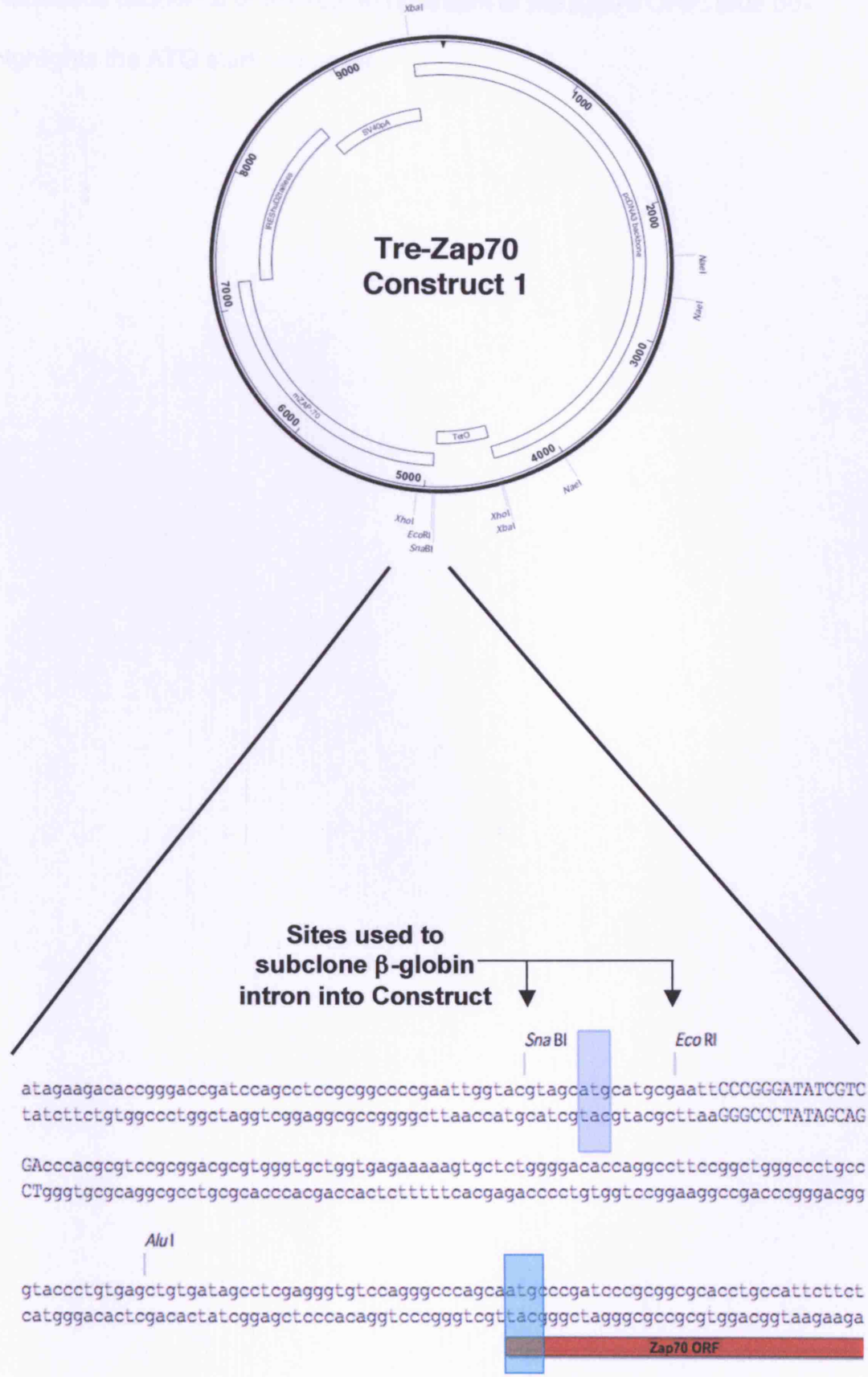
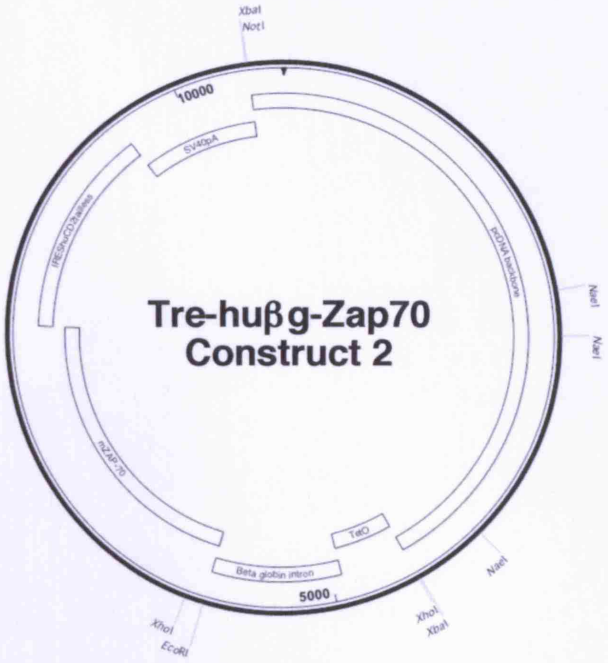

XbaI
9000
1000
8000
2000
7000
3000
6000
4000
5000
SV40pA
IRES/hCD2Marker
pCDNA3 backbone
mZAP-70
TetO
NaeI
NaeI
NaeI
XhoI
EcoRI
SnaBI
XhoI
XbaI
Tre-Zap70
Construct 1
Sites used to
subclone β-globin
intron into Construct
Sna BI
Eco RI
atagaagacaccgggaccgatccagcctccgcggccccgaattggtacgtagcatgcatgcgaattCCCGGGATATCGTC
tatcttctgtggccctggctaggtcggaggcgccggggcttaaccatgcatcgtacgtacgcttaaGGGCCCTATAGCAG
GAcccacgcgtccgcggacgcgtgggtgctggtgagaaaaagtgctctggggacaccaggccttccggctgggccctgcc
CTgggtgcgcaggcgcctgcgcacccacgaccactctttttcacgagaccctgtggtccggaaggccgacccgggacgg
AluI
gtaccctgtgagctgtgatagcctcgagggtgtccagggcccagcaatgcccgatcccgcggcgcacctgccattcttct
catgggacactcgacactatcggagctcccacaggtcccgggtcgttacgggctagggcgccgcgtggacggtaagaaga
Zap70 ORF

Figure B: Nucleotide sequence of Tre-huβg-Zap70 Construct 2.

Nucleotide sequence of the region upstream of the Zap70 ORF. Blue box highlights the ATG start sequence.



Eco RI

gctggtctgtgtgctggcccatcactttggcaaagaattcccgggatatcgtcgacccacgcgtccgcgg
cgaccagacacacgaccgggtagtgaaaccgtttcttaagggccctatagcagctgggtgcgcaggcgcc

Beta globin intron

acgcgtgggtgctggtgagaaaaagtgctctggggacaccaggccttccggctgggccctgccgtaccct
tgcgcacccacgaccactctttttcacgagacccctgtggtccggaaggccgacccgggacggcatggga

Xho I

gtgagctgtgatagcctcgagggtgtccagggcccagcaatgcccgatcccgcggcgcacctgccattct
cactcgacactatcggagctcccacaggtcccgggtcgttacgggctagggcgccgcgtggacggtaaaga

Zap70 ORF

Chapter 7

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