

*T cell deficiencies resulting from aberrant
pre-mRNA alternative splicing caused by
a novel splicing silencer hnRNP LL in an
ENU mutant mouse strain thunder*

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Statement

The research contained within this study was performed in the Immunogenomics Laboratory under the supervision of Dr. Gerard Hoyne and Professor Chris Goodnow. The data presented is my own work, with all contributions from others clearly stated in the acknowledgements and methods.

This thesis is dedicated to my father-in-law, Professor Dezheng Liang, who encouraged me to face challenges, but sadly passed away in my first year of PhD.

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Abbreviations

ACAD: activated cell autonomous death
AICD: activation induced cell death
ARS: activation responsive sequence
APCs: antigen presenting cells
BH: Bcl-2 homology
Bim: Bcl-2 interacting molecule
CD45R: CD45 restricted epitope
CFSE: Carboxyfluorescein succinamide ester
Csk: C-terminal Src kinase
DISC: death inducing signalling complex
DN: CD4-CD8- double negative cells
DP: double-positive cells
Egr3: Early growth response gene 3
ELISA Enzyme-linked immunosorbance assays
EMS: ethylmethane sulfonate
ENU: N-ethyl-N-nitrosourea
ES: embryonic stem cells
ESE/ISE: exonic/intronic splice enhancers
ESS/ISS: exonic/intronic splice silencers
FACS: Flow cytometry analysis
FADD: FAS-associated death domain protein
hnRNPs: heterogeneous nuclear ribonucleoproteins
Hnrp11: heterogeneous nuclear ribonucleoprotein L-like (protein semble: hnRNP LL)
IL-7/IL-7R: Interleukin-7/Interleukin-7 Receptor
ISP: immature single-positive
ITAMs: immunoreceptor tyrosine-based activation motifs
ITIM: inhibitory tyrosine immunoreceptor motifs
KH: hnRNP K homolog
MAPK: Mitogen activated protein kinase
PCD: programmed cell death
PKC: Protein kinase C

PTB: Polypyrimidine Tract Binding protein (hnRNP I)

PTP: protein tyrosine phosphatases

ROR γ t: orphan nuclear hormone receptor

RRM: RNA recognition motif

SCID: severe-combined immunodeficiency

SF: splicing factor

SHP1: SH2-domain-containing PTP1

SLE: systemic lupus erythematosus

SNPs: single nucleotide polymorphisms

snRNPs: small nuclear ribonucleoprotein particles

snRNAs: small nuclear RNAs

SR proteins: Serine/Arginine rich proteins

SSLP: simple sequence length polymorphism

TCR: T-cell antigen receptor

T_{FH}: T follicular helpers

Tregs: regulatory T cells

Tap2: Transporter of Antigen Presentation 2

ZAP70: ζ chain associated protein kinase of 70 kDa

Abstract

ENU mutagenesis screening is a phenotype-driven approach to identify genes in a nonbiased manner. Through this approach we identified a previously uncharacterised gene, *Hnrpll*, which is involved in nascent mRNA alternative splicing. *Thunder* strain carries a point mutation in the *Hnrpll* gene (407T->A) in its first RNA recognition motif (RRM) which changes its function in regulating nascent mRNA alternative splicing.

One of *Hnrpll* target genes is CD45 that undergoes alternative splicing in T cells depending on their development stages and activation status. We demonstrated that *Hnrpll*^{thu/thu} T cells fail to silence 3 variable exons of CD45 and result in constitutive expression of CD45RA, B, and C epitopes on the cell surface. Retroviral based expression of wild-type *Hnrpll* cDNA in the *Hnrpll*^{thu/thu} T cells compensates the effects of loss of function in the mutation. The mutated RRM1 domain remains the ability to bind the regulatory element of activation responsive sequence (ARS) within CD45 pre-mRNA but cripples the protein function by destabilising the proteins to unfold in a thermolabile sensitive manner.

We also found that *Hnrpll*^{thu/thu} mutation disrupts peripheral T cell subsets. *Thunder* mice have normal T cell development in the thymus but specifically lost naïve T cells in the peripheral lymphoid tissues, whereas memory T cells are not affected. *Hnrpll*^{thu/thu} naïve T cells can homeostatically proliferate but fail to persist for a long term *in vivo*, suggesting that *thunder* mutation influences naïve T cell longevity.

We observed that *Hnrpll*^{th^u/th^u} naïve T cells express lower level of IL-7Ra and lower Bcl-2, together with the stronger pro-apoptotic BimS isoform due to alternative splicing. This highlights the nonredundant role of the *Hnrpll* gene in regulating peripheral T cell homeostasis.

CD45 isoforms are widely used markers to distinguish naïve and memory T cell subsets. CD45 splicing does not account for the loss of naïve T cells in the *thunder* mice, however, CD45RABC shows stronger phosphatase activity than CD45RO when the amount of CD45 is dramatically reduced to 5% remaining on T cell surface, suggesting jointly regulation of CD45 catalytic activity by its expression and alternative splicing.

Immunological memory is the hallmark of the adaptive immune system. Through Affymetrix mouse all exon arrays, we found that hnRNP LL protein plays a critical role in controlling an extensive program of alternative splicing as naïve T cells differentiate to the memory cell fate. It acts as an either trans- or cis- acting factor in regulating multiple nascent mRNA alternative splicing. This study provides an unprecedented insight into the extent of alternative splicing in the generation of immune memory.

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~Chapter 1~

INTRODUCTION

1.1 ENU mutagenesis to screen immune regulators

The mouse has been an ideal genetic model for a long time, with many elegant approaches developed and tremendous resources set up especially since human and mouse genomic sequence were revealed. The similarity between mouse and human in genetics and biology provides golden opportunities in biomedical research. Among these, chemical mutagenesis is a powerful protocol to elucidate genome functions.

Immunity is composed of the innate and the adaptive responses, which are different in the speed and specificity of the reaction. The innate immune system include physical, chemical, and microbiological barriers and more usually encompass the elements of neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins which provide immediate host defence. Innate response is highly conserved across species. It provides important protection against pathogens in simple animals and interacts with adaptive immune system in higher animals to facilitate more efficient protection. Adaptive immunity is the hallmark of the immune system of higher animals, characterised with the immune homeostasis and immune memory. The major players are a spectrum of antigen-specific T lymphocytes and B lymphocytes composing the immune repertoires whose overall numbers are kept constant. Whereas the innate response is rapid but sometimes damages normal tissues due to lack of specificity, the adaptive response is precise, but takes several days or weeks to develop the primary reaction. However, upon second encountering the same antigen, the adaptive response has immune memory to develop more vigorous and rapid response, although not immediate (Janeway, C.A. et al. 2006).

Despite almost half a century's of research in cellular and molecular immunology, many important questions have not been completely answered. For example, how is the number of immune cells so tightly and precisely regulated during the course of immune responses? How does the immune system control the fate of autoreactive lymphocytes and resting lymphocytes in unstimulated animals? What controls the lifespan of different lymphocyte subsets and contribute to the longevity of memory T cells versus naïve T cells? Given the functional complexity of the immune system and the fact that there are much larger numbers of proteins than that of the genes encoding them because of a process of post-transcriptional modification termed pre-mRNA alternative splicing, how does alternative splicing control immune functions? To answer these questions, ENU mutagenesis is a powerful approach to provide ideal mouse models enabling us to study genes regulating immune functions.

1.1.1 Genomic approaches to identify immune regulatory genes

Two genomic approaches have been widely applied in biomedical researches for many years. Gene knock out (KO) technique establishes the base of reverse genetics studies by completely or partially deleting target gene(s). It is readily applied to study the pattern of the gene expression and the functions of the protein encoded by a known gene (Nelms, K.A. & Goodnow, C.C. 2001). One limitation of gene knockout approach is the possibility of embryonic lethality in some situations. The gene knockout may affect the breeding potential of mice and they may not produce viable progeny. On the other hand, some knockout animals show no phenotypes due to compensation by other related proteins. Forward genetics based on ENU mutagenesis

has obvious advantages in identification of immune regulatory genes. Firstly, it is very efficient in inducing a mutation in a given locus at the rate of approximately one in every 1000 sperm and a spread of point mutations every 1-2 Mb throughout the genome (Hitotsumachi S. et al. 1985; Lyon, M. F. & Morris, T. 1966; Beier, D. 2000; Chen, Y. et al. 2000; Concepcion, D. et al. 2004; Coghill, E. L. et al. 2002; Kile, B.T. and Hilton, D, J. 2005). Secondly, as there is no prior assumption of the roles of a gene in a particular trait, it generates non-biased observations. Thirdly, ENU is a germline point mutagen and affects single loci with the mutation that can be inherited by the offsprings. Most importantly, it generates new alleles of known proteins and this may provide further insights into the functional role of a protein in a given biological function.

Bruce Beutler's group and our laboratory have identified a series of ENU mutations in novel genes or with novel alleles of known proteins governing different aspects of immunity e.g. Myd88, Lps2, CD14, CD36, TLR2, 4, 6, 7, 9, TNF- α etc (Hoebe, K. and Beutler, B. 2006; Hoebe K, and Beutler B. 2008.) and ZAP70, Slp76, Ikaros, Carma1, p53, NF- κ B2 (Jun, J.E. et al. 2003; Miosge, L.A. et al. 2002; Papathanasiou, P. et al. 2003; Vinuesa, C.G. et al. 2005; Siggs O.M, et al. 2007; reviewed by Cook, M.C. 2006). Details are summarised in table 1.1 and table 1.2.

1.1.2 ENU mutagenesis

N-ethyl-N-nitrosourea (ENU) is a chemical mutagen that causes random point mutations in a variety of organisms firstly reported 3 decades ago (Russell, W.L. et al 1979). The ethyl group can be transferred to some reactive oxygen or nitrogen

Table 1.1 Identification of TLR-deficient germline mutant mice (Hoebe K, and Beutler B. 2008.)

Locus	Allele	Dominant/recessive	Mutation characteristic
Unc93b1	<i>3d</i>	Autosomal recessive	1273 T → C transition of the <i>Unc93b1</i> gene, in exon 9 of 11 total exons
Cd36	<i>Oblivious</i>	Autosomal recessive	1283 T → A transversion of the <i>Cd36</i> gene. Results in a premature stop codon
Cd14	<i>Heedless</i>	Autosomal recessive	1013 C → T transition of the <i>Cd14</i> gene, creating a premature stop codon
Tlr2	<i>Languid</i>	Autosomal recessive	487 Asn → Ile of the LRR module in the TLR2 protein
Tlr4	<i>Lps3</i>	Autosomal codominant	709 Asp → Val of the TLR4 protein, affecting the BB loop of the TIR domain
Tlr6	<i>Insouciant</i>	Autosomal recessive	327 Val → Ala of TLR6
Tlr7	<i>Rsq1</i>	X-linked recessive	68 Thr → Ile of TLR7, located within the first LRR
Tlr9	<i>CpG1</i>	Autosomal codominant	499 Leu → Pro of the TLR9 protein (within the sixteenth LRR module of the ectodomain)
	<i>CpG2</i>	Autosomal codominant	985 Gln → Leu of the TLR9 protein, which lies in α -helix D of the TIR domain
	<i>CpG3</i>	Autosomal recessive	214 Val → Glu of the TLR9 protein, which lies in the predicted sixth LRR module of the TLR9 ectodomain
Trif/Ticam-1	<i>Lps2</i>	Autosomal codominant	deletion of a G at position 2258, causing a frameshift deleting 24 a.a. and replacing with 11 unrelated amino acids
MyD88	<i>pococurante</i>	Autosomal recessive	179 Ile → Asn, which exists near the center of the $\alpha\alpha$ -helix of the MyD88 TIR domain
	<i>lackadaisical</i>	Autosomal recessive	116 Tyr → Cys of MyD88 in the intermediate domain between the death domain and the TIR domain
Tirap	<i>torpid</i>	Autosomal recessive	a splice site mutation, the last 6 C-terminal amino acids of Tirap replaced by 3 unrelated residues
Irak-4	<i>otiose</i>	Autosomal recessive	327 Ile → Thr, located in subdomain 7 of the kinase domain, outside of the ATP-binding site
Tnf	<i>Panr1</i>	Autosomal dominant	138 Pro → Thr substitution in one of the α -helical elements and exposed at the surface of the molecule

Table 1.2 Summary of novel alleles identified through ENU mutagenesis by C.C. Goodnow's laboratory

Strain	Mutation	Gene	Phenotype
<i>Plastic</i>	Missense	<i>Ikaros</i>	T-cell lymphoma (dominant); aplastic anaemia (homozygotes)
<i>Xander</i>	Stop	<i>Nfkb2</i>	Reduced follicular B cells; disrupted splenic architecture
<i>Unmodulated</i>	Missense	<i>Carma1</i>	Increased IgM on peripheral B cells; defective polysaccharide antibody response; dermatitis and hyper IgE
<i>Sanroque</i>	Missense	<i>Roquin</i>	Lupus; increased T _{FH}
<i>Aire</i>	Missense	<i>Aire</i>	Type 1 diabetes; central tolerance
<i>Murdock</i>	Missense	<i>Zap70</i>	Decreased TCR signalling and thymic selection
<i>Mrtless</i>	Missense	<i>Zap70</i>	Immune deficiency;
<i>thunder</i>	Missense	<i>Hnrpll</i>	T cell homeostasis; pre-mRNA alternative splicing

radicals of purine or pyrimidine, causing mistaken identity through alkylation of nucleic acids and resulting in mispairing during DNA replication in both somatic and germ cells (Figure 1.1.1) (Singer, B. and Dosahjh, M.K. 1990). It was recommended that an effective $3 \sim 4 \times 100 \text{mg/kg}$ dose is tolerated well by several inbred mouse strains (Russell, L.B. et al 1982a, 1982b; Justice, M.J. et al 2000). Pre-meiotic spermatogonial stem cells have the highest mutation rates with single locus mutation frequencies of $\sim 1 \times 10^{-3}$, about 100 fold higher than spontaneous mutation rate (Hitotsumachi, S. et al 1985, Justice, M.J. et al. 1999). After a sterile period of about 10 weeks, the males regain fertility, allowing males to sire mutant offspring for extended period. A dominant trait can be recovered in a single generation with visible phenotypes or detectable changes, while recessive trait requires 3 generations breeding to pick up the homozygous (Favour, J. & Neuhauser-Klaus, A. 2000, Nelms, K.A. & Goodnow, C.C. 2001).

An ENU treated male mouse was bred with wild-type female to generate multiple mutation carrying G1 offsprings. Each G1 mouse was used as a founder of a pedigree, backcrossed to wild-type, and paired with its G2 daughters potentially carrying the same mutation. For a recessive trait we would expect 25% of G3 offspring to be homozygous mutants expressing specific phenotypes.

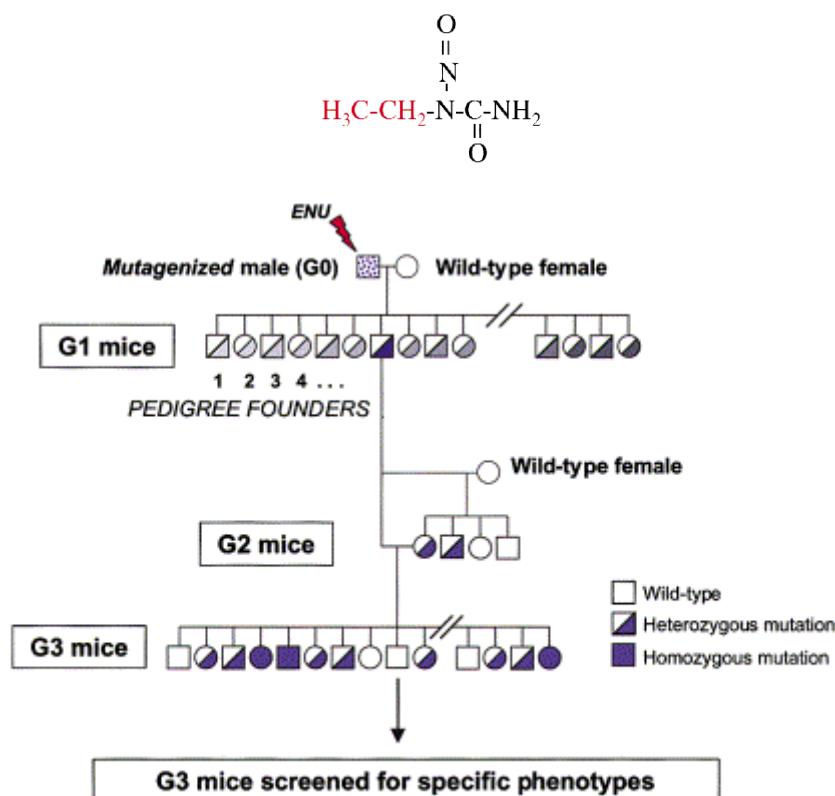


Fig. 1.1.1 Top: Chemical structure of ENU. Transferable ethyl group is highlighted in red. Bottom: A typical 3 generation screening pedigree for a recessive trait. (From Nelms, K.A. and Goodnow, C.C. 2001).

1.1.3 Phenotype screening in immune system

To identify genes involved in lymphocyte development and homeostasis, phenotypic screens can be performed routinely on peripheral blood samples. It is a convenient cost-efficient method to carry out large scale and high throughput analyses on multiple samples. The success of an ENU screen is underpinned by having a reliable and robust screening assay that can be used to measure quantitative and qualitative changes in cellular and humoral immunity under steady state conditions or following an immunogenic challenge.

Flow cytometry analysis (FACS) provides accurate and rapid detection of immunological cell populations and expression of both cell surface and intra-cellular proteins with a range of fluorescent antibodies (Ab). It is now possible to simultaneously measure 12 different cell surface markers with the development of new fluorochromes. This multi-parameter approach can identify either qualitative or quantitative changes in immune cell development and regulation that affect the adaptive and innate immune system.

Enzyme-linked immunosorbance assays (ELISA) can be used to measure changes in the basal levels of immunoglobulin isotypes in the serum. Any changes in basal antibody levels could indicate a defect in B cell differentiation. Alternatively ELISA assays can be used to measure the production of antigen specific antibodies following immunization. Defects in T cell help and/or B cell signalling could influence the production of immunoglobulin isotypes and the ability of B cells to undergo isotype switching. Finally ELISA assays can be used to measure the presence of auto-antibodies in the serum that can help identify defects in immune tolerance. Mutations that affect tolerance mechanisms could lead to the activation of autoreactive T cells and B cells and predispose to the development of systemic autoimmune diseases such as systemic lupus erythematosus (SLE) where high affinity antibodies are generated to nuclear and cytoplasmic self proteins as well as DNA and RNA.

Once a phenotype is observed, mice carrying the variant phenotypes are used for breeding to establish the heritability of the phenotype. When heritability is confirmed, further breeding is required to establish the mode of inheritance (dominant or

recessive), and then a mapping cross needs to be setup to identify the gene mutation which will be discussed later.

1.1.4 Spectrum of ENU-induced mutations

A/T base pairs are the most frequently affected by ENU, with 87% of 62 pre-meiotic germline mutations (44%A/T->T/A transversions, 38%A/T->G/C transitions, 5%A/T->C/G transitions) in contrast with only 13% G/C base pairs modified (Justice, M.J. et al 1999). This effect is possibly due to alkylated O4- or O2-thymidine. Those ENU mutations that have phenotypic effects are 64% missense, 10% nonsense, and 26% splicing errors. By contrast, spontaneous mutations in mouse germline reporter assays show approximately equal chance of A/T and G/C mutation rate, with 38% A/T->T/A transversions and 37% G/C->A/T transitions regardless of pre- or post-meioses (Noveroske, et al. 2000). A/T base pair preference in ENU mutagenesis is confirmed by Sakuraba and colleagues. They identified 143 mutations by sequencing approximate 200 Mb genomic DNA and found that 67.6% of the mutations hit A/T sites, despite 52.7% A/T content in the sequenced region (Sakuraba, Y. et al 2005). ENU treatment yields a new loss of function allele in any given gene, on average , once per 1000 gametes and therefore screening 10,000 first generation (G1) offspring should yield at least one heterozygous loss-of-function mutation in the majority of immune regulatory genes (Hitotsumachi, S. et al. 1985, Lyon, M.F. & Morris, T. 1966).

Recently Barbaric and co-workers assembled published phenotypic screens and found that functional ENU mutations tend to be more frequently found in genes that

encode large proteins and that contain large numbers of exons. The majority of ENU induced point mutations are located in coding sequence and splice sites, and surprisingly, tend to have higher %G+C content (54%) in the coding sequence compared to 45% for the average of all genes in the entire genome (Barbaric, I. et al. 2007). Longer coding length provides a larger target for missense or nonsense mutations while more exons means more splice site targets to be mutated, but it is still not clear if G+C content in the coding sequence correlates to mutation preference.

1.1.5 Mapping and sequencing of ENU induced mutations

As opposed to the functional mutation spectrum, several groups have reported that unselected (underlying) mutation rate induced by ENU ranges from 1 base per 1.9 Mb (Coghill, E.L. et al. 2002) to 1 base per 1.0 Mb (Quwailid, M.M. et al. 2004) by DNA based screening of 2000-6000 generation 1 (G1) mice. To find the functional point mutation, the mouse carrying the variant genes is intercrossed or backcrossed to a different inbred mouse strain. This provides DNA polymorphisms between the mutation-bearing chromosomes and those from the mapping partner strain that can be distinguished at the molecular level by single nucleotide polymorphisms (SNPs) or simple sequence length polymorphism (SSLP) (Silver, L. 1995). Meiotic mapping is then used to identify which chromosomal location segregates with the mutant phenotype of interest in F2 intercross or N2 backcross offspring.

Mapping normally starts with a genome scan testing 2-3 markers on each chromosome in a group of 10-20 phenotypically mutant mice. Theoretically, the more frequently a marker is associated with the mutant mice, the closer the mutation is to

the loci of the marker. Fine mapping with higher resolution then enables to narrow down the chromosome interval by testing more affected mice with a panel of molecular markers in the region to find out informative meiotic recombination events. The chromosomal linkage is easy to distinguish if the mutant mice have clearly different phenotypes from wild type.

Sequencing of genes within an interval to identify the causative mutation must take into consideration of the length of the chromosomal interval, the number of candidate genes in the region, the availability of extra polymorphic markers, and the likelihood of more recombinations. Candidate genes can be picked up by checking if the function of the gene in the interval or its family members in the same biochemical pathway is associated with a similar phenotype, or by lymphocyte-specific expression pattern of the gene. Given the fact that most ENU mutations identified so far locate in protein coding region or exon-intron boundaries, it is efficient to sequence only these regions.

1.1.6 Recent progresses and future prospects

Whole-animal vs. ES mutagenesis, Phenotype-driven vs. gene-driven screen:

As an alternative to whole-animal ENU mutagenesis, chemical treatment with ENU or ethylmethane sulfonate (EMS) of embryonic stem (ES) cells provides an alternative way to generate mutations in genes of interest. This enables a gene-driven dissection of gene functions, as opposed to a phenotype driving strategy. Cultured ES cells can be inexpensively screened for phenotypes, although this is limited to those

processes that can be interrogated in tissue culture and to heterozygous defects (Chen, Y. et al 2000, Munroe, R.J. et al 2000, Vivian, J.L. et al 2002).

Insertional mutagenesis and gene trapping:

The majority of ENU mutations are single base pair substitution, with a few small deletions reported (Shibuya, T. and Morimoto, K. 1993), while insertional mutagenesis provides an alternative by interrupting gene function through integration with plasmid or retroviral constructs. Transposon-mediated mutagenesis has been achieved in mouse ES cells and in the mouse germline (Luo, G. et al 1998; Ivics, Z. & Izsvák, Z. 2005; Ding, S. et al. 2005). However, this approach has specific requirements such as transgenic mice able to harbour mobile elements like transposons and limited expression of transposonase in the sperm stem cells and their progeny. The mutation is then tagged with a transposon, so that it is easier to identify the insertionally mutated gene by inverse PCR (polymerase chain reaction). Like knockout mice, transposon mutagenesis creates primarily null alleles, as opposed to the missense mutations in discrete domains typically resulting from ENU mutagenesis.

1.2 T cell development

B and T lymphocytes play the major role in the adaptive immune system of mammals. Despite similarities between the two populations, they are distinct in many biological aspects. Contrasted with B cell's continual production in the bone marrow (BM) throughout the life, mature T cells are generated in the thymus in large numbers in the juvenile and kept in a relatively constant level after the thymus involutes by homeostatic division of mature T cells in adults. Although the T cell and B cell progenitors both originate from common lymphoid progenitor cells in the bone marrow, T and B cells undergo rearrangements of the antigen receptor genes that allows them to express a clone specific receptor and during their development the receptors are tested for binding to self antigens and depending on the affinity of the receptor for self antigens these cells will be allowed to finish their maturation known as positive selection, or alternatively cells with a receptor with high affinity for self antigens will be induced to die through a process termed negative selection. The work presented in this thesis will focus on T cell development, particularly $\alpha\beta$ T cells.

Immature T cells follow a well-defined programme of development in the thymus characterised by the expression of a variety of specific cell surface molecules driven by T-cell antigen receptor (TCR) gene rearrangement. The change in surface marker expression includes the TCR:CD3 antigen receptor complex and CD4 and CD8 co-receptors (Figure 1. 2.1).

Haematopoietic progenitor cells that migrate from the bone marrow and enter the thymus express c-kit (CD117, receptor for stem cell factor SCF), CD127 (interleukin-7 receptor α chain, IL-7R α), and lack rearranged TCRs and T cell co-receptors CD4 and CD8. On colonizing the thymus they are termed CD4-CD8- double negative cells (DN) (Ciofani, M, and Zúñiga-Pflücker J.C. 2007). They represent approximately 5% total thymocytes and give rise to the major subset of $\alpha\beta$ T cells and the minor subset of $\gamma\delta$ T cells. DN cells begin to express T cell specific molecule Thy1 in mouse about one week after their relocation in the thymus and interaction with thymic stroma. Thy1⁺ DN cells can be subdivided into four phases on the basis of the expression of adhesion molecule CD44 and Interleukin-2 receptor α chain, CD25: DN1, CD44^{hi} CD25⁻; DN2, CD44^{hi} CD25⁺; DN3, CD44^{lo} CD25⁺; DN4, CD44^{lo} CD25⁻. DN3 cells lose expression of kit and start to rearrange TCR β chain (Jackson AM, and Krangel MS. 2006; Zediak VP, et al. 2005). Only those making productive TCR β chain rearrangement can survive and proceed to DN4. DN4 cells subsequently become immature single-positive (ISP) cells that transiently express small amounts of CD8. The importance of immature single-positive cells is unclear, but it seems that most cells rapidly upregulate CD4 and enter the next stage of double-positive (DP) CD4⁺CD8⁺ population that constitutes the majority of thymocytes (Hayday, A.C. and Pennington, D.J. 2007).

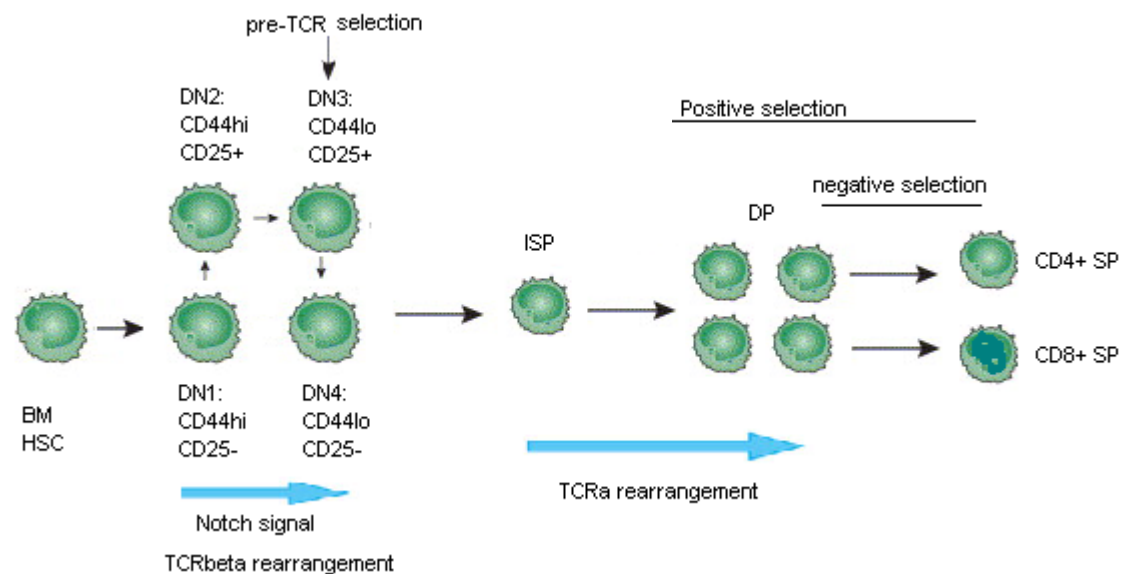


Figure 1.2.1 Schematic view of T cell development within the thymus.

The rearranged TCR β chain initially pairs with the pre-T-cell receptor α chain (pT α), forming a complete pre-TCR that associates with CD3 as a complex expressed at the cell surface. Signals from the pre-TCR/CD3 complex lead to cell proliferation and expression of both CD4⁺ and CD8⁺ coreceptors to become CD4⁺CD8⁺, double positive (DP) cells. At this point the TCR α chain locus undergoes rearrangement and only if the rearrangement is productive can a mature TCR $\alpha\beta$ receptor be formed. DP cells stop proliferating and become small resting DPs, initially with low TCR expression at the cell surface.

Pre-TCR $\alpha\beta$ serves as a surrogate for TCR α at an early stage of thymocyte development (DN3) before rearrangement at the TCR α locus. pT α has only a single extracellular Ig-domain without the variable region and a larger cytoplasmic domain in contrast with mature TCR α , which are associated with pre-TCR signalling (Groettrup M, et al. 1993; Saint-Ruf C, et al. 1994; Borowski C, et al. 2004; Aifantis I, et al. 2002). Structural models of pre-TCR $\alpha\beta$ and TCR $\alpha\beta$ are shown in Figure 1.2.2.

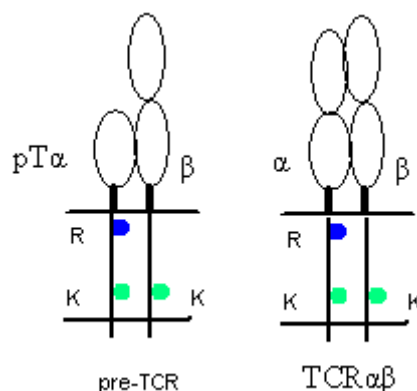


Figure 1.2.2 Structure models of pre-TCR and TCRαβ.

Heterodimeric pre-TCR and TCRαβ both have conserved transmembrane regions with charged basic residues highlighted (R in blue and K in green). Pre-Tα has a single extracellular Ig fold domain whereas TCRαβ has two Ig domains. The larger cytoplasmic domain of pTα is not shown here (Call, M.E. & Wucherpfenning, K.W. 2005).

In accordance with its extracellular structure, pre-TCR signalling is mediated by ligand-independent oligomerisation of pre-Tα chains, leading to constitutive internalisation of the pre-TCR complexes (Irving, B.A. et al. 1998; Yamasaki, S. et al. 2006). It then induces transient expression of the transcription factor Egr3 (Early growth response gene 3) which is a member of Egr family and leads to the upregulation of Id3, a RORγt (orphan nuclear hormone receptor RORγt) inhibitor. This results in a rapid proliferation and expression of CD4, CD8 coreceptors on the thymocytes. Transient expression of Egr3 establishes a period of proliferation in response to pre-TCR signals by inhibition of RORγt expression and RORγt function (Xi, H. et al. 2006). RORγt is an isoform of RORγ that is predominantly expressed in lymphocyte compartment and lymphoid tissue inducer cells. It promotes survival and inhibits proliferation of DP thymocytes (Shao, H. et al. 1997; He, Y.W. et al. 1998; 2002; Sun, Z. et al. 2000). E2A, a member of E proteins that are basic helix-loop-

helix (bHLH) containing transcriptional regulators, encodes both E12 and E47 isoforms and is inhibited by Egr1 (Bain, G. et al. 1997, 1999, 2001). E proteins can positively regulate ROR γ t expression, resulting in cell cycle arrest, TCR α gene rearrangement and survival of resting DP cells by inducing expression of Cpeb4, RAG-2 and Bcl-XL, respectively (Xi, H. et al. 2006; Moisse, L. & Zamoyska, R. 2007). Signal transduction by the pre-TCR is shown in figure 1.2.3.

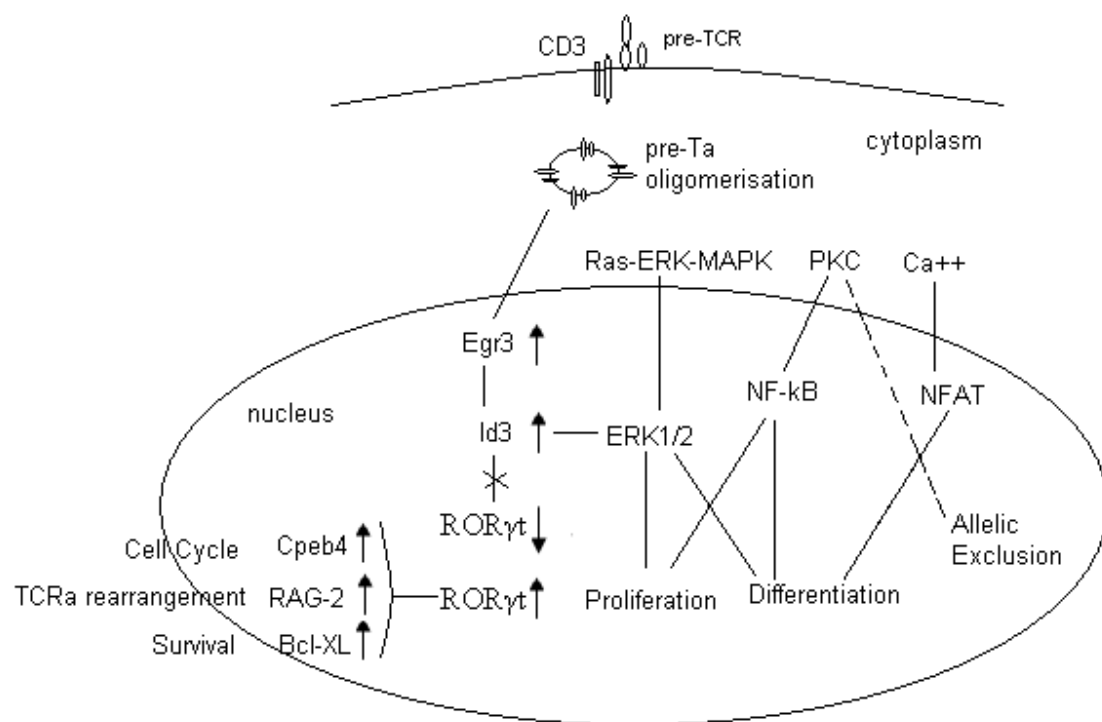


Figure 1.2.3: pre-TCR signal transduction

Over the last decade, several key transcription factors have been identified playing essential roles in pre-TCR responses (Michie, A.M. and Zúñiga-Pflücker J.C. 2002). Among them NF- κ B and NFAT are essential for mediating pre-TCR signalling and thymocyte proliferation and differentiation (Aifantis, I. 2001, 2002, Voll, R.E. 2000) as well as their roles in mature T cell activation with TCR stimulation. NFAT and NF- κ B are central coordinators of adaptive immune system with broad roles in regulating

gene expression of numerous cell types during proliferation and apoptosis, inflammation and tumour genesis (Michie, A.M. and Zúñiga-Pflücker J.C. 2002; Karin, M. and Greten, F. R. 2005). Other transcriptional factors include transcription factors T cell factor-1 (TCF-1)/lymphocyte enhancer factor-1 (Lef-1) in the Wnt pathway via β -Catenin (Staal, F.J. et al. 2001), and Id proteins (Engel, I. and Murre, C. 2001).

Several studies have highlighted the important role for MAPK signalling in pre-TCR function on immature thymocytes (Crompton, T. et al. 1996, Swat, 1996, Michie, A.M. et al. 1999). It was also shown that E2A and Id3 are regulated by the Ras-ERK MAPK cascade (Bain, G. et al. 2001). In addition the pre-TCR can stimulate the mobilization of intracellular $[Ca^{2+}]_i$ and activation of a similar range of downstream effector proteins including NF- κ B and NFAT (Aifantis, I. et al. 2001, 2002, Voll, R.E. et al. 2000, Sommers, C.L. et al. 2002, Aguado, E. et al. 2002).

Pre-TCR signals inhibit further TCR β rearrangement through allelic exclusion, induce cell proliferation, and drive the thymocytes into DP stage in which the majority express mature TCR $\alpha\beta$ because of TCR α rearrangement. Most of those do not recognize self-peptide:MHC complexes and die from “neglect” in the thymic cortex. Those with high affinity TCRs for self-peptide presented by medullary epithelial and dendritic cells are deleted by apoptosis. Only those expressing a functional TCR $\alpha\beta$ receptor with intermediate affinity can survive, undergoing positive selection and mature to either CD4⁺CD8⁻ (MHC II restricted) or CD4⁻CD8⁺ (MHC I restricted) single positive cells (SP) (Hogquist, K.A. et al. 2005; Holländer G, et al. 2006; Hayday, A.C. & Pennington, D.J. 2007).

TCR signal transduction is critical for clonal selection in the thymus and for clonal expansion of antigen-specific cells in the periphery (Davis, M.M. et al., 1998). However, TCR molecules have only a very short intracellular domain that is insufficient to transmit signals into cytoplasm. TCR $\alpha\beta$ expression on T cells depends upon the association with CD3 $\gamma\epsilon$, CD3 $\delta\epsilon$, and CD3 $\zeta\zeta$ signalling subunits (Wegener, X. et al., 1995; Call, M.E. and Wucherpfenning, K.W. 2005). The intracellular domains of the CD3 chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) that function as the nucleating point for the intracellular signal transduction machinery upon TCR ligation to the peptide/MHC complex on antigen presenting cells (APCs) (Kane, L.P. et al. 2000). The CD3 δ , γ , and ϵ chains each contain one ITAM, and CD3 ζ contains three ITAMs (Figure 1.2.4).

TCR on thymocytes and mature T cells activates three major signalling pathways: 1. Mitogen activated protein (MAP) kinase pathways (ERK, Jnk, p38); 2. Intracellular calcium mobilization [Ca^{++}]i; and 3. Protein kinase C (PKC). TCR $\alpha\beta$ stimulation activates a cascade of tyrosine phosphorylation which involves: 1. Lck-mediated recruitment and activation of ZAP-70/Syk; 2. ZAP-70/Syk-mediated phosphorylation of LAT, SLP-76, and PLC γ 1; 3. recruitment of adaptor molecules with guanine nucleotide exchange proteins to LAT and SLP-76; and 4. subsequent Ca^{2+} mobilization, PKC activation and Ras activation followed by MAPK activation (Michie, A.M. and Zúñiga-Pflücker JC. 2002). Figure 1.2.5 shows the current understanding of proximal TCR signal transduction.

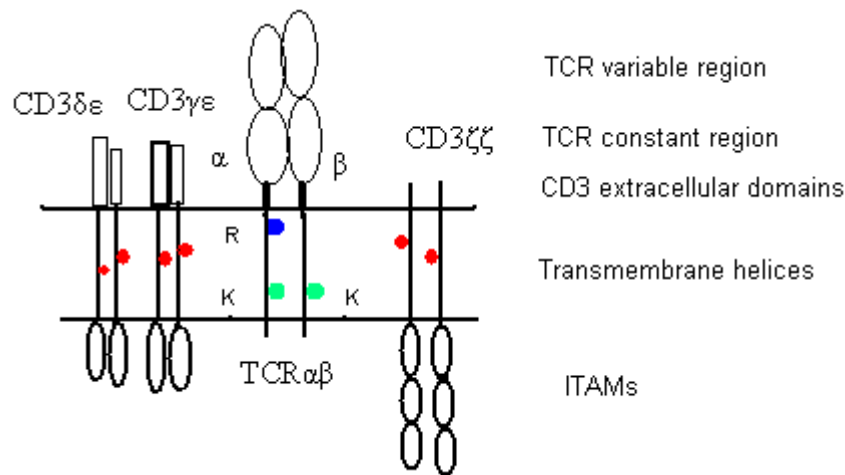


Figure 1.2.4 Structural model of TCR:CD3 complex

The “anatomy” of $\alpha\beta$ TCR/CD3 shows extracellular, transmembrane, and intracellular regions. The extracellular domains contain TCR variable region which is the antigen binding site and constant regions that may have some interaction with CD3 extracellular domains. Acidic residues in the CD3 dimer transmembrane regions highlighted in red have ionisable interaction with the basic residues in the TCR transmembrane residues in blue, which contributes to the formation of the complex. The two acidic-one basic electrostatic interactions are the driving forces to form TCR $\alpha\beta$:CD3 complex (Kuhns, M.S. et al, 2006).

The fate of a DP cell is dependent on the affinity of the TCR $\alpha\beta$ it expresses. If the TCR has no affinity for self-peptide/MHC complexes presented on the surface of cortical epithelial cells they will die by neglect. Low –medium avidity of the TCR will promote positive selection whereas a high affinity interaction of the TCR with self-peptide/MHC results in clonal deletion (Jameson, S.C. et al 1995). Thus in the same population of cells the TCR can transmit signals that can lead to distinct outcomes, i.e. survival and differentiation or cell death depending on the nature of the interaction between the TCR with peptide/MHC ligand. Studies have shown that positively and negatively selecting peptides can induce both JNK and p38 signalling with similar kinetics. In contrast positive selection leads to a slow but sustained activation of ERK signalling, whereas negative selection coincides with a intense and

transient burst of ERK signalling which precedes activation of p38 & JNK (Bommhardt, U. et al. 2000, Mariathasan, S. 2001). The kinetics of activation of the various MAPKs may induce a specific set of transcription factors and this could have an important outcome of survival versus apoptosis (Swan, K.A. et al 1995, Alberoll-Ila, J. et al. 1996, O'Shea, C.C. et al. 1996, Pages, G. et al. 1999, Dower, N.A. et al. 2000, Rincon, M. et al. 1998, Sugawara, T. et al. 1998; Dong, C. et al. 2000, Gong, Q. et al. 2001, Takagaki, Y. et al. 2001, Mora, A.L. et al. 2001),

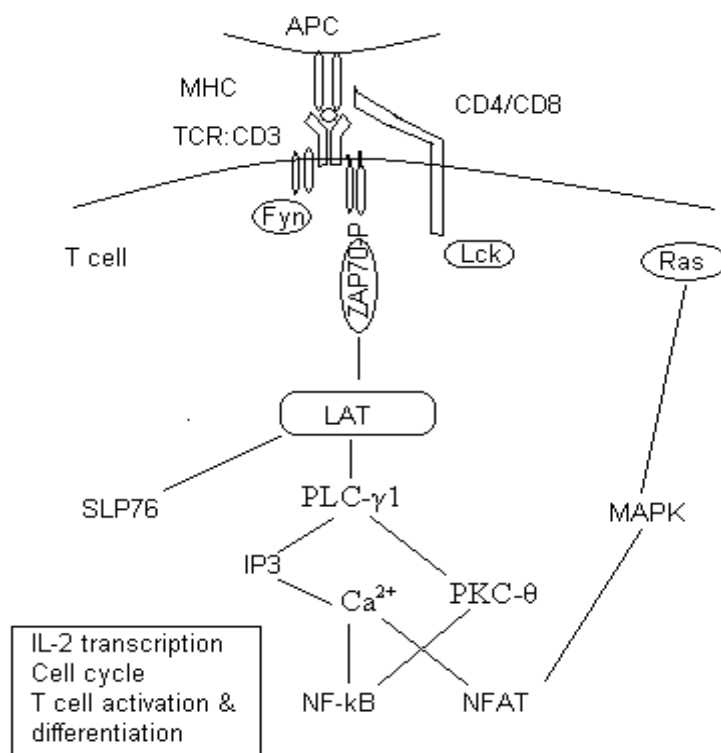


Figure 1.2.5 TCR signal transduction pathways

Following TCR engagement, key transcription factors NF-κB and NFAT are activated through intracellular calcium mobilization, MAPK and PKC. This initiates the expression of a set of genes including IL-2, resulting in the entry of cell cycle and T cell activation (Abraham, R.T. and Weiss, A. 2004).

The linker for activation of T cells (LAT) protein helps to recruit the Grb2 protein

and this in turn help in activation of RAS and ERK signalling. Mutations in Lat and Grb2 or deficiencies in Jnk and p38 can lead to autoimmunity as a result of failure in negative selection of autoreactive thymocytes indicating that each of these signalling components downstream of the TCR play critical roles in negative selection (Genton, C. et al. 2006; Salojin, K. et al. 1997; Johnson, G.L. & Lapadat, R. 2002). The transcription factors Nur77 and NOR-1 are both induced in thymocytes in response to TCR triggering and calcium mobilization and both appear to be important in clonal deletion of autoreactive thymocytes. The expression of a dominant negative version of Nur77 blocks thymocyte apoptosis (Calnan, B.J. et al. 1995, Lee, S.T. et al. 1995), while transgenic expression of NOR1 in thymocytes leads to unbridled apoptosis (Cheng, L.E. et al. 1997). Bim is a member of the Bcl2 protein family that plays a critical role in inducing apoptosis in thymocytes (Marsden, V.S. & Strasser, A. 2003). Bim deficient mice have defective negative selection but also mature T cells fail to undergo activation induced cell death (Bouillet, P. et al. 2002).

1.3 T cell activation and differentiation

Macfarlane Burnet's clonal selection theory is the central principle of adaptive immunity, not only to explain the generation of Ab specificity, but also the fate of T cells bearing specific TCRs for antigen. When a naive T cell encounters its specific foreign Ag presented by antigen presentation cells (APCs), it is activated and starts to proliferate, generating a clone of identical progeny all with the same binding specificity. The progeny proliferate and differentiate into effector cells to eliminate the Ag. The cell numbers decrease in contract phase and a proportion of the cells remain as memory T cells to retain immunological memory.

In contrast with B cells recognizing extra-cellular antigen, T cells detect intracellular Ag generated inside infected cells or digested by professional APCs. Foreign Ag must be presented to T cells as peptide fragments bound to MHC on the surface of APCs, providing the first signal of TCR binding together with the costimulatory signals mediated by interactions of CD28 on T cells and CD86/CD80 on APCs or other co-stimulatory signals. Too strong or too weak signals cause death of the T cell. MHC-I molecules collect peptides derived from synthesized protein in the cytosol and display fragments of viral proteins on the surface so that they can be taken up by cytotoxic CD8 T cells (Pamer, E. & Cresswell, P. 1998; Cresswell, P. et al. 2005). MHC-II molecules bind peptides derived from exogenous proteins internalized and degraded in intracellular vesicles of macrophages and B cells, and present them to CD4 cells (Menéndez-Benito V, Neefjes J. 2007). MHC-I restricted Ag cross-presentation of DCs to CD8 T cells provides important pathways in maintaining tolerance and protective immunity (Rock, K.L. and Shen, L. 2005).

1.3.1. TCR signal pathways in T cell activation

When a mature T cell binds to specific peptide-MHC, co-receptor CD4 or CD8 clusters to the TCR:CD3 complex. Src family protein tyrosine kinases (PTKs) p56Lck and p59Fyn are associated with the cytoplasmic domains of CD4/CD8 and CD3 ζ chain respectively, and their clustering activates them to phosphorylate the ITAMs of CD3 ϵ and ζ chain. Doubly phosphorylated ITAMs create the docking site for ZAP70 (ζ chain associated protein kinase of 70 kDa). Lck also activates ZAP70 by phosphorylation. Activated ZAP70 then activates signal pathways through PLC- γ , GEF, and Tec kinases, and ultimately leads to the induction of new gene synthesis including IL-2 by activating transcriptional factors NF κ B, NFAT and AP-1 (Gallo, E.M. et al. 2007; Sundrud MS, Rao A. 2007).

A wide range of factors can modulate TCR signals. First of all, the affinity of peptide-MHC complex binding to TCR determines the signal strength; the antigen concentration and the duration of T cell and APC interaction also determine the amount of TCR engagement and the duration of signalling respectively (Iezzi G, et al. 1998; 1999). TCR signal is enhanced by co-receptor CD4/CD8 that binds the same ligand of MHC II/MHC I class molecule respectively and initiates Lck tyrosine kinase and ITAM (Denny MF, et al. 2000). Costimulatory molecules like B7.1 (CD80) and B7.2 (CD86) engage CD28, determining the rate of TCR triggering and signal amplification and playing a role in activating NF κ B to provoke survival and proliferation (Viola A, et al. 1999). In addition to MHC-TCR signalling, survival of

naïve T cells also requires a signal from cytokines, particularly IL-7 (interleukin-7) (Schluns, K.S. et al. 2000).

Following T-cell activation, several attenuating mechanisms operate to avoid over-activation. The TCR is internalized following ubiquitylation by c-Cbl (Casitas B-lineage lymphoma) and Cbl-b and degraded mainly in the lysosome (Wiedemann, A. et al. 2005; von Essen, M. et al. 2004). An alternative receptor for B7.1 and B7.2, CTLA4 (cytotoxic T-lymphocyte-associated antigen 4), is induced and phosphorylated on inhibitory tyrosine immunoreceptor motifs (ITIM) that recruit protein tyrosine phosphatases SHP1 (SH2-domain-containing PTP1) and SHP2 to dephosphorylate the CD3 ζ -chain, protein kinases and other substrates. CD45 has a dual effect to dephosphorylate either activating or inhibitory residues of Lck to activate or attenuate TCR signalling respectively (McNeill, R.J. et al. 2007). We will discuss this issue further in chapter 1.4 of T cell homeostasis.

1.3.2. Differentiation of activated T cells

When a T cell encounters its specific Ag, it undergoes clonal expansion and differentiation into effector or memory T cells with same TCR specificity.

Activated CD8 cells differentiate to cytotoxic T cells either alone or with the help of CD4 cells. Cytotoxic CD8 cells produce two broad classes of effector molecules, cytotoxins and cytokines, in addition to some membrane associated proteins like Fas ligand. Cytotoxins are stored in specialised lytic granules and include granzymes and perforin. Release of these granules is tightly focused at the site of cell contact and

triggers an intrinsic cell death program of the target cells. IFN- γ is the major cytokine produced by CD8 effector cells that can block viral replication. TNF- α and TNF- β from CD8 cells can deliver activating signals to innate immune system and have cytotoxic effects.

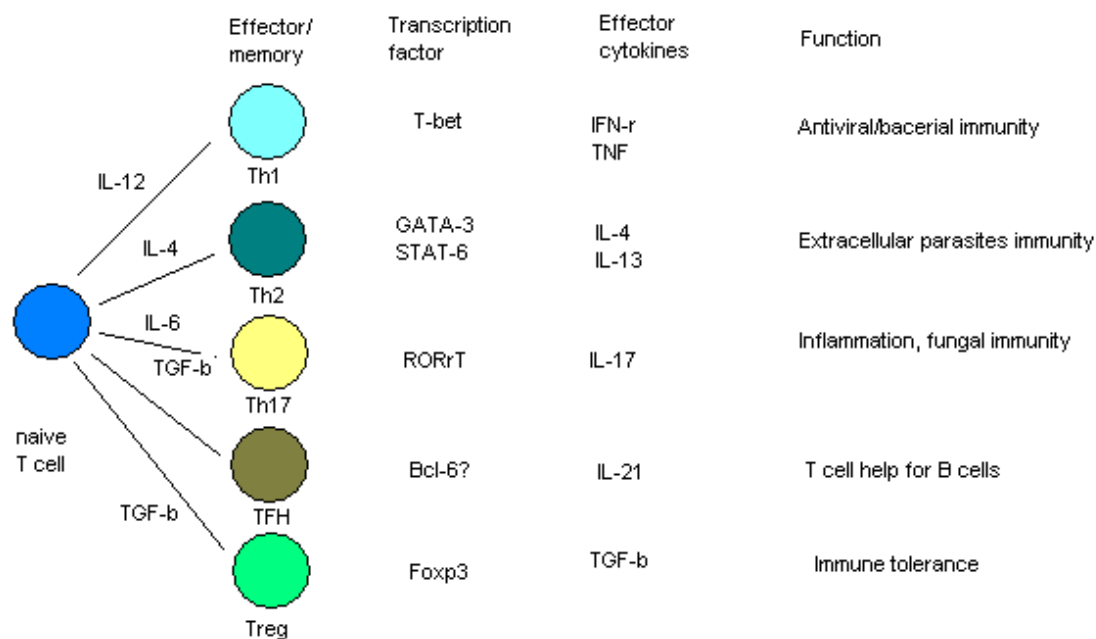


Figure 1.3.3 Program of T helper cell differentiation

Differentiation of memory/effector T cell subset from naïve CD4+ T cells is induced by specific cytokines secreted by innate immune system cells. Through the activation of specific transcription factors, T cells are programmed to differentiate into 5 subsets of effector cells with a spectrum of cytokine production.

CD4 cells can differentiate upon activation into T_H1 or T_H2 cells, which differ in cytokine production and regulate cellular and humoral immunity respectively, and more recently identified subpopulations including T follicular helpers (T_{FH}), induced regulatory T cells (Tregs), and T_H17 (Mosmann TR, Coffman RL. 1989; Tong, Q. & Bluestone, J.A. 2008; Dong, C. 2008; King C, Tangye SG, Mackay CR. 2008). Despite different cytokines produced by the innate immune system, it is not yet fully

understood what factors determine a proliferating CD4 T cell to differentiate into one or other subset of effectors.

T_H1 cell differentiation depends on the presence of IL-12. It expresses membrane-associated proteins (CD40 ligand and Fas ligand) and a range of soluble cytokines (IFN- γ , IL-2, TNF etc) conferring immunity to intracellular bacteria. IFN- γ secreted by T_H1 cells activate macrophages to kill engulfed bacteria; IL-2 induces T cell proliferation, increasing effector cell numbers and potentiating the release of other cytokines; IL-3 and GM-CSF stimulate the production of macrophages and granulocytes; TNF α and TNF β act on endothelium to recruit macrophages to the site of infection; a chemokine from T_H1 with macrophage chemotactic activity (CCL2) also causes accumulation of macrophages at the infection site (Mosmann, T.R. & Sad, S. 1996, Szabo, S.J. et al. 2003). The key transcription factor for T_H1 differentiation is T_{bet}, a member of T_{box} transcription factor family (Szabo, S.Z. et al. 2001).

Under the control of IL-4, T_H2 cell differentiation is important for T cell dependent B cell activation. Ag-binding B cells can present peptide:MHC complexes to the TCR of T_H2 cells, leading to expression of the B cell stimulatory CD40 ligand on T_H2 cells and the secretion of B cell stimulatory cytokines IL-4, IL-5, IL-6, IL-10, and IL-13, which in turn drive B cell proliferation and differentiation into Ab-secreting plasma cells (Glimcher, L.H. & Murphy, K.M. 2000). Gata3 is the master regulator for T_H2 cell differentiation (Zheng, W. & Flavell, R.A. 1997).

Recently a distinct IL-17 producing lineage of CD4 effector cells have been identified in mouse and human which is different from T_H1 and T_H2 cells in terms of

the cytokines they produce and the programmes governing their differentiation (Harrington, L.E. et al. 2006, Park, H. et al. 2005, Chen, Z. et al. 2006, Reiner, S.L. 2007). T_H17 differentiation depends on $TGF\beta$ and IL-6 (Bettelli, E. et al. 2006, Mangan, P.R. et al. 2006), not IL-23 as previously reported (Aggarwal, S. et al. 2003), although IL-23 is important for maintaining the T_H17 response. It was originally thought that T_H17 effector cells exclusively secrete IL-17 but new evidence appears that T_H17 cells can also secrete IL-22 (Zheng, Y. et al. 2007). T_H17 cells produce TNF but not $IFN\gamma$, which is different from T_H1 cells (Infante-Duarte, C. et al. 2000). T_H17 have critical roles in immunity to fungi and tissue inflammation. $ROR\gamma_t$ has been proposed to be the essential transcription factor for T_H17 differentiation (Ivanov II, et al. 2006).

Natural regulatory T cells differentiate in the thymus, while induced Treg cells differentiate in the periphery in response to antigen signals and in the presence of $TGF-\beta$. The master transcription factor of Treg cells is Foxp3 and the effector cytokines secreted are IL-10 and $TGF\beta$. Tregs have important immunomodulatory activity and play key roles in immune tolerance (Tang, Q. & Bluestone, J.A. 2008).

T_{FH} was named from their location or migration to the B cell follicles in the lymphoid tissues. T_{FH} cells are distinguishable from other T cell subsets by the expression of chemokine receptor CXCR5. They function to provide help to B cells which is essential to humeral immunity. The major effector cytokine generated from T_{FH} cells is IL-21 that potentially helps B cells to differentiate into Ab-forming cells (King, C., Tangye, S.G. & Mackay, C.R. 2008).

1.3.3 Memory T cells

Immunological memory is the ability of immune system to respond more rapidly and effectively to previously encountered pathogens, reflecting the persistence of clonally expanded Ag specific lymphocytes as “memory T cells”. Most effector cells die at the end of immune response, and the differentiation of memory cells is still poorly understood.

A linear pathway of memory commitment was proposed in 1999, suggesting sequential differentiation of naive T cells to effectors and then to memory T cells (Opferman, J.T. et. al. 1999). In this theory, naive T cells become activated following Ag stimulation, differentiate into effectors and quickly expand to clear pathogens. After Ag clearance most effectors die, the cell numbers contract, and only a proportion of Ag experienced cells revert to a slowly cycling long-lived memory T cells. This is supported by lymphocytic choriomeningitis virus (LCMV) infection experiments showing that virus specific memory CD8 cells are derived from expanded effector cells (Murali-Krishna, K. et al 1998; Kaech, S.M. et al. 2001, 2002), and by adoptive transfer experiments showing that memory CD4 cells originate from Ag-activated effector T cells in vivo (Swain, S.L.1994; Garcia, S. et al. 1999).

However, accumulating evidence indicates the independence of memory T cell generation from effector differentiation. Lauvau G et al reported memory CD8⁺ T cell generation in vivo in the absence of an overt effector response (Lauvau, G. et al. 2001). Manjunath and colleagues showed that effector differentiation is not a

prerequisite for memory CD8⁺ cells (Manjunath, N. et al. 2001). Wu CY et al reported distinct effector cells have differential capacities for memory generation (Wu, C.Y. et al 2002). Farber's group proved that memory T cells have functional plasticity in cytokine production (Ahmadzadeh, M. and Farber, D.L. 2002). They also showed that memory CD4 cells can be produced at different states of differentiation upon Ag stimulation from activated precursors, which have gradations of effector functions (Moulton, V.R. et al. 2006).

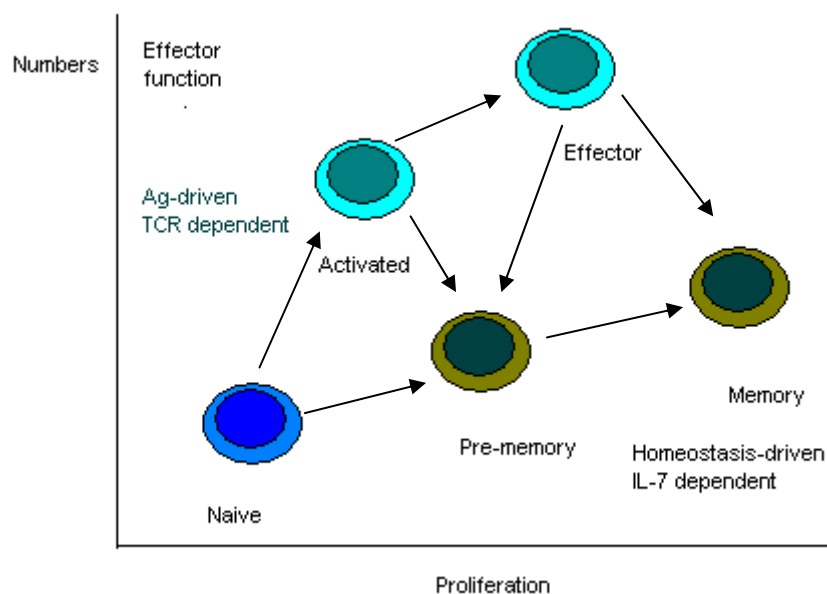


Figure 1.3.4 The intersecting pathway model of memory T cell formation

This model takes into consideration proliferative turnover, acquisition of effector function and cell survival in memory T cell generation driven from two pathways: antigen dependent activation and independent homeostasis. Naive T cells upregulate IL-2R and downregulate IL-7R receptor expression after Ag stimulation, resulting in rapid proliferation and differentiation to effectors, then contract to memory subset after Ag clearance. Alternatively, naive T cells ($CD25^{\text{lo}}IL-7R^{\text{hi}}$) undergo slow proliferative turnover in the expose of homeostatic factors like IL-7 and differentiate to memory-phenotype cells with effector capacity in the absence of specific antigen (Moulton, VR. & Farber, DL. 2006).

Memory-like T cells also arise without effector differentiation during homeostatic expansion in T cell depleted hosts to restore the circulating T cell pool. When naive T cells are adoptively transferred into lymphopaenic hosts, they acquire memory T cell phenotypes and functional properties during homeostatic proliferation, which is referred to as homeostasis driven memory T cell differentiation (Cho, B. et al. 2000; Goldrath, A.W. et al 2000; Murali-Krishna, K. and Ahmed, R. 2000).

Farber proposed a revised “intersecting pathway model” for memory T cell generation (Moulton, V.R. and Farber, D.L. 2006) (Figure 1.3.4). In this model, memory T cells can be generated through either Ag activation or Ag independent homeostatic factors. Naive T cells (CD25^{low} IL-7R^{hi}) undergo rapid IL-2 driven proliferation by up-regulated CD25 (IL-2R α chain) and down-regulation of CD127 (IL-7R α chain), and differentiate into effectors when further exposed to Ag. In the absence of further Ag stimulation, activated T cells undergo slow proliferative turnover driving to memory phenotype cell differentiation by homeostatic factors especially by IL-7. The intersection of these two pathways occurs at Ag clearance due to down-regulation of IL-2R α and up-regulation of IL-7R α on intermediate “pre-memory” cells that can homeostatically differentiate into stable memory T cells.

Memory T cells have high heterogeneity. Sallusto firstly reported two subsets of T cells with distinct homing potentials and effector functions in human (Sallusto, F. et al. 1999). Central memory T cells (T_{CM}) express CCR7 and CD62L that are required for T cells to recirculate into the T cell area of secondary lymphoid organs. T_{CM} do not have immediate effector functions but they are sensitive to TCR stimulation and independent on costimulation. After priming, T_{CM} produce IL-2 and then efficiently

differentiate into effector cells producing IFN γ or IL-4 (Sallusto, F. et al. 2004). Effector memory T cells (T_{EM}) do not express CCR7 and only a proportion of T_{EM} express CD62L. Instead T_{EM} express a set of chemokine receptors and adhesion molecules required for homing to inflamed tissues. T_{EM} have rapid effector functions and produce large amount of perforin from CD8 T_{EM} and IL-4, IFN γ , and IL-5 from both CD4 and CD8 T_{EM} (Williams, M.A. & Bevan, M.J. 2007). Even T_{CM} and T_{EM} are heterogeneous in expression of costimulatory molecules, adhesion molecules, and chemokine receptors, which discriminate T_{CM} and T_{EM} into functional subsets in resting status (Table 1.3).

Transcription factor T-bet has been linked with long-term renewal of memory CD8⁺ T cells and their responsiveness to IL-15 (Intlekofer, A.M. et al. 2005). It was recently revealed that the amount of inflammatory cytokines (i.e., IL-12) during T cell priming determines the fate of short-lived effector cells (SLECs) and memory precursor effector cells (MPECs) through a gradient expression of T-bet transcription factor, which potentially regulates memory commitment from activated CD8⁺ T cells (Joshi, N.S. et al. 2007).

In summary, memory T cells function as a dynamic repository of Ag experienced T lymphocytes that are generated from different precursors in multiple pathways. They are highly heterogeneous and mediate both protective and reactive immunity over the life time of the individual.

Table 1.3 Comparison of T cell subsets

properties	naïve	effector	memory	
homogeneity/heterogeneity	homogeneous	heterogeneous	heterogeneous	
		TH1, TH2, TH17, Treg	TCM	TEM
phenotype:				
cell size	small	large	small	
adhesion molecules	CD44lo	CD44hi	CD44hi	CD44hi
	CD11a (LFA-1)+	LFA-1 hi	LFA-1 hi	LFA-1 hi
	CD62Lhi	CD62Llo	CD62Lhi	CD62Llo
activation markers	CD69lo	CD69hi/lo	CD69lo	CD69lo
	CD25-	CD25+	CD25-	CD25-
chemokine receptors	CCR7hi	CCR7lo	CCR7hi	CCR7lo
others	CD45RA/RB/AC	CD45RO	CD45RO	CD45RO
	IL7Rhi	IL7Rlo	IL7Rhi	IL-7Rhi
	CD43lo	CD43hi	CD43hi	CD43hi
	Ly6c-		Ly6C+	Ly6C+
effector functions	mainly IL-2	IL-2, IFN γ , TNF (TH1)	effectors	effectors
		IL-4, IL-5, IL-10, IL-13 (TH2)		
		IL-17, IL-22 (TH17)		
		IL-10, TGF β (Treg)		
		Cytotoxicity of CD8 cells		
maximum cytokine secretion	days	hours (Rapid)	days	hours (rapid)
homing	lymphoid tissues	lymphoid and non-lymphoid tissues	Lymphoid tissues	non-lymphoid tissues
telomerase	long	long	shorter	shortest
half life	weeks	hours ~ days	years	Weeks-months
requirement of immune response	Ag at high concentration	Ag at high concentration	Ag at low concentration	Ag at low concentration
		IL-10, TGF β (Treg)		

1.4 T cell longevity and homeostasis

1.4.1. Homeostasis: balance of production and loss, survival and death

Maintaining a constant number of T cells is an important aspect of adaptive immunity. In spite of variable T cell production by thymus as a function of age and clonal expansion/contraction during the course of infection, circulating T cell numbers remain remarkably fixed. In young animals, the input of circulating T cells depends largely on the number of thymic emigrants, while in adults this relies on the longevity of mature T cells and their division in the periphery. Both processes, thymic emigration and peripheral expansion, determine the initial size of the mature T cell pool. Meanwhile, generation of new lymphocytes is balanced by cell loss. In intact animals, cell death is the major reason of T cell loss as displacement into other T cell subsets is relatively minor.

Each subset of lymphocytes appears to have independent niches for homeostasis. For example, loss of T cells is not compensated with increasing B cells, nor is loss of CD4 cells compensated by an increase in CD8 cells. Naive and memory T cells also have their own niches, with different survival signals, proliferative conditions, and death mechanisms.

1.4.2. Factors influencing T cell homeostasis

(1) MHC-peptide complex

It has been controversial about the roles of MHC molecules in naive T cell survival and homeostatic proliferation. Shimizu's group first proposed that long term survival of naive CD4 cells requires contact with MHC-II molecules (Takeda, S. et al. 1996). Rocha's group showed a similar requirement for MHC-I molecules from naive CD8 cells by demonstrating that these cells disappeared in the absence of MHC-I expression (Tanchot, C. et al. 1997). After that, there were accumulating evidence to favour the concept that homeostatic expansion and / or survival of naive T cells requires recognition of MHC complex in the periphery (Kirberg, J. et al. 1997; Beutner, U. et al 1998; Kieper, WC. 1999; Nesic, D. 1998; Murali-Krishna, K. 1999), although there were conflicting reports about whether the peptide ligands were the same for thymic selection or from environmental antigens (Ernst, B. et al. 1999; Bender, J. et al. 1999). There is also argument about the requirement of MHC for naive T cell homeostasis in intact mouse, as most of previous studies were done in lymphopaenic hosts which may be affected by altered level of trophic factors like IL-7, and the inability to monitor both proliferation and persistence in the same experiment.

In contrast with naive T cells, memory T cells survive quite well without the requirement of peptide/ MHC molecules. Research from different centres all suggest that memory CD4 and CD8 T cell homeostasis is independent on peptide/MHC complexes (Lau, LL. et al. 1994; Mullbacher, A. 1994; Garcia, S. et al. 1999; Murali-Krishna, K.1999; Swain, SL. 1999; Hu, H. 2001). However, this seems not the case for all memory T cells. John Sprent's group found that memory CD8 T cells are actually composed of the majority of CD122^{hi} and a small proportion of CD122^{lo}

cells based on the expression of β subunit of IL-2R/IL-15R recognized by anti-CD122 (Zhang, X. et al. 1998). Later on, they found that IL-15 is responsible to the survival and homeostatic proliferation of CD122^{hi}CD8⁺ cells, as these cells are absent in IL-15 KO mice and disappear rapidly after adoptive transfer to IL-15 deficient mice (Judge, AD. et al. 2002), while CD122^{lo} memory CD8 cells rely on the contact with MHC-I molecules and presumably together with self-peptide ligands as these subset of memory CD8 cells failed to proliferate and persist in MHC-I knockout mice (Boyman, O. et al. 2006). Others suggest that memory CD8 cells depend on their TCR engagement with foreign Ags such as in chronic viral infections (Wherry, EJ. et al. 2004).

For CD4 memory T cells, it was initially suggested that memory CD4 cell generation and survival is independent on MHC-II molecules (Swain, SL. et al. 1999), but later evidence showed that TCR ablation reduced homeostatic proliferation of memory CD4⁺ T cells although the initial size of naturally occurring memory CD4 cell pool was normal (Polic, B. et al. 2001). Furthermore, it was shown that a TCR signal from MHC is required for memory CD4 cell homeostasis when the IL-7 signal is absent, suggesting peptide MHC complexes do play a role in memory CD4 cell homeostasis even though it is not the only factor or the primary governor (Seddon, B. et al. 2003). Therefore, at least some proportion of memory T cells rely on TCR signals generated from continuous contact with either self or foreign peptide complexed to MHC.

Affinity/avidity of TCR engagement by peptide-MHC may influence homeostasis by providing weak or intermediate signals to maintain the viability of naïve T cells

(Marrack, P. & Kappler, J. 2004). This is supported by the finding that only T cells with proper TCR affinity proliferate in T cell deficient mice (Ernst, B. et al. 1999; Goldrath, AW. 1999). This is further evidenced by a recent report showing that MHC-I restricted CD8⁺ cells carrying low affinity transgenic TCRs have pronounced homeostatic proliferation, and that deficiency in a negative regulator of TCR signalling, SIT (Src homology domain containing tyrosine phosphatase 2-interacting transmembrane adaptor protein), lowers the TCR mediated activation threshold and enhances homeostatic proliferation of a range of Ag specific CD8⁺ cells with a range of TCR affinities (Posevitz, V. et al. 2008).

(2) Costimulation

It has been established that costimulatory signals mediated by interactions between B7(CD80/CD86)-CD28 is essential for T cell activation in addition to Ag-TCR binding during a primary immune response (Harding, FA. et al. 1992; Lenschow, D.J. et al. 1996). The second signal through CD28 enhances T cell activation, proliferation, and survival through amplifying TCR signal to lower the threshold of activation, upregulating anti-apoptotic proteins, and increasing IL-2 production to promote cell survival (Viola, A. et al. 1996; Tuosto, L. et al. 1998; Sperling, AI. et al. 1996; Kirchhoff, S. et al. 2000; Yu, X.Z. et al. 2003). In spite of the paradigm that naïve rather than memory CD8⁺ cells require CD28 costimulation, evidence suggests that costimulatory signals are also required during recall responses of memory CD8⁺ T cells (Bertram, E.M. et al. 2002; Mittrucher, H.W. et al. 2001; Borowski, A.B. et al. 2007). More recently, Usherwood and colleagues show that vaccinia virus specific memory CD8⁺ cells in CD28 deficient mice have lower expression of differentiation

markers CD27 and CD122, suggesting a role of CD28 in memory CD8⁺ cell differentiation (Fuse, S. et al. 2008).

Bluestone's group have shown that CD28 costimulation is critical for Treg development in thymus and their survival and homeostasis in the periphery (Salomon, B. et al. 2000; Tang, Q. et al. 2003). They demonstrated that CFSE labelled CD4⁺CD25⁺ Tregs cannot proliferate in anti-B7 Ab treated intact hosts and failed to survive in CD28 knock out recipients, indicating the requirement of CD28 costimulatory signal in Treg homeostasis.

However, it is not clear whether or not costimulation affects other subsets of mature T cell homeostasis. Although CD28 deficient mice have normal overall T cell numbers in peripheral lymphoid tissues (Shahinian, A. et al. 1993; Yu, X. et al. 2000), there are reports showing that costimulation may influence thymic negative selection (Noel, P.J. et al. 1998; Punt, J.A. et al. 1997) and effector/memory differentiation and function (Lenschow, D.J. et al. 1996; Salomon, B. et al. 2000; Mostbock, S. et al. 2007 a and b). Yu et al examined B7 transgenic and deficient mice and found that over expression of B7 result in peripheral T cell hyperplasia and decreased CD4/CD8 single positive cell ratio in the thymus which depended on TCR and CD28, while B7 deficiency showed the reciprocal observation, suggesting a substantial role of costimulation in regulating T cell development and homeostasis (Yu, X, et al. 2000). Costimulatory signals may contribute to T cell homeostasis by modulating TCR signalling threshold in TCR ligation with various affinities (Mostbock, S. et al. 2007 b).

(3) Cytokines

In addition to TCR ligation (signal 1) and costimulation (signal 2), a third extrinsic signal is from specific cytokine, especially common γ -chain cytokines such as IL-7 (and IL-15), for T cell survival, differentiation, memory development, and homeostasis (Curtsinger, J.M. et al. 1999; Mescher, M.F. et al. 2006; Boyman, O. et al. 2007). Cytokine signals have been extensively studied in T cell homeostasis, especially the common γ chain family comprised of interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15, and IL-21. It was named from the common γ chain (CD132) shared by the receptors of all these cytokines (Kovanen, P.E. & Leonard, W.J. 2004). The heterodimeric receptor of IL-4, IL-7, IL-9, and IL-21 comprises the common γ chain and their specific subunits, namely IL-4R α , IL-7R α (CD127), IL-9R α and IL-21R α respectively, while IL-2 and IL-15 receptors are heterotrimeric of the common γ chain, shared β chain IL-2/IL-15R β (CD122), and their specific α chains, IL-2R α (CD25) or IL-15R α (Alves, N.L. et al. 2007).

IL-7 is generated by the stromal cells in the lymphoid tissues in a constant rate. It is believed that the level of IL-7 in the serum is determined by the rate of consumption by the circulating lymphocytes. IL-7 was initially defined as a B cell trophic factor, but both IL-7 and IL-7R deficient mice showed additional key roles in T cell development in central and peripheral lymphoid organs (von Freedon-Jeffry, U. et al. 1995; Peschon, J.J. et al 1994; Maraskovsky, E. et al. 1996). IL-7 acts as a growth stimulus to promote T cell proliferation through the JAK/STAT pathway (Yao, Z. et al. 2006, Suzuki, K. et al. 2000) as well as a survival factor to prevent T cells from death by regulating the balance of anti-apoptotic and pro-apoptotic proteins of

the BCL-2 family (Maraskovsky, E. et al. 1997; Pellegrini, M. et al. 2004). Through these effects, IL-7 mediates both naïve and memory T cell homeostasis.

Boursalian and Bottomly firstly reported that IL-7 jointly with self-peptide/MHC complex is required for naïve T cell survival. They showed that naïve T cell numbers decreased following anti-IL-7 Ab treatment in adult thymectomized mice (Boursalian, T.E. and Bottomly, K. 1999). More reports after that have set up our current view that IL-7 is the primary cytokine for naïve T cell survival and proliferation, and that the basal level of IL-7 determines the overall size of naïve T cell pool (Schluns, K.S. et al. 2000; Tan, J.Y. et al. 2001; Surh, C.D. & Sprent, J. 2005). For memory T cell homeostasis, IL-7 seems to have less of a role than IL-15, as over expression of IL-7 can overcome the dependence of memory CD8⁺ cells on IL-15 when IL-15 is deficient (Kieper, W.C. et al. 2002). It was initially thought that memory CD4⁺ cells do not require IL-7 (Lantz, O. et al. 2000; Tan, J.T. et al. 2002), but new evidence suggests that memory CD4⁺ cells do depend on IL-7 for survival and homeostatic proliferation in the absence of TCR signalling (Seddon, B. et al. 2003; Kondrack, R.M. et al. 2003). The dependence on IL-7 of naïve and memory T cells is correlated to the high expression of IL-7R on these cells.

An “altruism theory” was proposed to explain the observation that IL-7R expression can be transcriptionally suppressed by IL-7 or other cytokines like IL-2, IL-4, IL-6, and IL-15, to benefit other cells that have not received the limited prosurvival cytokines (Park, J.H. et al. 2004). However, the study was mainly performed *in vitro*, and recent *in vivo* data suggested that IL-7R expression is independent of IL-7 itself (Klonowski, K.D. et al. 2006) and may be induced by IL-12

(Takemoto, N. et al. 2006) and IL-2 (Dooms, H. et al. 2007). TCR triggering down-regulates IL-7R expression in double positive thymocytes and peripheral effector T cells (Franchimont, D. et al. 2002). Recent studies show that there is interplay between TCR signal and cytokine signals. Alfred Singer's group suggested that the IL-7 signal can transcriptionally increase CD8 coreceptor expression and promote TCR signalling in a transgenic mouse model, whereas TCR signals impairs IL-7 signalling and downregulates CD8 expression (Park, J.H. et al. 2007). Farrar's group reported the linkage of TCR, costimulation, and cytokine signalling govern the process of Treg development (Burchill, M.A. et al. 2008).

IL-15 has overlapping functions with IL-7 in naïve T cell homeostasis. IL-15 plays a role in supporting naïve CD8⁺ cell survival but not naïve CD4 cells. This is correlated with the expression of IL-2/IL-15R β chain (CD122) on naïve CD8⁺ but not naïve CD4⁺ cells (Surh, C.D. et al. 2006). As mentioned earlier, IL-15 plays a key role in the turnover and survival of CD8⁺CD122⁺ memory cells. Low levels but not absence of IL-15R β chain expression on CD4⁺ memory T cells suggests some role of IL-15 in regulating memory CD4 cell homeostasis (Ilangumaran S, et al. 2003).

1.4.3. Life and death of peripheral T cells

Life and death of peripheral T cells is determined by the signals received by the cells such as TCR triggering, costimulation, and cytokines through several mechanisms: extrinsic cell death receptor and caspase dependent apoptosis; intrinsic mitochondria and caspase independent apoptosis; or caspase independent cell death.

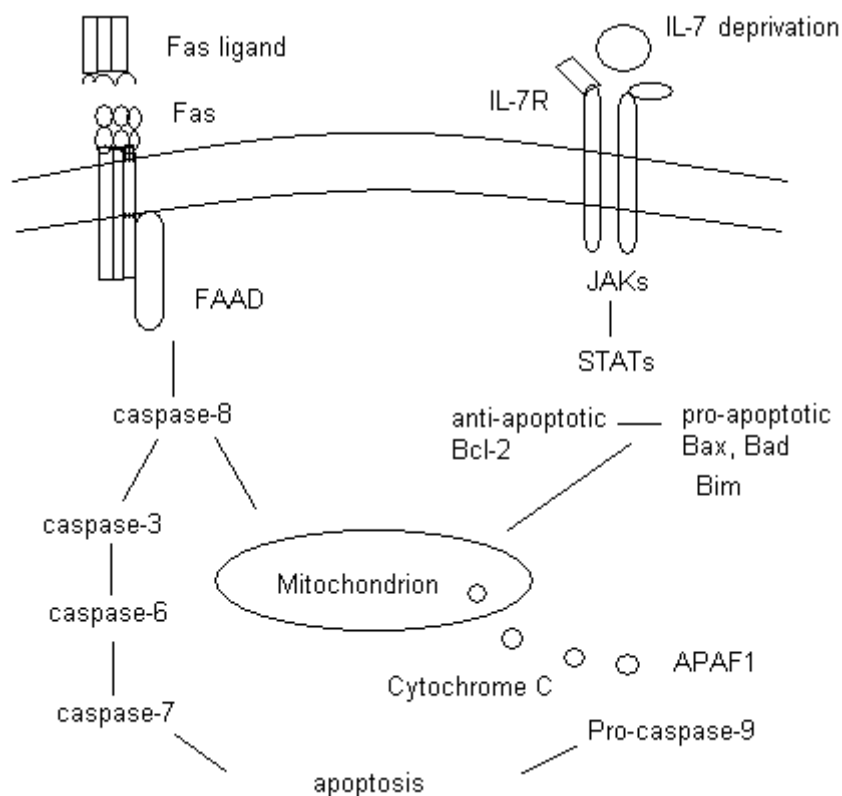


Figure 1.4.1 Two distinct cell death pathways from cytokine withdrawal and activation of Fas

Lymphocyte death occurs either in the contraction phase of immune responses or during homeostasis, through death receptors such as Fas (CD95) or Bcl-2 family responses to trophic factor deprivation. Ligand binding trimerizes Fas inducing the binding of adaptor FADD (FAS-associated death domain protein) to the death domain in Fas, resulting in the activation of caspase and eventually rapid apoptosis. By contrast, trophic-cytokine withdrawal causes loss of signaling through the JAK–STAT and PI3K–AKT pathways and disrupts mitochondria through favouring the balance of pro-apoptotic Bcl-2 members (Khaled, AR. & Durum, SK. 2002).

The extrinsic apoptotic pathway is triggered by ligand binding to cell surface death receptors of the TNF receptor family including TNF receptors (TNFR), CD95 (FAS or APO-1), TRAIL receptors (TRAILR), and death receptors (DR), which are defined by the presence of an intracellular death domain (DD). This results in the formation of death inducing signalling complex (DISC) and activation of caspase leading to cell death (Green, DR. 2005). The intrinsic apoptotic pathway is induced by

a variety of stimuli like TCR stimulation, DNA damage, endoplasmic reticulum (ER) stress, and cytokine deprivation that cause break down of the balance of anti-apoptotic and pro-apoptotic members of BCL-2 family and permeabilization of the mitochondrial membrane which consequently release cytochrome C and result in cell death (Youle, R.J. & Strasser, A. 2008).

Activated T cells die through either activation induced cell death (AICD) through FAS or activated cell autonomous death (ACAD) due to cytokine withdrawal. It is still obscure why memory cells die but it is assumed that memory T cells die in different ways (Marrack, P. & Kappler, J. 2004). Naïve T cells die through intrinsic pathway rather than extrinsic pathway because naïve T cells express lower levels of TNFR and FAS, and deficiency in these molecules does not affect naïve T cell survival. There is evidence suggesting that naïve T cell viability is controlled by the ratio of anti-apoptotic Bcl-2 and pro-apoptotic molecule Bim (Bcl-2 interacting molecule) (Wojciechowski, S. et al. 2007). Bim deficiency or Bcl-2 overexpression promotes the accumulation of naïve T cells in the periphery (Bouillet, P. et al. 1999; Hildeman, D.A. et al. 2002; Strasser, A. et al. 1991). Bim deficiency prevented the death of CD127^{lo} CD8⁺ effector cells during the contracting phase after LCMV infection (Wojciechowski, S. et al. 2006).

1.4.4. Survival signalling in naïve T cells

Once a mature single positive thymocytes leaves the thymus, it enters the blood stream as a naïve T cell and is carried into the peripheral lymphoid tissues which have highly organised architecture providing naïve T cells the opportunity to interact with

other cell types like dendritic cells and B cells. Naive T cells continually circulate through lymph nodes and spleen via lymph and blood until they encounter their specific antigens, become activated and differentiate into effectors, or die after a period of 4-8 weeks on average.

Recent thymic emigrants continue to undergo peripheral selection through their TCR. Strong binding to self-peptide-MHC complex eliminates autoreactive clones (i.e. through peripheral deletion), or renders the T cells hyporesponsive. Repeated TCR contact with low avidity self/peptide-MHC complexes is required to maintain naïve T cells in the peripheral circulation as these cells cannot survive when transferred into recipients that lack MHC molecules (Murali-Krishna, K. et al. 1999; Kassiotis G. et al. 2002).

Mature naive T cells are metabolically quiescent and have infrequent division by contact with self-peptide-MHC complex in unstimulated animals; therefore they have a prolonged life span (Sprent, J. & Tough, DF. 1994). In the past a couple of decades, tremendous efforts have been put in defining T cell functions during activation and differentiation. T cells have different functions and biological features at different stages of development, characterised by specific cell surface markers and intracellular molecules. The comparison of the properties of naive, effector, and memory T cells is summarized in table 1.3.

1.5 Pre-mRNA alternative splicing

Shortly after exons and introns were discovered in RNA transcript precursors (Berget, S.M. and Sharp, P.A. 1977; Chow, L.T. et al. 1977; Gilbert, W. 1978) it was found that some exons are variably included in the mature transcript (Early, P. et al. 1980; Rosenfeld, M.G., et al. 1982). This process is termed pre-mRNA (precursor messenger RNA) alternative splicing, referring to the differential removal of introns and inclusion of exons (Gilbert, W. 1978). Genome and cDNA sequencing projects have revealed that human (and mouse) genes generate complex nascent mRNAs, with an average eight exons and large introns making up 90% of the precursor transcript (Lander, E.S. 2001). Recent splicing arrays and bioinformatics studies show that 70% ~ 80% human genes express multiple mRNA isoforms due to alternative splicing of exons or exon segments (Johnson, J.M. 2003; Kampa, D. 2004; Clark, T.A. 2007), much larger than initially estimated 5% (Sharp, P.A. 1994) or 35-65% (Mironov, A.A. 1999; Modrek, B and Lee, C. 2002).

Alternative mRNA splicing expands protein complexity and functional diversity. Many protein isoforms encoded by splicing variants have distinct or even antagonistic biological activities. It correlates to the disparity between lower number of protein-coding genes and the large number of proteins in human (approximately 25,000 vs 900,000) (Woodley, L. and Valcárcel, J. 2002). Comparative analysis shows that most human and mouse genes are orthologues sharing the same intron/exon arrangement as well as a high degree of conservation in homologous exon sequences and indistinctive splicing patterns (Waterston, R.H. 2002; Modrek, B. and Lee, C. 2003).

1.5.1. Regulation of alternative splicing

For an individual nascent mRNA, introns must be removed and exons have to be precisely joined together to maintain the reading frame. Constitutive exons are constantly included in mature transcripts, while there are several types of variable exons (Figure 1.5.1a). A cassette exon is one that is either included or excluded (skipped) from mature mRNA. As opposed to exon skipping, some introns can be variably retained. Two neighbouring exons can be included in a mutually exclusive pattern. Differential splice sites at the 5' or 3' end can change the exon length. Transcript can have alternative initiations and first exons or termination and 3' exon (Smith, C.W. and Valcárcel, J. 2000; Xing, Y. and Lee, C. 2006; Hu, A. and Fu, X.D. 2007). Exon skipping is the most frequent alternative splicing events in mammals (Sugnet, C.W. et al. 2004).

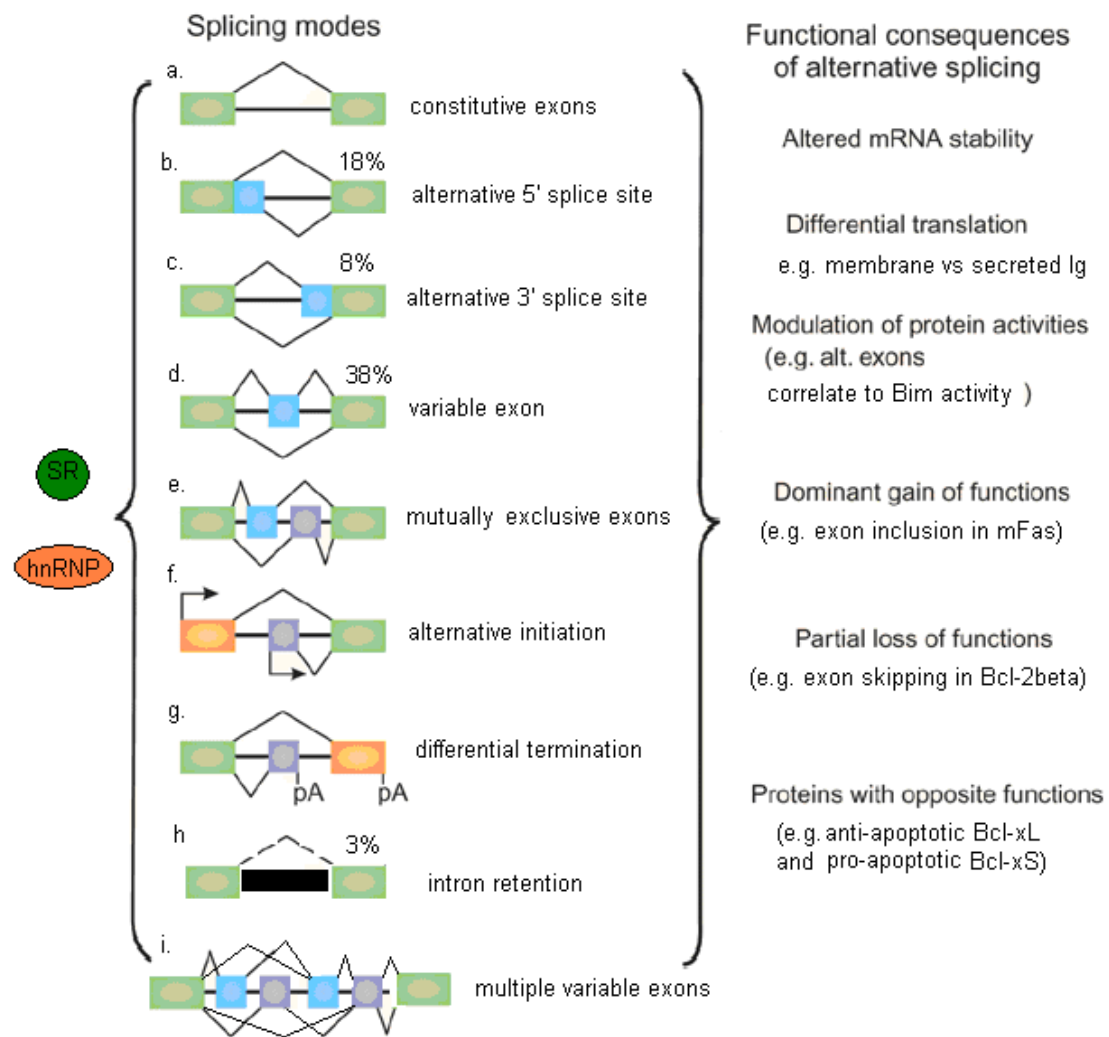


Figure 1.5.1a: pre-mRNA splicing patterns and functional complexity

Splicing regulators, e.g. SR proteins and heterogeneous nuclear ribonucleoproteins, modulate alternative splicing of nascent messenger RNAs in different patterns in a gene specific, tissue dependent, or cell signal dependent manner, which alters the structure of the mature mRNA transcripts as well as the function of proteins isoforms (Hu, A. and Fu, XD. 2007).

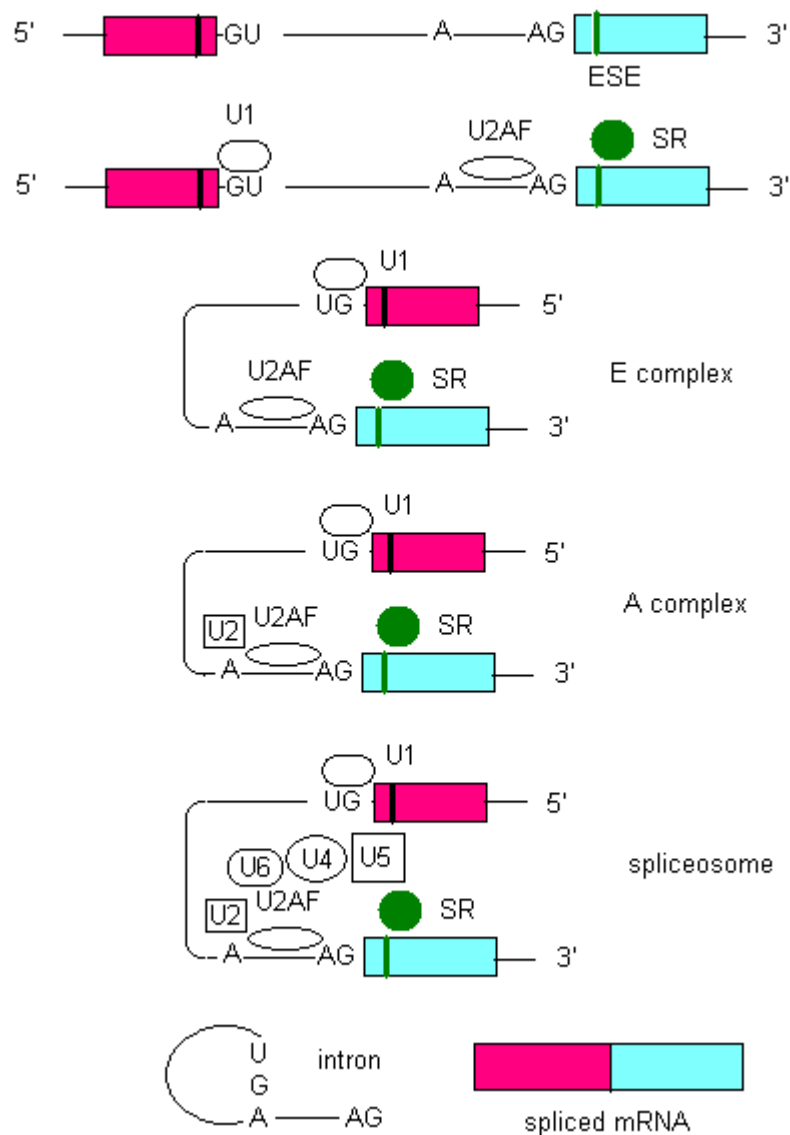


Figure 1.5.1b: Splicing machinery

Stepwise assembly of spliceosome, a multiple snRNP complex, to the target exon-intron boundary to cleave the intron promoted by SR protein binding to the exonic splice enhancer (Li, Q. et al. 2007).

Alternative splicing is directed by a complex set of splicing codes consisting of consensus sequences of splicing sites at exon-intron boundaries and exonic or intronic regulatory elements called exonic splice enhancers (ESE) or intronic splice enhancers (ISE), and exonic or intronic splice silencers (ESS/ISS) (Black, D.L. 2003). The

splicing sites are critical for all exons and were identified from alignment of intron-exon boundary sequences (Berget, S.M. 1995). The splice site sequences are recognized by small nuclear RNAs (snRNAs) or small nuclear ribonucleoprotein particles (snRNPs) through specific base pair interaction (Jurica, M.S. & Moore, M.J. 2003). During splicing, five snRNA / snRNPs assemble in a stepwise manner to form the splicing machinery referred to as the spliceosome. U1 snRNP binds to 5' splice site at a conserved GU dinucleotide, U2AF (U2 snRNP auxiliary factor) recognizes the 3' splice site polypyrimidine tract and a conserved AG dinucleotide with its 65 and 35 kD subunits respectively (E complex) (Lim, S.R. & Hertel, K.J. 2004; Chiara, M.D. & Reed, R. 1995). U2AF65 then recruits U2 which is specific for the branch point sequence at the 3' splice site (A complex). Subsequently the U4/U6-U5 complex joins (B complex) and remodelling of RNA and protein components in the B complex forms an enzymatically active C complex to catalyse the RNA-RNA transesterification reactions to cleave the intron and connect the exons (Martin, A.J. et al. 2005) (Figure 1.5.1b).

Conserved sequences at about 10 nucleotides serve as splicing enhancers and / or silencers, and occur near the exon/intron boundaries. This directs the spliceosome machinery to the right splice site rather than potential cryptic sites of the pre-mRNA. The action of two classes of splicing regulatory proteins, Serine/Arginine rich proteins (SR proteins) and heterogeneous nuclear ribonucleoproteins (hnRNPs) are best studied (Zhu, J. et al. 2001). SR proteins bind to splicing enhancers to initiate exon recognition and inclusion (Fairbrother, W.G. 2002; Reddy, A.S. 2007), although they may inhibit splicing as well (Barnard, D.C. 2002). SR proteins have two common domains: the RNA recognition motif (RRM) and the RS domain with repeated

arginine/serine dipeptides. The RRM (also referred to as the RNA binding domain, RBD) targets the protein to a particular RNA sequence, whereas the RS domain modifies the affinity of RNA binding. SR proteins bind both ESE and ISE to positively regulate alternative splicing through recruiting the spliceosome to the adjacent exon (Black, D.L. 2003; Graveley, B.R. 2000). SR protein binding sites are found in both constitutive and variable exons suggesting their roles in both constitutive and alternative splicing (Mayeda A, et al. 1999; Stark, J.M. et al. 1999).

The hnRNP proteins are a large group of proteins associated with mRNA precursors that were termed heterogeneous nuclear RNAs (hnRNAs) to describe their size heterogeneity and cellular location (Dreyfuss, G. et al. 1993). hnRNPs are highly conserved among vertebrates. Most hnRNP proteins have one or more RRMs which are also found in many other RNA binding proteins. The RRM domain comprises approximately 90 amino acids with two consensus sequences termed, RNP1 and RNP2 submotifs. RNP1 is the most conserved octapeptide segment in RRM that is comprised of Lys/Arg-Gly-Phe/Tyr-Gly/Ala-Phe-Val-XPhe/Tyr. RNP2 is a less conserved hexapeptide sequence of Ile/Val/Leu-Phe/Tyr-Ile/Val/Leu-X-Asn-Leu. The aromatic amino acids Phe/Tyr in RNP1 and RNP2 are essential for RNA binding (Dreyfuss, G. et al. 1993; Birney, E. et al. 1993). From studies on the best characterised hnRNP A1 and PTB (Polypyrimidine Tract Binding protein, or hnRNP I), it has been shown that hnRNPs repress spliceosomal assembly either by multimerization along exons or by blocking the recruitment of snRNPs to loop out exons (Zhu, J. et al. 2001; Tange, T.O. et al. 2001; Sharma, S. 2005; Martinez-Contreras, R. 2006). Roles of some hnRNPs in alternative exon splicing have been established, as well as evidence that hnRNPs regulate constitutive splicing events

(Pozzoli, U. and Sironi, M. 2005). Major splice regulators identified so far are listed in table 1.4.

	Name	Domains	Binding Sites	targets
SR / SR related proteins	9G8/Sfrs7	RRM, RS, C2HC Znf	(GAC) _n	Tau, GnRH, 9G8
	SC35/Sfrs2	RRM, RS	UGCUGUU	AChE
	SRp55/Sfrs6	RRM, RS	GGCAGCACCUG	cTnT, CD44
	SRp75/Sfrs4	RRM, RS	GAAGGA	FN1, E1A, CD45
	SRm160/Srm1	RS, PWI	AUGAAGAGGA	CD44
hnRNPs	hnRNP A1	RRM, RGG	UAGGGA/U	Hipk3, Smn2, c-H-ras
	hnRNP H	RRM, RGG, GYR, GY	GGGA, G-rich	PLP, HIV tat, Bcl-x
	hnRNP I / PTB	RRM	UCUU, CUCUCU	nPTB, c-SRC, Fas, cTnt, hnRNP A1 etc
	hnRNP L	RRM	C/A-rich	eNOS, CD45
	hnRNP LL	RRM	?	CD45, Nalp1, etc
others	TIA1	RRM	U-rich	Fas, MYPT1, IL-8, etc
	Sam68	KH	A/U-rich	Bcl-x

Table 1.4: Some key alternative splicing regulators (Gabut, M. et al. 2008)

The three dimensional structure of the RRM of many RNA binding proteins has been solved by X-ray diffraction and NMR. Most RRM structures consist of similar four strands and two helices arranged in an alpha/beta sandwich ($\beta 1$ - $\alpha 1$ - $\beta 2$ - $\beta 3$ - $\alpha 2$ - $\beta 4$), although in some cases there is a third helix present. The RNP1 and RNP2 submotifs

are located in the central $\beta 3$ and $\beta 1$ strands with conserved aromatic residues contacting RNA (Dreyfuss, G. et al. 1993; Birney, E. et al. 1993).

RRM structure and its binding studies revealed that it can interact with target RNA or protein via its β sheet surface; it also interacts with proteins via $\alpha 2$ helix on the other side of RRM. The $\alpha 3$ helix of U2AF65's interaction with the β sheet surface of RRM can occlude RNA binding. The $\alpha 3$ helix swings away upon RNA binding, facilitating protein dimerization and RNA binding in U1A RRM (Singh, R. and Valcárcel, J. 2005)

It has been an interesting issue how hnRNPs and SR proteins specifically bind to degenerate nucleic acid sequence and regulate target nascent mRNA splicing (reviewed by Singh, R. and Valcárcel, J. 2005). Splicing enhancer and silencer sequences have been defined for recognition by major SR proteins and hnRNPs respectively. However, these specific regulatory elements are wide spread throughout the genome and still provide complex potential high-affinity binding sites for alternative usage. Additional protein factors are required to build up the specificity of splicing including tissue or cell-type specific expression of RNA binding proteins and/or their intracellular localization; effects of their cofactors or interaction with other snRNPs; the splicing isoforms of hnRNPs and SR proteins themselves and the sensitivities to the specific context comprised of other cofactors; usage of multiple RRM domains and KH (hnRNP K homolog), another abundant RNA binding motif, and domain tethering; and alternative splicing in responding to specific cell signals (Singh, R. and Valcárcel, J. 2005; Lareau, L.F. et al. 2007; Shin, C. and Manley, J.L. 2004; Rothrock, C. et al. 2003).

Dysregulation of alternative splicing resulting from mutations in splice sites or regulatory elements has been implicated in a wide spectrum of human diseases including cancer and autoimmune diseases. More than 15% of mutations causing genetic diseases affect pre-mRNA alternative splicing (Krawczak, M. 1992). There is increasing attention being given to developing therapeutic approaches that modulate alternative splicing in target cells.

1.5.2. CD45 alternative splicing

CD45 is the prototypic receptor-like protein tyrosine phosphatase. It is abundantly expressed on all nucleated hematopoietic cells, occupying up to 10% of the cell surface (Thomas, M.L. 1989). This transmembrane glycoprotein consists of an extracellular domain with cell-type and developmental-specific isoforms generated from alternative splicing of three variable exons, a single transmembrane domain, and cytoplasmic tandem phosphatase (PTPase) domains termed D1 and D2.

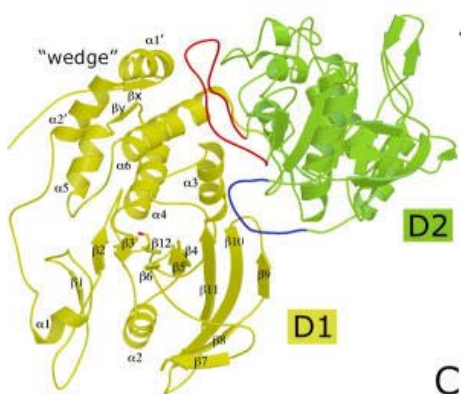


Figure 1.5.2a: Ribbon diagram representations of the CD45 D1D2 domains (from Nam, H.T. et al. 2005).

The D1 and D2 domains are shown in yellow and green, respectively. The viewer is looking straight into the D1 active site with the putative “wedge” site marked, and the corresponding active site of D2 is facing upward. The acidic and basic loops are shown in magenta and blue, respectively.

From molecular modelling studies based on the crystal structure of PTP α D1 domain, it has been proposed that a wedge structure in the juxtamembrane region of CD45 favours dimerization and inhibition of PTPase activity (Bilwes, A.M. et al. 1996; Xu, Z. & Weiss, A. 2002). However, three dimensional studies on human CD45 cytoplasmic domains recently revealed that this may not apply to CD45. Frederick, C.A. and colleagues (Nam, H.J. et al. 2005) reported the crystal structure of CD45 cytoplasmic region containing D1 and D2 domains in native form and in substrate interactions. They found that the CD45 D2 domain has the same $\alpha 1$ helix-loop- $\alpha 2$ helix motif as in D1 domain, suggesting strong D1-D2 interaction and that the steric hindrance from the D2 domain prevents D1 dimerisation. This study confirms that phosphopeptides only bind D1 active site but D2 may have auxiliary effects. Based on these recent studies, structure models of CD45 molecule have been significantly changed. The comparison of the two models is shown below.

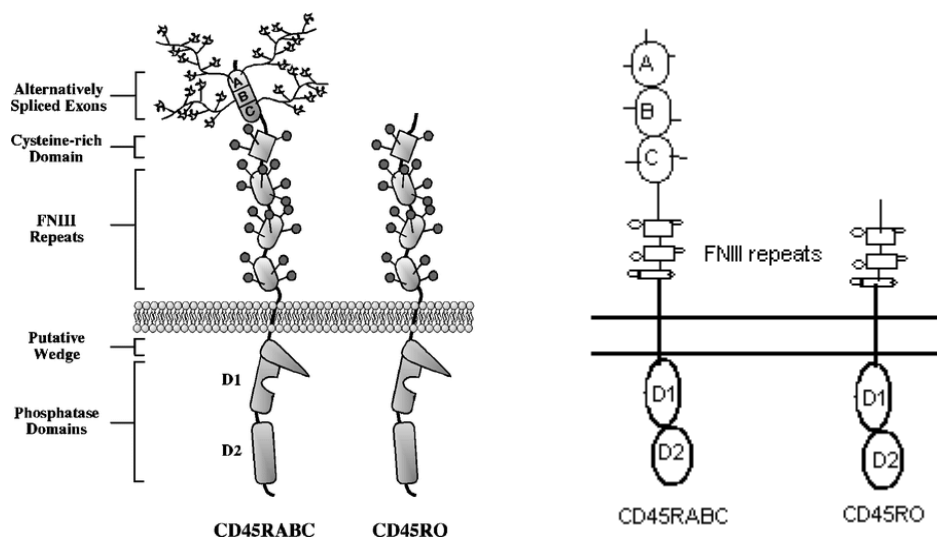


Figure 1.5.2b: CD45 Models

Left: old model with wedge structure to D1 domain (Hermiston, M.L. et al. 2003).
 Right: CD45 model based on the 3-D crystal structure study of the cytoplasmic regions showing no wedge structure. Short lines and small circles represent O-linked glycosylation in the variable regions and N-linked glycosylation in the Fibronectin type III respectively. For more information, please see texts in this chapter.

The CD45 extracellular domain contains 3 fibronectin type III repeats and a cysteine-rich region that are both heavily N-glycosylated. The membrane distal N-terminus of CD45 molecule is encoded by 3 variable exons, exon 4, 5, and 6 encoding segments A, B, and C that are heavily decorated by O-linked glycosylation. Thus through alternative splicing multiple isoforms can be produced depending on the developmental stage, and activation status of the cell. The splicing pattern and expression of CD45 is highly conserved across species over hundreds of millions years of evolution (Hermiston, M.L. et al. 2003; Holmes, N. 2006).

Although alternative splicing of CD45 exon 7, 8, and 10 have been reported in cell lines, alternative splicing of exons 4, 5, and 6 is most well established in both human, mouse, and other mammals (Chang, H.L. et al. 1991; Virts, E. et al. 1998). Isoforms that lack exon 7 (Virts, E. et al. 1998) and a novel exon encoding 41 amino acids between exon 3 and exon 4 termed exon D has been reported of the mRNA level (Li, D. et al. 2004), but none of these have been detected at protein level in either human or mouse. So far only five isoforms have been observed at protein level (Hermiston, M.L. et al. 2003): CD45RO (lacks exons 4, 5, 6); RB (exon 5); RAB (exons 4 and 5); RBC (exons 5 and 6); and RABC (exons 4, 5, and 6). B cells typically express CD45RABC (B220). Naïve T cells primarily express CD45RB, CD45RAB, or CD45RBC, whereas activated and memory T cells primarily express CD45RO. Variable splicing of CD45 also occurs in other blood lineages such as dendritic cell subsets (Lynch, K.W. 2007).

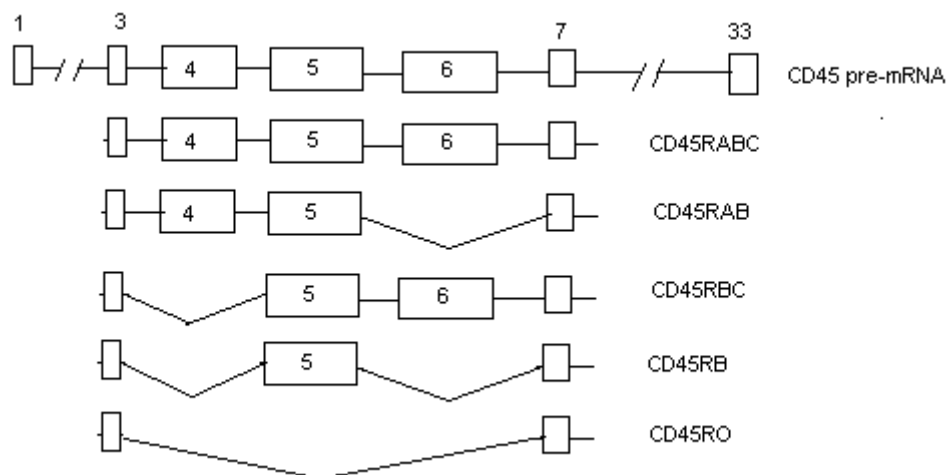


Figure 1.5.2c: CD45 pre-mRNA splicing variants

The dependence of CD45 alternative splicing on cell types, developmental stage and activation status implies that this process is under the control of both external signals and intrinsic regulatory mechanisms (Lynch, K.W. 2007). K.W. Lynch and colleagues delimited an exonic splicing silencer to a 60 nucleotide fragment within exon 4 designated ESS1 containing an activation responsive sequence (ARS) that is responsible partially for basal exon silencing in resting T cells and is sufficient to confer activation-induced exon exclusion upon T cell stimulation (Lynch, K.W. and Weiss, A. 2001; Rothrock, C. et al. 2003). The ARS sequence has been further refined to a minimal sequence of which occurs three times in CD45 exon 4, twice in exon 6, and once in exon 5 (Tong, A. et al. 2005). The relative strength of exonic splicing enhancers (ESEs) and silencers (ESSs) of each of the three variable exons determines the differential expression of CD45 isoforms (Tong, A. et al. 2005). Recently Lynch and co-workers used RNA affinity chromatography with a human CD45 minigene template to identify that the hnRNP proteins: hnRNP L, PTB and hnRNP E2 associate as a complex to regulate splice site silencing of exon4 of CD45 pre-mRNA in a human T cell line (Rothrock, C. et al. 2005). They demonstrated that ESS1 bound by

hnRNP L represses exon 4 inclusion by blocking spliceosome assembly after ATP-dependent exon recognition by U1 and U2 and the formation of spliceosome E-A complex, resulting in failure to progress to a final catalytic B-C complex (House, A.E. and Lynch, K.W. 2006). Recently, the same group found that cellular stimulation causes post-translational modification of hnRNP L in mobility and activity, and more importantly, the activation-specific addition of PSF (PTB associated splicing factor) with increased activity, suggesting combinatorial control of signal induced alternative splicing (Melton, A.A. et al. 2007). This series of studies provides important evidence of exonic/intronic regulatory elements in CD45 mRNA as well as identifies some of the key trans/cis-acting regulatory hnRNPs/SR proteins that regulate CD45 alternative splicing in vivo. One caveat is that most of these results were based on exogenous protein expression. Considering the high degree of conservation amongst hnRNP proteins, hnRNPs other than hnRNP L may have more important or complementary roles in modulating CD45 alternative splicing. More works need to be done to elucidate the functions of other hnRNPs in the regulation of CD45 alternative splicing.

CD45 Function

CD45 has been identified as an important regulator of T cell development. Initial studies of CD45 deficient T cell lines identified CD45 as a positive regulator of TCR signal transduction (Pingel, J.T. et al. 1989; Koretzky, G.A. et al. 1990; Volarevic, S. et al. 1993; Hovis, R.R. et al. 1993). Subsequent observations of severe-combined immunodeficiency (SCID) were reported in both CD45 deficient mice (Kishihara, K. et al. 1993) and humans (Kung, C. et al. 2000) showing greatly diminished peripheral T lymphocytes, unresponsiveness to mitogen stimulation, and decreased serum

immunoglobulin despite normal B-lymphocyte numbers. Molecular studies revealed that CD45 deficiency causes dysfunctional pre-TCR and TCR signalling resulting in enhanced basal apoptosis and arrest of T cell development at DP to SP transition (Byth, K.F. et al. 1996; Kishihara, K. et al. 1993). CD45 deficiency bred into TCR transgenic mice proved that CD45 plays a positive role in promoting both positive and negative selection of thymocytes and regulates the strength of signal to a fixed Ag stimulation (Mee, P.J. et al. 1999).

The earliest event in the TCR signal transduction cascade is the activation of Src family protein tyrosine kinases Lck and Fyn. Lck has an inhibitory pTyr505 site that is phosphorylated by Csk (C-terminal Src kinase) and dephosphorylated by CD45. Lck is the most important substrate for CD45 in T cells as CD45 is the only known phosphatase to dephosphorylate the negative regulatory pTyr505 site of Lck, causing a conformational change leading to increased kinase activity (Veillette, A. et al. 1992; Latour, S. and Veillette, A. 2001). Full activation of Lck requires a second Y394 residue to be autophosphorylated in the activating loop, displacing the loop to fully open the catalytic cleft of the kinase. CD45 dephosphorylates this activatory pTyr394 site to suppress Lck activity (Doro, U. and Ashwell, J.D. 1999). CD45 thus plays opposing roles, both promoting and inhibiting Lck activity (Zamoyska, R. 2007).

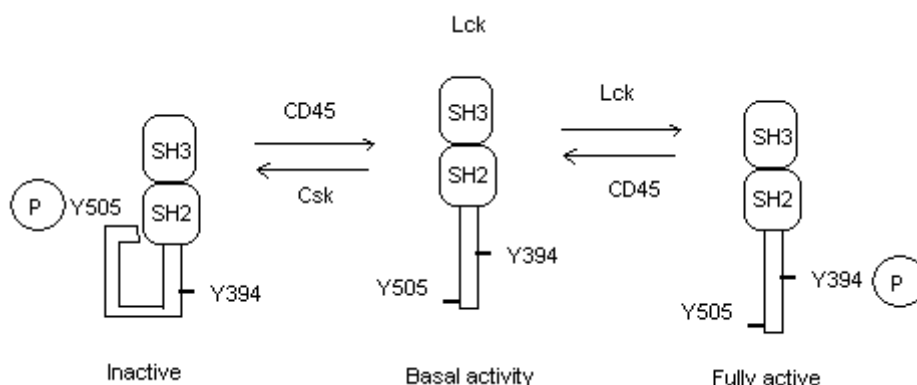


Figure 1.5.2d: Antagonistic regulation of Src-family kinase activity by Csk and CD45

Recently Alexander and colleagues generated transgenic mice expressing varying amounts of CD45RO and other isoforms, using CD45 mutant mice with low or null PTPase activity (Ogilvy, S. et al. 2003; McNeill, L. et al. 2007). They showed that only 3% of wildtype CD45RO activity was sufficient to fully restore thymic development from DP to SP cells. In the periphery CD45 had a rheostat effect: low CD45 activity reduced TCR signalling and intermediate CD45 activity caused hyperactivation. They proposed that CD45 differentially dephosphorylates pTyr505 and pTyr394, such low amounts are sufficient for activating Y505 whereas large amounts of CD45 are necessary to dephosphorylate pTyr394 and suppress T cell hyperactivity (McNeil, L et al. 2007). Together with an earlier report showing that different levels of CD45 expression affects thymic selection (Wallace, V.A. et al. 1997), these results suggest that titration of CD45 levels or activity may have non-linear effects on T cell physiological functions.

The functions above apply to all CD45 isoforms but an important unresolved question is the role of different CD45 isoforms on T cell function. CD45 isoform

expression is tightly and precisely regulated by alternative splicing through the course of development and activation. What the exact roles the CD45 isoforms play have remained obscure despite several decades of intensive investigation. One reason is that it has not been possible to generate mice or cells with normal CD45 density when they are forced to transgenically express different CD45 isoforms. It is quite controversial as some reported that CD45RO is more efficient in restoring TCR signalling in CD45 deficient cells, whereas others suggested that large isoforms were active (Novak, T.J. et al. 1994; McKenney, D.W. et al. 1995; Chui, D. et al. 1994).

Another important aspect of CD45 is the dimerization and its impact on TCR signalling. Xu and Weiss. found that three CD45 isoforms (CD45RO, CD45RAB, and CD45RABC) can form homodimers in transfected T cells (Xu, Z. & Weiss, A. 2002). They found CD45RO isoform was more efficient to dimerise therefore was less active to reconstitute TCR signalling than CD45RABC. The process of dimerization of CD45 isoforms was independent on their intracellular domains but was modulated by sialylation and O-linked glycosylation.

Acuto's group tested the roles of CD45 ectodomain in TCR signalling using CD45 chimeras fused with different ectodomains. They found that a large ectodomain can rescue TCR signals in CD45 deficient T cells, whereas chimeras with small ectodomain were inefficient (Irles, C. et al. 2003). This correlates to the proposal that CD45 large isoform (CD45RAB) is more active in PTPase activity than CD45RO. The size of CD45 ectodomain as well as the levels of charges from glycosylation may have impacts in TCR synapse formation. CD45RAB could be excluded from TCR synapses in which the short CD45RO protein may be involved. To some extent this

could diminish the activity of CD45. Y394 is most sensitive to decreases in CD45 activity, this suggests that CD45RABC could enhance Lck activity in the TCR synapse, whereas CD45RO would better penetrate the synapse and blunt Lck signalling.

Polymorphisms of PTPRC/CD45 that influence differential isoform expression has been found in man and linked to various human diseases including multiple sclerosis (MS), type 1 diabetes (T1D), Graves' disease, systemic lupus erythematosus (SLE), human immunodeficiency virus (HIV) infections and hepatitis B (Jacobsen, M. et al. 2000; Vorechovsky, I. et al. 2001; Johanneson, B. et al. 2002; Vogel, A. 2003; Tchilian, E.Z. et al. 2001), although others reported no such linkage to T1D and Graves' diseases (Wood, J.P. et al. 2002; Thude, H. et al. 2004). These polymorphisms lie within or near the exon 4 or 6 and reduce their activity. Analysis of T cells from individuals with constitutive exon 4 expression showed increased Lck activity, decreased Y505 phosphorylation, and increased TCR signalling (Do, H.T. et al. 2006), consistent with the idea that CD45RABC has increased activity.

1.5.3. Apoptosis controlled by alternative splicing

Members of the Bcl-2 protein family are the central regulators of the intrinsic mitochondrial apoptotic pathway. At least 12 core Bcl-2 family proteins which contain 3 or 4 Bcl-2 homology (BH) domain are identified in mammals. These are classified into 2 sub-groups (Table 1.3) based on their activities either opposing apoptosis, like Bcl-2 itself, or promoting apoptosis, like Bax and Bak. A divergent group of proteins containing only the BH3 domain includes Bim, Bad, Bik, and makes up the third sub-group of the Bcl-2 family (Youle, R.J. and Stasser, A. 2008). Pro-apoptotic Bax and Bak are essential for inducing permeabilization of the outer mitochondrial membrane (OMM) and releasing apoptogenic molecules like cytochrome C to activate caspase, whereas anti-apoptotic members like Bcl-2 and Bcl-xL inhibit Bax and Bak. The BH1, BH2 and BH3 domains of Bcl-2 members fold to line a hydrophobic pocket that can bind BH3-only peptides. The BH3 domain, particularly among the BH3-only proteins, mediates interaction between the BH3-only proteins and core BCL-2 family proteins. BH3 only proteins may either bind and inhibit Bcl-2 anti-apoptotic members resulting in the derepression of Bax and Bak (Willis, S.N. et al. 2007; Hinds, M.G. et al. 2007), or, directly activate Bax and Bak leading to apoptosis (Youle, R.J. and Stasser, A. 2008).

	Name	Domains
Anti-apoptotic	Bcl-2	BH1-4, TM region
	Bcl-XL	BH1-4, TM region
	Bcl-W	BH1-4, TM region
	A1	BH1-2
	Mcl1	BH1-3, TM region
	Bcl-RAMBO	BH1-4, TM region
	Boo/Diva/Bcl-b/Bcl2l10	BH1-2
	Bcl-G	BH2-3
Pro-apoptotic	Bax	BH1-3, TM region
	Bak	BH1-3, TM region
	Bok	BH3, TM region
BH3 only	Bid	BH3
	Bim/Bod	BH3
	Bad	BH3
	Bmf	BH3
	Noxa	BH3
	Hrk/Dp5	BH3, TM region
	Puma	BH3
	Bik/Blk/Nbk	BH3, TM region
	Mule	BH3, UBA, WWE, HECT

Table 1.5: Bcl-2 family members

Bcl-2, Bcl-xL, Bcl-W, A1 and MCL1 are considered to have anti-apoptotic functions in vivo. The three proteins of Bcl-RAMBO, Boo, and Bcl-G are less well studied and therefore not categorized. Bax, Bak, Bok, Bid and BH3 only proteins are considered pro-apoptotic. MULE contains a ubiquitin-associated domain (UBA), the Trp-Trp-Glu interaction module (WWE) and a HECT ubiquitin ligase domain. Bid has a unique role as both a Bcl-2 homologue and a BH3-only protein and links the intrinsic and extrinsic apoptosis pathways (Youle, R.J. and Stasser, A. 2008).

Apoptosis plays essential roles in regulating T cell development. Developing thymocytes are subject to apoptosis if they fail to bind self-peptide/MHC with low avidity, or bear strongly autoreactive TCR.

Almost every Bcl-2 family member has evidence of multiple mRNA splicing variants. The prototypic member, Bcl-2, has two isoforms. The larger Bcl-2 α form contains a hydrophobic transmembrane domain within the C terminus, enabling it to target mitochondrial membranes and prolong cell survival. The short Bcl-2 β isoform has no transmembrane domain, resides in the cytosol and exerts no effect on survival or apoptosis (Tsujimoto, Y. and Croce, C.M. 1986; Tanaka, S. et al. 1993).

Another important anti-apoptotic molecule, Bcl-x, has been reported with 3 splice variants. Bcl-xL contains four BH domains and the C-terminal transmembrane domain that helps to localise the molecule to the mitochondria and facilitate its anti-apoptotic function (Boise, L. et al. 1993). Bcl-xS is the smaller isoform lacking the BH1 and BH2 domains due to utilising an alternative splice donor site in exon 2. Bcl-xS also has the transmembrane domain and localises to mitochondria, but antagonises cell survival and induces apoptosis (Boise, L. et al. 1993). A third Bcl-x isoform lacks the transmembrane domain, but its function has not been defined (Ban, J. et al. 1998).

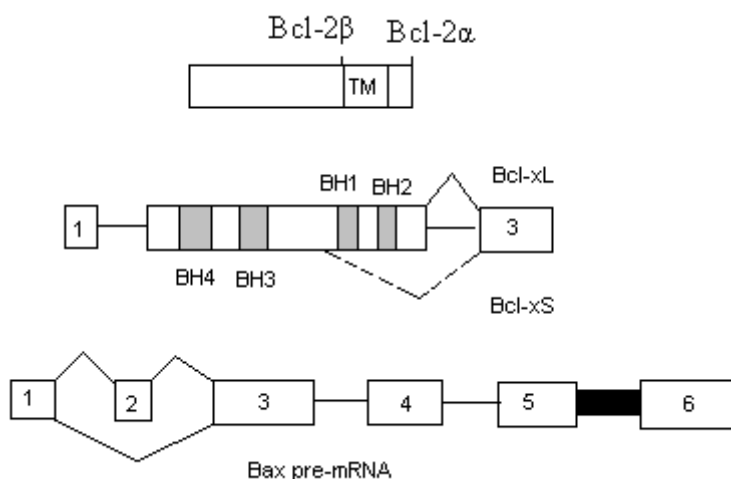


Figure 1.5.3a: Alternative splicing of Bcl-2, Bcl-x and Bax (Akgul, C. et al. 2004; Jin, K.L. et al. 2001; Shin, C. and Manley, J.L. 2004;)

The pro-apoptotic gene Bax also has multiple isoforms. Bax α contains BH1, BH2, BH3, and the membrane anchor domain and colocalises with mitochondria to prolong apoptosis; Bax β has no transmembrane domain, therefore localises in cytoplasm, but still induces apoptosis. Other Bax isoforms have been reported but their functions are still not clear (Oltvai, Z.N. et al. 1993; Wolter, K.G. et al. 1997; Apte, S.S. et al. 1995; Zhou, M. et al. 1998; Schmitt, E. et al. 2000).

Among the eight BH3 only proteins Bim exhibits a variety of splice variants showing different apoptotic activity (Bouillet, P. et al. 2001; Adachi, et al. 2005; Figure 1.5.3b). The best studied isoforms are the three initially identified: Bim_{EL}, Bim_L, and Bim_S (O'Connor, L. et al. 1998). In the human transcript, Bim_S contains exon 2 as the initial transcript site, exon 8 encoding BH3 domain, and exon 11 with the stop codon. Bim_L has an additional exon 4 encoding the Dynein-binding domain; Bim_{EL} is the largest of the 3 isoforms and includes both exon 4 and exon 3 plus the shared regions. Absence of the Dynein-binding domain in Bim_S results in failure to

bind Dynein-light-chain (DLC) proteins and the Dynein motor complex. This could be the reason why Bim_S has the strongest activity to induce apoptosis because mutation in Bim_L abolishing the interaction with DLC1 functions like Bim_S (Ley, R. et al. 2004). Bim_L has moderate pro-apoptotic activity and Bim_{EL} has the weakest apoptotic activity. More Bim isoforms have been reported in different cell lines, some of them have been demonstrated with pro-apoptotic effect when over-expressed, but only Bim_S, Bim_L and Bim_{EL} are readily detectable at the protein level (Marani, M. et al. 2002; Chen, J.Z. et al. 2004; Miao, J. et al. 2007).

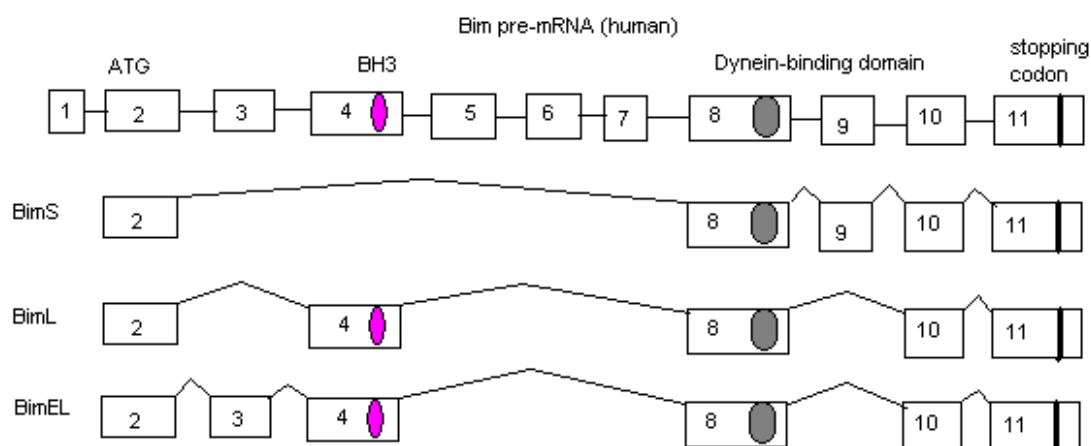


Figure 1.5.3b: Bim transcript isoforms (Adachi, M. et al. 2005)

The death receptor Fas (CD95/APO-I) is a major player in the extrinsic apoptosis pathway in T cells to help mediate activation induced cell death in peripheral T cells. Fas mRNA undergoes alternatively splicing to generate two isoforms. Inclusion of exon 6 encoding the transmembrane domain encodes membrane bound Fas (mFas) which mediates apoptosis upon ligation of Fas ligand (FasL). By contrast, silencing of exon 6 generates a soluble Fas (sFas) receptor that can bind to FASL and prevent

programmed cell death (PCD) through inhibition of Fas signalling (Krammer, P.H. 2000; Cheng, J. et al. 1994; Cascino, I. et al. 1995).

Transcriptional and post-translational regulation of the expression and activity of apoptotic molecules have been extensively studied, while post-transcriptional modification is still poorly understood (Puthalakath, H. and Stasser, A. 2002). Transcriptional regulation of Bim is complex, cell-type specific, and responds to several signalling pathways. Bim is transcriptionally up-regulated by FOXO3A in haemopoietic cells upon cytokine withdrawal. In the presence of survival cytokines, FOXO3A is repressed by PI3K/Akt mediated phosphorylation (Dijkers, P.F. et al. 2000). Bim is also kept low in growth factors stimulated cells by ERK-mediated phosphorylation, which targets Bim for ubiquitination and proteasomal degradation (Ley, R. et al. 2005).

HnRNPs and SR proteins play critical roles in alternative splicing of apoptosis regulators. Garneau demonstrated that hnRNP F/H is responsible for alternative splicing of Bcl-x and the shift between anti-apoptotic Bcl-xL and pro-apoptotic Bcl-xS (Garneau, D. et al. 2005). Massiello identified two cis-elements for Bcl-x exon 2 splice donor site selection (Massiello, A. et al. 2004) and showed that the splicing factor SAP155 can bind to the cis-element 1 at a purine rich sequence which is a typical SR-protein dependent ESE (Massiello, A. et al. 2006).

Also implicated in regulating Bcl-X splicing and apoptosis is Sam68, a member of Signal Transduction and Activation of RNA (STAR) metabolism family and a target of Src tyrosine kinases (Taylor, S.J. and Shalloway, D. 1996; Lukong, K.E. and

Richard, S. 2003). Sam68 contains a KH domain (hnRNP K homolog) and a highly conserved GSG (GRP33/Sam68/GLD1) domain which is required for sequence specific RNA binding and homodimerization (Lin, Q. et al. 1997; Chen, T. et al. 1999). Sam68 has been shown to induce CD44 exon V5 inclusion (Matter, N. et al. 2002). It was also shown that Sam68 has a role in inducing apoptosis (Taylor, S.J. et al. 2004). Recently, Paronetto, M.P. et al showed that Sam68 modulates apoptosis through binding to Bcl-x pre-mRNA and favouring the selection of Bcl-x upstream 5' splice site to produce pro-apoptotic Bcl-xS isoform. Furthermore, interaction with hnRNP A1 is required for Sam68's effect on Bcl-x alternative splicing, whilst ASP/SF2 counteracts Sam68's splicing activity (Paronetto, M.P. et al. 2007).

Fas pre-mRNA alternative splicing involves another RNA-binding protein, TIA1. TIA1 recruits U1 SnRNP to a weak 5' splice donor site in Fas exon 5 and activate exon 6 inclusion of Fas transcript, facilitating generation of pro-apoptotic mFas isoform (Forch, P. et al. 2000). Izquierdo found that TIA1 and PTB have antagonistic effects on regulating Fas exon 6 splicing. TIA1 binds to an intronic splicing enhancer to promote U1 SnRNP binding to the adjacent 3' splice acceptor site of exon 6, whereas PTB represses U2AF binding to the polypyrimidine tract upstream of Fas exon 6 resulting in exon skipping (Izquierdo, J.M. et al. 2005).

1.6 Focus of this thesis

In this thesis I will describe the characterization of a novel ENU variant mouse strain called *thunder*. The strain was identified through a peripheral blood leukocyte screen using multicolour flow cytometry and the affected mice had significantly reduced numbers of peripheral CD4⁺ and CD8⁺ T cells. Subsequent mapping revealed a mutation in a previously unknown member of the hnRNP protein family, *Hnrpll*. Further analysis of the *thunder* mice revealed that the *Hnrpll* gene is a critical trans-acting regulatory factor that coordinates the alternative splicing of the three variable exons of CD45 mRNA encoding exons 4, 5 and 6.

The aims of the thesis were:

1. To characterize the cellular phenotype of the *thunder* mice to help identify a cellular biomarker that would facilitate mapping and identification of the mutant gene.
2. To map and identify the ENU variant gene responsible for the loss of peripheral T cells and the change in CD45 alternative splicing.
3. To investigate the effect of the *Hnrpll* mutation on T cell homeostasis in vivo
4. To investigate if the *Hnrpll* mutation has any effect on positive selection of antigen-specific T cells.

The studies presented in this thesis provide new insights into the regulation of mRNA splicing in T cells and the regulation of T cell homeostasis. The *thunder* strain has provided a unique resource that has enabled us to address a fundamentally important question within the immune system which until now has been intractable to

analysis at the molecular and cell biological level. The *thunder* strain has allowed us to gain insight into the diversity of alternative splicing and how it is used to control T cell homeostasis as well as the generation of T cell effector responses and long lasting immune memory.

~Chapter 2~

Material and Methods

2.1 Mice

Some major mouse strains used in this study are described below:

C57BL/6S (B6): inbred mouse strain was originally obtained from Stanford University (CD45.2 or Ly5b).

C57BL/6S:Ly5a: B6 strain congenic for CD45.1 (Ly5a).

CBA: inbred mouse strain.

RAG1^{-/-}: RAG1 deficient mouse strain were bred on the C57BL/6 background and are devoid of mature T and B cells due to a null mutation in the RAG1 gene which prevents antigen-receptor rearrangement to occur in immature lymphocytes.

Lochy mice: A CD45 (*Ptp^{rc}*) mutant mouse strain identified by Adele L. Yates from an ENU mutagenesis screen carried out to identify mutations that affect lymphocyte development and homeostasis (Nelms, K.A. & Goodnow, C.C. 2001). It contains a T to A conversion of the intronic nucleotide 93085 of the genomic DNA sequence in the 5' end of exon 17 boundary resulting change of splice site, insertion of 5 base pairs to the transcript, shift the open reading frame in the PTPase D1 domain, and eventually cause CD45 low expression because of nonsense mediated decay (NMD) (Yates, A.L. 2003).

Jasmine mice: This is a novel ENU variant strain that was identified in the same screen as the *thunder* and *lochy* strains. It was determined that the *Jasmine* mice have a missense mutation in the Transporter of Antigen Presentation 2 (*Tap2*) gene and these mice have been maintained on a C57BL/6 background. The *Tap2* transporter plays critical roles in delivering antigenic peptides from the cytoplasm into the endoplasmic reticulum where they can be eventually loaded onto MHC class I molecule. The *Jasmine* mice have defective CD8 T cell development because MHC class I complexes cannot be formed and presented on the surface of antigen-presenting cells in the thymus or periphery. In contrast CD4⁺ T cell development is unimpeded since the *Tap2* gene does not affect peptides loaded onto the MHC class II molecules.

3A9 TCR transgenic mice: TCR $\alpha\beta$ transgenic mice produced in C57BL/6J mice were backcrossed more than seven generations to B10.Br/SgSnJ (B10k JAX). It expresses a rearranged TCR $\alpha\beta$ chain (V β 8.2 and V α 3) and directs thymocyte development towards CD4 lineage bearing transgenic TCR, which is specific for the immunodominant peptide of HEL (Hel₄₆₋₆₃) complexed with the major histocompatibility molecule (MHC) I-A^k.

InsHEL transgenic mice: The ILK-3 transgenic mice express the hen egg lysozyme (HEL) protein under the control of the rat insulin promoter and this directs expression of the HeEL proteins to pancreatic islet β cells acting as a neo-self antigen. The ILK-3 strain produced in C57BL/6J mice was backcrossed more than seven generations to B10.Br/SgSnJ (B10k JAX).

3A9 TCR:insHEL double transgenic mice are generated by breeding 3A9 TCR tg mice x Ilk-3 Hel tg mice. Most of the Hel-specific CD4⁺ T cells are deleted during negative selection in the thymus and residual circulating transgenic T cells are functionally anergic. This mouse model is ideal to study the regulation of organ-specific autoimmunity (Hoyne, G.F. and Goodnow, C.C. 2006).

All mice were bred and purchased from the Australian Phenomics Facility, Australian National University. All mice were housed in specific pathogen free conditions and all animal procedures were approved by the Australian National University Animal ethics and Experimentation Committee.

2.2 Flow cytometry

2.2.1 Cell surface staining

Cell suspensions of spleen or thymus were prepared by sieving and gentle pipetting. For surface staining, cells were maintained in the dark at 4°C throughout. Cells were washed twice in ice-cold FACS buffer (2.5%FCS, 0.02% NaN₃ in PBS), then incubated with each antibody and conjugate layer for 30 min and washed thoroughly with FACS buffer between each layer. Data were acquired on a FACS Calibur[®] flow cytometer, and analysed using FlowJo software (Treestar). Cells were sorted on a FACSVantage (BD).

The commercial antibodies were used (all from BD except where otherwise indicated): CD3ε-FITC, CD4-PerCP, CD4-APC, CD4-FITC, CD8-FITC, CD8-PE (Caltag), CD8-PerCP, CD44-FITC, CD44-PE, CD44-APC, CD45.2-FITC, CD45.2-

APC, CD45.1-FITC, CD45.1-PE, CD45RA-biotin, CD45RB-FITC, CD45RC-PE, CD62L-biotin, B220-PerCP, B220-APC, B220-PE, B220-biotin, CD19-PE, AA4.1-FITC, CD19-PE, CD25-APC, CD69-FITC, CD69-biotin, CD90-PE, CD95-PE, CD5-PE, CD127-PE, CD122-FITC, CD132-PE, anti-TCR β -FITC, 7AAD (Molecular Probes), Ly5a-PE, Ly5a-biotin, Ly5b-FITC. Secondary antibodies and streptavidin (SA) conjugates were: rat-anti-mouse IgG1-APC, rabbit anti-rat-IgG-biotin (DAKO), SA-PE, SA-APC and SA-Cy-chrome.

The 1G12 monoclonal antibody is a clonotype specific mouse IgG raised against 3A9 TCR produced from the 1G12 cell-line that was a gift of E. Unanue and D. Peterson from Washington University St. Louis, MO. The cell-line was grown in RPMI with 10% FCS and PGS for 3 days post-confluence. The supernatant was purified and used at 1 in 5 dilution for cell surface staining. Rat-anti-mouse IgG1-APC was used as the secondary antibody.

Solutions used for flow cytometry:

FACS wash: PBS + 2.5%FCS +/- 0.1% azide.

Red Blood Cell Removal Buffer (Tris-Ammonium Chloride, TAC):

Tris Base 2.06g

NH₄Cl 7.47g

MilliQ H₂O make up to 1 L before adjust pH to 7.2.

2.2.2 Intracellular staining

Antibodies include IL-4, IL-5, IL-17, IFN- γ , Foxp3-FITC, Lck-FITC, pLck505-FITC, Tyr416 (Cell signalling), and Bcl-2-Biotin. Isotype control and

suitable secondary antibodies were performed. Foxp3 intracellular staining kit (BD Pharmingen) was used following manufacturer's instruction. Data were acquired on a BD LSRII flow cytometer, and analysed using FlowJo software.

2.2.3 Cell sorting

The whole spleen cells from 6-8 weeks old female mice were stained with CD4-FITC, CD8-PE, and CD44-APC in sterile FACS wash buffer. Naive and memory T cells were sorted on a FACS Vantage (BD), gated on CD44^{lo} and CD44^{hi} respectively and collected in sterile medium. 2-5 millions of cells were collected from a pool of 3 sibling mice with same genotype. Total RNA were isolated immediately after sorting for Real-time PCR and exon array experiments.

2.3 Genome mapping and sequencing

2.3.1 Genomic DNA isolation from mouse tails:

Approximately 0.5 cm tails were collected and cut into small pieces in 1.5 ml eppendorf tubes, add 300 µl tail DNA lysis buffer with 3 µl of 20 mg/ml proteinase K (Merck catalog: 1.24568.05000, added before use) and incubated at 56 °C for at least 2 hours. The samples were centrifuged at 13000 rpm for 10 minutes, and the supernatant was transferred to new tubes. Saturated NaCl (6M) 150 µl per tube was added and mixed by inversion till the sample turned uniformly cloudy. The sample was centrifuged at 13000g for 10min and the supernatant was added to a new tube to which 450µL of ice cold isopropanol. The samples were vortexed and centrifuged as above for 1 minute. The supernatant was discarded and the pellet was washed with 1

ml 70% ethanol. The DNA pellet was resuspended in 100 µl TE buffer or ddH₂O. DNA samples were quantitated using Nanodrop Spectrophotometer (ND-1000, Nanodrop Technologies, USA).

Solutions:

Tissue Lysis Buffer:

[50mM] 1M Triris pH 8.0	50mL
[100mM] 0.5M EDTA	200mL
[0.5%] 10% SDS	50mL
MilliQH ₂ O (Sterile) make up to 1000mL	

TE buffer:

1M Tris, pH8	10ml
0.1M EDTA	1ml
MQ water make up 1L, use after autoclaved.	

2.3.2 Genome linkage screening by PCR using SSLP markers:

DNA from (B6 x CBA)F₂ intercross affected mice was analysed by PCR amplification of Microsatellite (MS) markers obtained from the MIT database based on genomic spacing and reliable discrimination between B6 and CBA strains. PCR using MS markers can be used to amplify approximately 6500 different Simple Sequence Length Polymorphisms (SSLPs) commonly dinucleotide repeats throughout the genome within inbred mouse strains to identify different genomic regions (Rust, S. et al. 1993; Schlüter, B. et al. 2002). Figure 2.1 shows an example of a SSLP in a particular region between B6 and CBA strain that can be amplified by MS-PCR and visualised by different product size when read on gel electrophoresis.

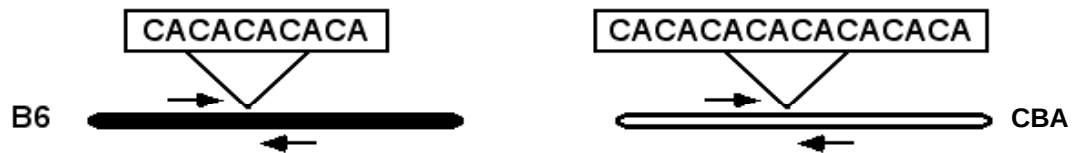


Figure 2.1 an example of SSLP between B6 and CBA strain

Whole genome linkage screening was performed by Mr. Adam Hamilton (Genotyping and mapping group, IGL) using a set of markers spaced 30-40 cM distance from one another. Primers and PCR conditions are listed in below.

Table 2.1 Microsatellite markers (SSLP) used for B6xCBA genome mapping:

Markers	cM	F primer	R primer	B6 (bp)	CBA (bp)
D1 Mcg163	35	AGCCTGACTCAACAAGA CAG	GTGAACTGTGTACTCTCC TC	261	289
D1Mit42	78	CTCAGGCACCATTCTAAA CATG	ATAGGGCAAAAAACATT CTGC	180	200
D1Mit155	112	ATGCATGCATGCACACGT TATTTCCCTGTCACTCCT CCC	ACCGTGAAATGTTCACCC AT	252	216
D2Mit102	52	ATGCCTTAGAAGAATTTG TTCCC	TGTCTTTATGCTCAGACAT ACACA	162	180
D2Mit15	50	TTTTTGCAACTTTGTAA GAATTCC	CTTGAAAAACACATCAAA ATCTGC	145	160
D2Mit226	96	GGGATTTTAAAGCAAAGC CC	AAAACACCCTCCCACCCT T	102	124
D3Mit268	13.8	CAGTCCCCAAGACACAC ATG	TCATGCACACACATGAAC ATG	106	122
D3Mit120	28	GGCTCGCTGGTTGGTTTT AC	TACAGGATTGAGGCTAGT TTTCG	148	160
D3Mit102	49.7	CCTTCTGACAAGCAGGAT TTG	GGGCTCCTACATGCATGA AT	152	142
D3Mit127	70.3	TGCAGGTAGGGGCTCAA G	TTTCTAGCATCTCCAAGC AGG	176	262
D4Mit172	8.6	TATGCATTAGTCTAGGTG GTAACAGC	AAGCAGATCTGGCTTCCT CA	137	123
D4Mit288	28.6	GATCACCCAAGGCTGGC TCCTCTGCCAGCTCTCTC TG	TTAGCCATGTAGGCAATG CA	116	100
D4Mit16	59.1	TCCCTGCTGCAAGCTCTCTC TG	TCCCCGTGAACTTCCATC CTGCAAGGAGGTGATGTT CA	217	235
D4Mit62	79	CTGACCAGAACACAGCA TAGTACA	TTTAAAATAGGAAAAGCAT TCTTTCC	190	170
D5Mit348	8	AATAAGAAAATTCCACCA AGTCTACA	CTTGATGGGTCTGATGCC TT	123	115
D5Mit391	26	AAAGGGGGTCTTCTTTG GAA	TCTCCTGTAGTGGGTGGT TT	148	178
D5Mit7	45	CTGAGGTGTATGTATGTT GCATATATG	TCCGGTCTCAGTACATGT ACAA	119	149
D5Mit320	70	AGTCAGAATATGGCAAGT CAGTG	TTTCAGAGTCTTTCTTTCA GTATCTCC	125	111
D6Mit268	15.6			123	110

D6Mit61	62.5	TACAGAGGCTAGAACACT CCTGG	CACTTGTTGCTCCTGCTG AG	135	146
D7Mit230	24.5	GGGTAACTGCTTTTAA AAGTGC	ACTTCTGCATGTTGCCCT CT	107	91
D7Mit40	53	GTCAACAGTCAGGAAAG CTGG	CAGATGCTTGTATTTGCA AAGC	204	228
D8Mit224	17	ACGTCCCCAGTGCTTAAC AC	TGGTGTAGCATAAAGCAT ATGTCC	326	454
D8Mit56	75	AACTCAGAGACCATGA GTACACC	GAGTTCCTACCCACAAG TCTCC	160	180
D9Mit129	26	TTGTCTTTAACCTCCTG GAGC	TCCCATCTTTCTCCTTGTG G	132	150
D9Mit115	56	TCCAGACTCCTGGGAAC TACA	TTTCCCAGCCAGTAAAGG C	145	137
D10Mit3	21	GTTGATAGTCCCACCTCA CTCA	TGAGAAATTCCATCTGTG GC	260	210
D10Mit70	59	ACCTTTCTTCCTACCTAC CTACCT	TGGCACTTAGAACTGAT AAATGG	144	148
D11Mit21	20	GAGAGCTCTTTCACACTT GAAGG	CAGAAAGGCTCCTACCTC AGG	156	146
D11Mit123	58	AGAAGAAACAAGAGCTG ATGGC	GCTTCTTGCCAAGTCAGG AC	310	335
D12Mit236	22	TGTTGCTAATCACAGTCC ATATCC	GTTGGCTTCTGGCTTCTG AC	121	109
D12Mit132	52	CCATATACATTTCTAACA CCCTTGC	AGAACTTACTTCTAGTGG AGACAATGC	131	112
D13Mit88	21	ACTGATGGCTCATGAGA CCC	AAAATTAATAGGAACTGC AAGGG	170	180
D13Mit211	58	TTTGCAAAAGACAAGACT TATTGC	AAAAGACATCCAGTTCTC AATGG	118	126
D14Mit10	3	CTCCTCCCCTCCCTACCT C	AGCACGGAATTACGGAAC AC	114	132
D14Mit115	40	AACTCTGCACTGTGGCA GG	AGTTTGTAAAGAAAAGGAA GCAAATG	113	137
D15Mit122	34	ACCCAGTGCAGGTGCTC TAT	GGAGAATACAAGTTATGC CTTAGACC	150	146
D15Mit108	55.6	TCCCATGTTACTCAGGAA TGC	GCAGAAGACATTCACAGT CTTAGG	130	140
D16Mit103	22	GGTGTGCATACACACAT GCA	TGACTAGACTTGACTCCT CCACC	111	126
D16Mit6	63.2	CAGGTCCAAGAGGAGAA CCA	TTTGACCTGTGAGCCTGT GG	191	200
D16Mit70	57	GGATCTATATGCTATAGA ACCATTCA	GTCATCAATTCCATTTCT AATATAGA	189	169
D17Mit133	10.4	TCTGCTGTGTTACAGGT GA	GCCCCTGCTAGATCTGAC AG	180	155
D17Mit38	45.3	CCTCTGAGGAGTAACCA AGCC	CACAGAGTTCTACCTCCA ACCC	110	92
D18Mit202	22	CCCCTTGCAAAGATGGA GTA	CATTTGCTAGTTTAGCAG GAAGC	111	143
D18Mit184	41	CACACATGTGTAGGTAG GTAGGTAGG	CGCACAAGGACTACTGAA ACA	172	127
D19Mit16	15	TCTTAGGTAATCTCCCTT AGGGG	TGGTAAATGTAAAAGTGA AGCATG	118	136
D19Mit1	52	AATCCTTGTTCACTCTAT CAAGGC	CATGAAGAGTCCAGTAGA AACCTC	121	142

PCR mix:

DNA sample (10-20ng/ul):	2 µl
10X Buffer:	5 µl
dNTPs (10mM):	1 µl

Forward primer (10 μ M):	1 μ l
Reverse primer (10 μ M):	1 μ l
ddH ₂ O:	39 μ l
Taq Polymerase:	1 μ l

PCR program (optimised in some reactions):

Steps	Temp (°C)	Time (min)	# of cycles
1. Initiation/Melting	94	2	1
2. Denaturation	94	0:30	35 (steps 2-3-4 will cycle in sequence)
3. Annealing	55	0:20	
4. Elongation	72	0:30	
5. Finish (i.e., 10oC,)	10	hold	1

Solutions:

10x buffer: 10X PCR buffer

2M Tris.Cl pH 8.8 (Tris base Roche # 1 814 273)	3.35 ml
1M (NH ₄) ₂ SO ₄	1.6 ml
2 ME (mercaptoethanol)	70 μ l
1M MgCl ₂ (Final Conc = 2.0mM MgCl ₂ / PCR reaction)	200 μ l
MilliQH ₂ O (Sterile):	4.780ml

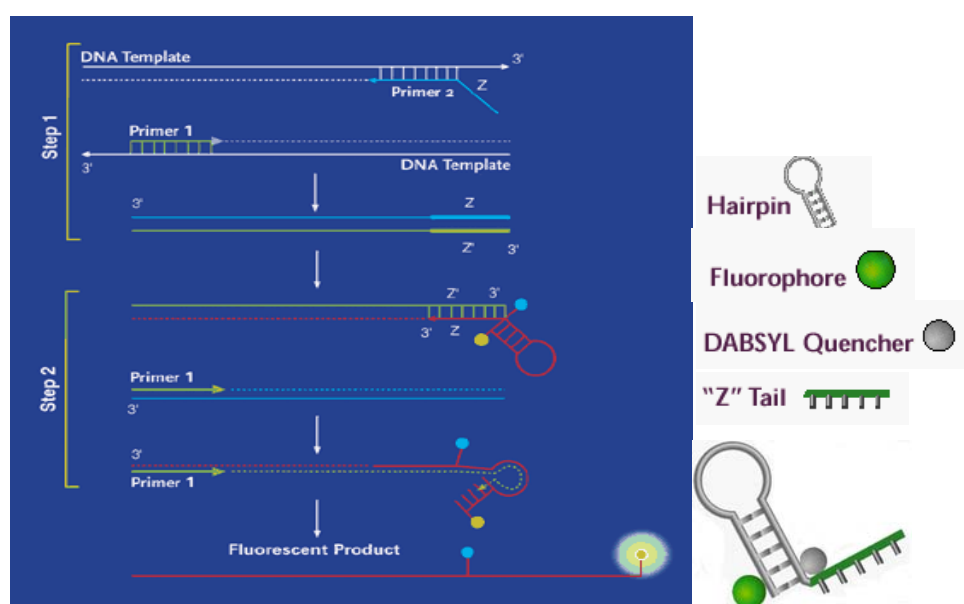
2.3.3 Fine mapping

Fine mapping was performed by expanding the Microsatellite marker panel, and also by using more markers including Single Nucleotide Polymorphisms (SNPs) markers between C57BL/6 and CBA inbred strains, Samples from a larger cohort of affected and unaffected F2 mice were used to delimit the locus containing the ENU variant gene. During the mapping process the Genotyping lab had moved toward using SNP markers routinely for fine mapping of ENU variant genes. The SNP

markers were also used to map the polymorphism of distal end of chromosome 17 between B6 and CBA strains using Amplifluor technology (Flowgen Bioscience).

Amplifluor® Universal Systems are based on energy transfer from an excited fluorophore to a complex acceptor moiety resulting in quenching of the fluorescence. The quenching is accomplished by linking the fluorophore and the acceptor 4-(dimethylamino) azo benzene sulfonic acid (DABSYL) to an oligonucleotide primer. Each reporter UniPrimer™ is composed of a 5' hairpin region with a unique energy transfer pair and a different 3' primer sequence. The primer sequences enable the UniPrimer to work with specific unlabelled primers that can be used to amplify specific sequence of interest. The target primers are synthesised with a 5' tail sequence complement to the 3' region of a particular Uniprimer™, allowing hybridisation of the Uniprimer served as a template for DNA polymerisation. The hairpin structure unfolds to double strands and disrupts the energy-transfer between the fluorophore and the quencher so as to emit fluorescence (Figure 2.2).

Figure 2.2 Schematic Amplifluor PCR (Adopted from <http://www.flowgen.net>)



Mrs. Belinda Whittle from the genotyping and mapping group of IGL helped to identify SNPs between C57BL/6 and CBA mouse strains from the Wellcome Trust Centre for Human Genetics database of mouse SNP genotype set (www.well.ox.ac.uk/mouse/INBREDS), and designed Amplifluor primers based on SNPs through Amplifluor™ Assay Architect™ at <http://apps.serologicals.com/AAA/>. SNP primers and more MS markers used for fine mapping of chromosome 17 were listed below in table 2.2 and table 2.3 respectively.

Table 2.2 SNP primers used for fine mapping of distal chromosome 17

JAX SNP ID (chr-position-source)	RefSNPs ID	New Chr position (bp)	Allele-specific primer 1/primer 2/reverse primer sequences
17-077624509-N	rs4231663	76,987,367	GAAGGTGACCAAGTTCATGCTCCAACTACAGCAGCATTTCACTGG
			GAAGGTCGGAGTCAACGGATTCCAACTACAGCAGCATTTCACTGA
			GACTTTCCACCTCAGAGTCTCAT
17-080241937-M	rs3676709	79,603,774	GAAGGTGACCAAGTTCATGCTACTTCTCCAGTTGGTCCTCACA
			GAAGGTCGGAGTCAACGGATTTTCTCCAGTTGGTCCTCACG
			CCCTGGCTACAGAAGATTCTGAGAGTCAAT
17-083294998-M	rs3707550	82,660,173	GAAGGTGACCAAGTTCATGCTTGCTGGGTTGATTAGCCAAGACA
			GAAGGTCGGAGTCAACGGATTTGCTGGGTTGATTAGCCAAGACT
			ATCCCTCACTGCGGAAGGAGAAT
17-086110517-N	rs4231722	85,479,538	GAAGGTGACCAAGTTCATGCTGCATCTGTGAGAGTGTGACAGC
			GAAGGTCGGAGTCAACGGATTGTGCATCTGTGAGAGTGTGACAGT
			GTCTTCACATGGAAGGAGGCTA
17-090405932-M	rs3675244	89,770,264	GAAGGTGACCAAGTTCATGCTTCAAGCTAACAGCCTAGCACAC
			GAAGGTCGGAGTCAACGGATTTCTTCAAGCTAACAGCCTAGCACAA
			GGACAGATTTGGTCAGCCATCCTCAA
17-091151542-M	rs3711314	90,508,757	GAAGGTGACCAAGTTCATGCTAAACCAGTCAAACAGATATAAATACAGTT
			GAAGGTCGGAGTCAACGGATTAAACCAGTCAAACAGATATAAATACAGTG
			CAAAGCATCACAGTGCTAATTGCTTT
17-093441700-M	rs3707114	92,846,090	GAAGGTGACCAAGTTCATGCTAGTACAGACTATCAAAGAATGTGCTTT
			GAAGGTCGGAGTCAACGGATTAGTACAGACTATCAAAGAATGTGCTTC
			CCACTTTGTTACTGAGACATTT

Table 2.3 MS markers used for chromosome 17

MS markers	cM	position (bp)	B6 (bp)	CBA (bp)
D17Mit133	10.40	23,455,567	180	155
D17Mit88	29.50	55,559,823	242	186
D17Mit38	45.30	72,746,147	110	92
D17Mit42	47.4	77,603,774	156	162
D17Mit123	56.7	91,943,428	133	155

Amplifluor PCR reaction:

DNA (10-20ng/ul)	2ul
DD H ₂ O	4.6ul
dNTP	0.8ul
10×Buffer	1ul
FAM primer	0.5ul
JOE primer	0.5ul
Allelic primer	0.5ul
Taq DNA polymerase	0.1ul

Amplifluor PCR program:

Steps	Temp (°C)	Time (min)	# of Cycles
1. Initiation/Melting	94	2	1
2. Denaturation	94	0:10	35 (steps 2-3-4 will cycle in sequence)
3. Annealing	55	0:20	
4. Elongation	72	0:40	
5. Elongation	72	3:00	1
6. Finish (i.e., 10oC,)	20	hold	1

2.3.4 Sequencing

Total RNA were isolated from spleen of individual wildtype and thunder mice using Trizol reagent (Invitrogen, Carlsbad, CA). Cells were homogenised or

resuspended in Trizol and then chloroform was added for phase separation. RNA was precipitated from the aqueous phase with isopropanol and 1ul Linear Acrylamide (Ambion, Austin Texas) and washed with 75% ethanol, and the RNA was dissolved in DEPC treated water. cDNA were synthesised using SUPERScript First Strand cDNA Synthesis kit (Invitrogen cat: 18080-051) following manufacturer's instruction.

Transcripts of candidate genes in the linkage interval were amplified by reverse transcription PCR using Elongase DNA polymerase kit (Invitrogen, Cat: 10480-010) following manufacturer's direction. Primers are listed below in table 2.4.

Table 2.4 Sequencing primers for genes in Chromosome 17 77.6-79.6 Mb

Genes	Forward primer	Reverse primer
Arl6ip2	gtttgacgtcagtgaggag	TAGCAGCACAGCCACTTTTG
Arl6ip2	CAAAAGTGGCTGTGCTGCTA	CTCAGCTGTTGCCTGAAGC
Arl6ip2	GAGAGGAACACACACCCAAA	TGTTCCACAGTGTTTCAGC
Arl6ip2	AGGAGTCCCAGGAAGGTGTT	GGAGAGTGGAGTCCATGAGC
46633	ACTGCCACCTCCTCCAGTT	TCAGTGCTTCAGGGGAAAAG
46633	CCTGACTCCTAACCGATGG	GGCTAACATCACCACATACTCG
66941	TGACCTTGAACACAGAGATCC	TGACAACTGTAGGACTGGAGAGA
Hnrpl	ACCGATCAGCGACATCTGAG	TCTGCAGCAAATGTCACACA
Hnrpl	AGGCTCTCGTGGAGTTTGAA	GAAGACGCTTGTGGTAATGGA
Hnrpl	TGGGCTCTAGAGATACACCTGA	GCAAGGCAACATTAGACCAA
Galm	AGAAGCTGCACACCCTGAAC	CATCTGCTGCAATGGTGACT
Galm	GCCAGACCACTCCAGTTAATC	TAAAGCAAGGCAGGTGAGTG
Rik	aaaaagcccaggatagtgactt	TGTAGGCCTGCTTCCAATTC
Rik	TACTGGGAGCTCATGTGGTG	cacagaagcacactgggttaag
sfrs7	GCCTATATAAGCGCGACTCC	TCGTGACCTGCTTCTTCTTC
sfrs7	TCGTCCCTTTGATCCTAATG	AGATGGTTGATGATCCTTCCTA
sfrs7	TCTCAAGTTCACCCGTTAGG	CCCTCTAAGCTTGACAATTCC
sfrs7	GAGTGCATCATGTCAGATCTCTA	AGGAGGAAAAACCCAAAACA
sfrs7	TTCTATTTTGCCAAGCTGGT	tcaatctctgcagatttattgaac
sfrs7	AGTAGCGCGAGGGGAAGAC	TGCGGGAGCGAGAGTATC
sfrs7	atgatggcgcttaagattcg	gggtggcctatcaaacgaga
sfrs7	cgctgccccttgatcctaa	ggttgatgatccttcttaggt
sfrs7	CTAGATCCCATTCCCGATCC	CCCAGTGATTAGGTGGCAGA
Mopt	CCTAGGGGAATCGCAGAGT	TGTGGGTGCATGACAACTTT
Gm941	TTGCATTGTAGGAAGAGCTGA	TCAGGATCAGCGAGATGTTG
Gm941	ACTGTGGCTCTGGAAC TGCT	ggcaccgtcacacacttact

Q8BSD7	TGCTTCGCGTGCTGTAGA	CTGGAGACTGGGAGCTGGAG
Sos1	GCAGCTGCCTTACGAGTTTT	GGTGAAGGCTCTTCATCAG
Sos1	TGTGCTGATAAGGTATTGATGG	GGGCTGTTATTGCTTGCTTC
Sos1	CCACTGTCTGCATTACTTTGAA	CTCCTCGGAGTCAGGTTCTG
Sos1	CTGCCCAGTGCTGAAGTGTA	TGCTTATGTGCCACTCAACTG
Sos1	AATGGGTCTGAATCCATCACT	GCCCTCTTCTGTCTTCAGGA
Sos1	ACCGTGTGTGCCTTTCTTTG	CTTCATCAGTGCCCTTGGAT
XP-355031.3	caagtcgaccggtacactaaaa	CGTGTCTCCCACGAGAAGTT
XP-355031.3	CACAGACTATGTTGCCACCAG	tcacgtggaaaagcagaaaa
Map4k3	GAACCCGGGCTTCGATCT	ATTGTACCCGCCTTTCCTCT
Map4k3	ACAATTGCCAAACGGAAGTC	CCTCTTCAGCGCCAAATATC
Map4k3	TGTGCCACATCCTGGATAAA	AATTGGGATTGACCGTCTCA
Map4k3	TACCCTCTGGTCTGTGTTGG	CTAGGTTCTGCAGGGTCTG
54957	acacccaccaggagaggag	GCAGTCTGGACGTAATGCTG
54957	TACGTAGCCGCGACATTACC	tcctggaaacttggtttt
66934	agcgagacatcaccagac	caatggtggagaagacacca
M05Rik	gaggccaagtGCATTGTGT	GGATTGGCTGAGAAAAGTGG
M05Rik	GACATCGACACCCTCATCCT	GGATAAGCGATGCAGAGACC
Thumpd2	TGAGCGCCATGGCTGTAG	TCACTCTTCTGCCTCCTGT
Thumpd2	CTCAGGACCAGGAGGACTTG	ACGCAGCACTCTTTCATTT
Thumpd2	TGACATTCCATTTGGGAAAAA	CCAAGGCAGGAAGCCTTATT
NM_178927.2	CCCAGTAGGTGACTTAAGGGTA	CTGCTCAAGGCATTCCAAGT
Slc8a1	ggttggagcagttggaagtc	GGCTGCTGTCAAAAGAACA
Slc8a1	GGTCCCTGATGGAGAGACAA	TTCAGCCATTTCCATGTTGA
Slc8a1	AGCTGATCAAGCAAGGAAGG	GAATTCGAGCTCTCCACAGG
Slc8a1	CAAACTATTGAAGGCACAGC	GGCAAACAGAACCTTCCAGA
Slc8a1	AGAAGCTGCCCTCCTGTTTT	ttgttcctTTAGAAGCCCTTT
ENSMUSG000006932	AAATCTCAGGTTTGTAGCATCC	ATGATGAGAAGGAAAGGAGTTG
66931	AAAGCCTAGGGAATGAAAGGA	AAGCTGGTTCCCAGTCTTCA
AW494914	GAACCTTGCATGACTAGGTGAA	AGAAACACTGGCGGAGAAGA
AW494914	GGCCCTGAGGATCTGTGAT	GGCCTTGCCATAAAGGAACT
AW494914	CCTACCTCTGGCTTGTCGTT	TCCCACATCCAGTCATACCA
AW494914	CAAGCACCAAGTGGTTGTCA	GCTCCCGTCTGTAAACACAT
AW494914	CAGTTTTTCTGGAGGATGC	CTCAGCAATGTTGGTGAAA
AW494914	TGAAACCTCCAATGGGTGTA	GGGAGACACAAAAGGGGACT
AW494914	GGACCCTGCTCTCACCATT	TAGGGGCTGTGCAAGTGTCT
AW494914	ATGTCCACATTACCCCTTCC	CAGTCTTCCGTGGGAGGTAG
Gmin6	cGGCGTGTGAGCTAAGTAGA	ATTGGAGCTGCTGCAGTTTT
Gmin6	AAATGGCTGGAGAAGAACCA	ccacccaggaaaaaccaata

PCR mix to amplify transcripts of candidate genes:

ddH ₂ O	34 ul
5x buffer B (or a mix of A and B for Mg ⁺ titration)	10 ul
dNTPs (10 mM)	1 ul
Amplification primer A (Forward) (10 uM)	0.5 ul

Amplification primer B (Reverse) (10 uM)	0.5 ul
<i>Elongase</i> DNA polymerase	2 ul
cDNA (10-20ng/ul)	2 ul

Programs: 94°C, 3 minutes, 1 cycle.

94°C, 30 seconds,

59°C, 60 seconds, (variable from primers)

72°C, 90 seconds, 36 cycles.

72°C, 5 minutes, 1 cycle.

Amplified cDNA products were then electrophoresed in 1.5% agarose gel, stained with EtBr, cut under UV light, and purified with Gel Purification kit (Qiagen) following manufacturer's instructions. cDNA were quantitated with Nanodrop Spectrophotometer and sequenced using BigDye terminator mix performed by Mr. Carmarone in the Biomedical Research Facility, JCSMR.

Sequencing reaction:

5x buffer (BRF)	3ul
Clean DNA (can use less if DNA product is short)	25-50 ng
Sequencing primer (10 uM)	1 ul
Big Dye mix (BRF)	2 ul (1/4x)
ddH ₂ O to 20 ul	

Program:

1. 94C – 5 min
2. 96C - 10 s
3. Ramp 1C/sec
4. 50C 5 sec
5. Ramp 0.5C/sec
- 6, 60C 4 min
7. Ramp 0.5C/sec
8. GOTO 2, 30 times
9. 10C hold

Sequencing were performed in both directions (forward and reverse), using the same primers for RT-PCR amplification of gene of interest. Sequencing results were analysed using Sequencher software.

2.3.5 Genotyping *thunder* mutation

Mrs. Belinda Whittle designed allele specific primers for distinguishing wildtype and thunder mutation in the *Hnrpl* gene to genotype the Thunder mice using mutagenically separated PCR (MS-PCR) strategy (Rust, S. et al. 1993; Schlüter, B. et al. 2002). Introduction of mutations into the PCR-primer binding regions in an allele specific manner prevents the formation of products by the error elongation of the wrong primer in subsequent cycles. The allele-specific primers introduce additional deliberate differences into the allelic PCR products in one reaction that can be identified by gel electrophoresis.

Table 2.5 Primer sequences for thunder genotyping

Primers	Sequence
F' Wt	GTCCATGTTTCGAGGGCTCTGTGAATCTCT
thu F' Mut	CTGTTTCCCCTGTTCAACATGTTTCGAGGGCTCTGTGAATGTGA
thu Rev	CATATTGTCCCAAATTTCTCCAGTGCC

Table 2.6 PCR mix of thunder genotyping:

Reagent/ Constituent	Volume (uL)
DNA Sample (ear punch)	0.5
2x Qiagen Multiplex Master Mix	5
Q solution	0.25
ddH ₂ O	3.5
Primer 1 (10 uM) Name: <i>thunder</i> F'WT	0.30
Primer 2 (10 uM) Name: <i>thunder</i> F'Mut	0.15
Primer 2 (10 uM) Name: <i>thunder</i> Rev	0.30
TOTAL VOLUME OF REACTION	10 ul

Table 2.7 MS-PCR program of *thunder* genotyping:

Steps	Temp (°C)	Time (min)	# of Cycles
1. Initiation/Melting	95	15	1
2. Denaturation	95	0:20	35 steps 2-3-4 will cycle in sequence
3. Annealing	60	0:20	
4. Elongation	72	0:30	
5. Finish	10	hold	1

The PCR products were separated by gel electrophoresis in 3% agarose gel that was ran at 400mV for about 10 minutes before staining and visualising wildtype band of 77bp and *thu/thu* band of 91bp of size using EtBr.

2.4 RT-PCR and real time PCR

2.4.1 Total RNA isolation, cDNA synthesis, and RT-PCR verifying alternative spliced transcript variants

Total RNA were isolated from thymus and lymph nodes of 3 individual wildtype and *thunder* mice using TRIZOL and chloroform, precipitated with isopropanol and 75% ethanol, and resuspended in DEPC treated water. cDNA were synthesised using SUPERScript First Strand cDNA Synthesis kit (Invitrogen) following manufacturer's instruction. CD45 transcripts were amplified with a forward primer (5'-GGC AAA CAC CTA CAC CCA-3'), and a reverse primer (5'-GCT TGC AAG GCC CAG AGT GGA TGG TGT AAG-3') both at 10uM reaction concentration using Elongase polymerase kit (Invitrogen). PCR was performed by heating the samples to 94°C for 3 minutes, followed by 36 cycles of 30 seconds at 94°C, 60 seconds at 59°C, and 90 seconds at 72°C, and finished with 5 minutes at

72°C. PCR products were electrophoresed in 1.5% agarose gel, stained with EtBr, and filmed with GeneSnap software from SynGene Company.

Some other genes were also tested including Nalp1, Bim, Fas, Mapk4, CD44, CD3 τ , Thada, etc. Primers were listed in figure 2.8.

Figure 2.8 Primers for validation of alternative splicing variants:

Primers	Sequence
Bim F	GCCAAGCAACCTTCTGATGT
Bim 1R	ACATCGACACAGTGCAGAGC
Bim 2R	AGGACTTGGGGTTTGTGTTG
Bim 3R	CCATTTGAGGGTGGTCTTCA
Bim 4R	GGCATCACCGTGGATATTTT
CD44 F	TCTTTATCCGGAGCACCTTG
CD44 R	CGCACTTGAGTGTCCAGCTA
Mapk7 F	ATACCAAAGCAGCCCTCAAA
Mapk7 R	TGGAGTGCCAGAGAACACAG
Nalp1 F	CGTGGCTGCCAACTAAAGAC
Nalp1 R	GATTGTCACTGCCCTTGTC
Thada F	AGCTTCTACTGCTGGCGATG
Thada R	GGGCGAGTGAATCTTTGTTC
CD3z F	GCACGATGGCCTTTACCAG
CD3z 1R	CCTTCTTTGAGCCACCTCTG
CD3z 2R	TATATCCGACGGGCTTTCTG
CD3z 3R	GTCATCACCGAGCTGAGCA
CD3z 4R	TTTGCTGCACAGTGTGTTAGG

2.4.2 Real time RCR

FACs sorted peripheral B cell and T cell subsets (naive and memory, CD44^{lo} vs CD44^{hi}) were sorted as described before. Total RNA was extracted using MiniRNA column (Qiagen) following manufacturer's instruction. RNA was

quantitated using the Nanodrop spectrophotometer. First strand cDNA was synthesised using SuperScript® First-Strand Synthesis System from Invitrogen (cat: 11904-018). Before cDNA synthesis, contaminating genomic DNA were removed by DNase treatment.

5× first strand buffer	2ul
RNA (50ng/ul)	7.5ul (~375ng)
DNase	0.5ul
Total	10ul

Incubate at 37°C for 20 minutes and then inactivate at 75°C for 5 minutes.

Then reverse transcript to cDNA using oligo dT primers:

RNA	10ul (~375ng) (at final conc. ~20ng/ul)
Oligo dT	1ul
DEPC treated H ₂ O	3ul
5× first strand buffer	2ul
DTT [0.1M]	2 ul
dNTP [10mM]	1 ul
RNase Inhibitor [40u/ul]	0.5ul
Superscript III [200U/ul]	0.5ul
Total	20ul

Incubate at 41°C for 1 hour and then inactivate at 90°C for 10 minutes.

Quantitative real-time PCR was performed using the TaqMan Gene Expression Assays system from Applied Biosystems (Foster City, CA), Hnrp1l and Hnrpl expression was examined relative to a housekeeping gene Ube2d1 for T cells.

TaqMan Universal Master mix	5ul
20× TaqMan Gene Expression Assay mix	0.5ul
cDNA	1ul (20ng)
DEPC treated H ₂ O	3.5ul
Total	10ul

PCR reaction was performed following manufacturer’s instruction with ABI SDS qPCR7900 machine in the Biomedical Research Facility in John Curtin School of Medical Research, using the universal thermal cycling parameters (Hold 2 minutes at 50°C, 10 minutes at 95°C, then melt at 95°C for 15 seconds and anneal/extend at 60°C for 1 minute for 40 cycles). Gene expression assays for *Hnrpll*, *Hnrpl*, and *Ube2d1* are shown as below in figure 2.3.

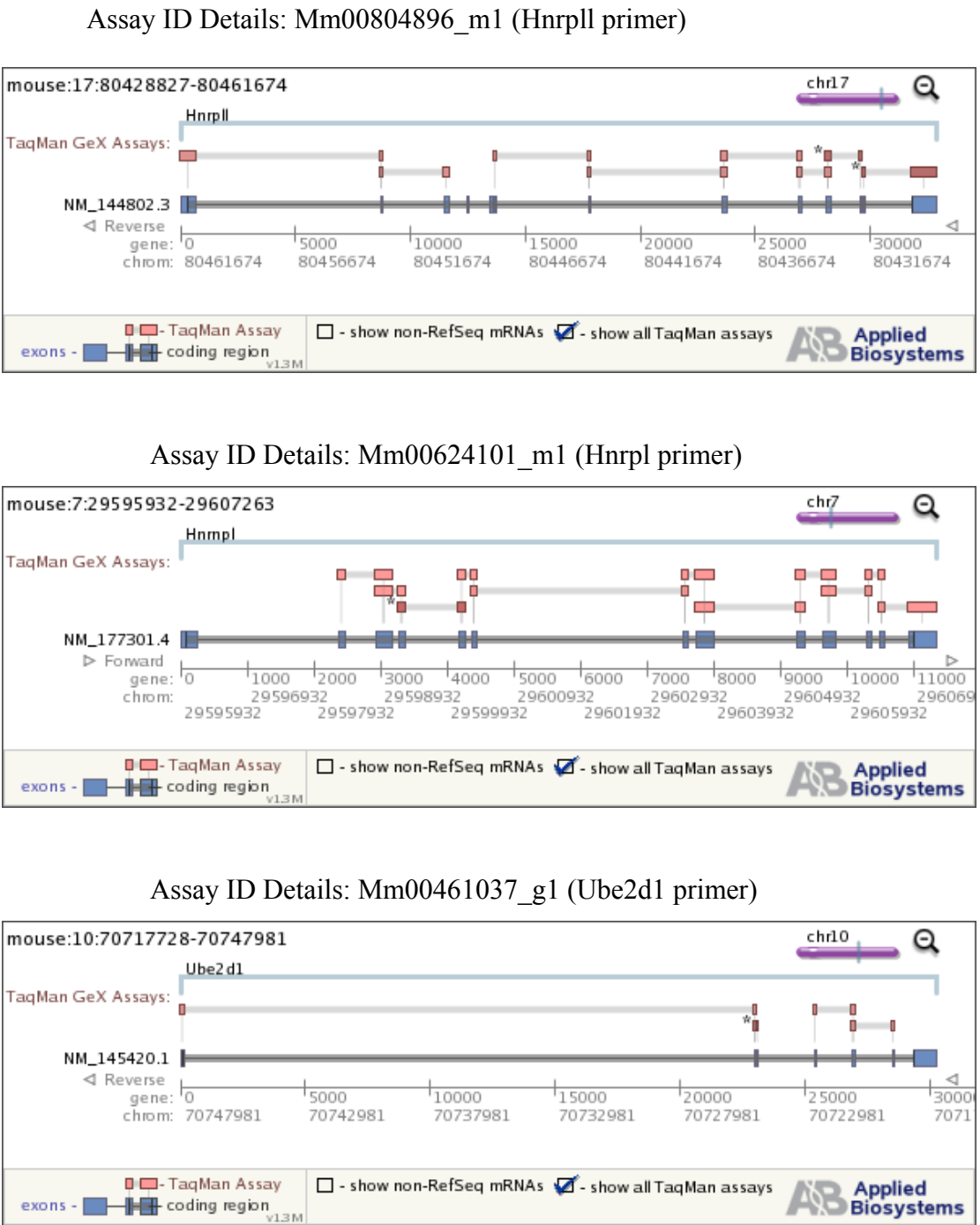


Figure 2.3 Realtime-PCR assays for *Hnrpll*, *Hnrpl*, and *Ube2d1*.

2.5 Retrovirus based gene transfer

2.5.1 Retrovirus vector

Retroviral vector pIRES-KMV is modified from pIRES bicistronic expression vector from Clontech laboratories, Inc. It contains the internal ribosome entry site (IRES) of encephalomyocarditis virus (ECMV) allowing translation of two open reading frames from a single mRNA. Ribosomes can enter the bicistronic mRNA either at the 5' end of the inserted gene of interest or at the ECMV IRES to translate the reporter gene GFP. The single cassette express both the gene of interest and GFP so all transduced cells expressing GFP also express the gene of interest. Another antibiotic resistant gene (β -lactamase) included in the vector has its own promoter so that the transfected colonies can be selected for ampicillin resistance (Figure 2.4).

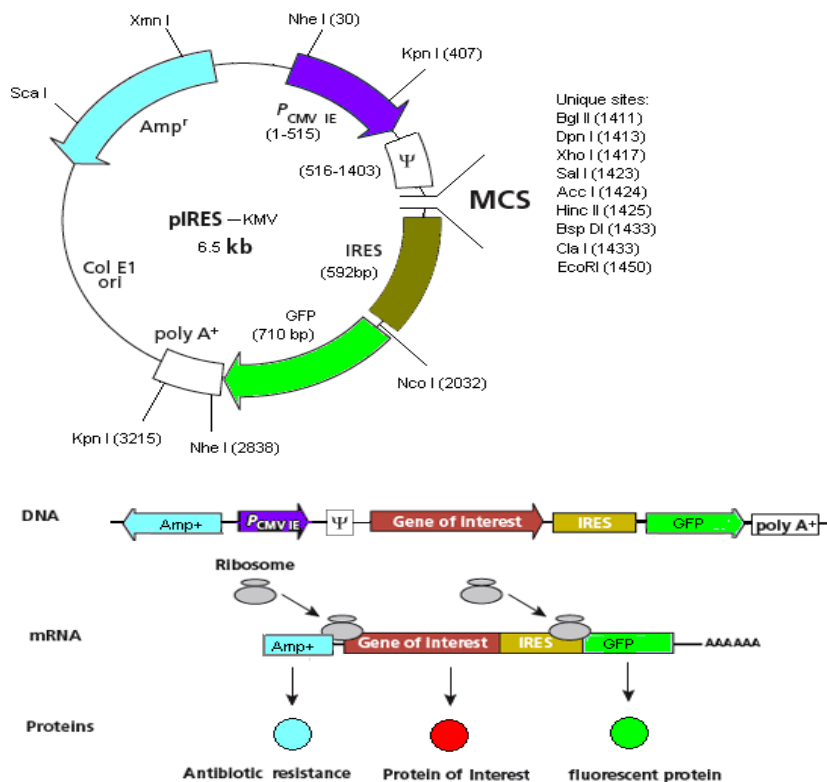


Figure 2.4 Schematic diagram of retroviral vector pIRES-KMV and bicistronic mRNA translation.

P_{CMV IE}: Human cytomegalovirus (CMV) major immediate early promoter enhancer; IRES: The internal ribosome entry site; GFP: Green Fluorescent Protein; Amp⁺: Ampicillin resistance; Ψ: packaging signal; Coli E1 ori: Coli E1 origin of replication.

Polylinkers: AccI ClaI BglII DpnI XhoI SalI HincII BspDI

AGATCTCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCGCCC

EcoRI

CCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCGGTGTGCG

2.5.2 Clone *Hnrpll* EST

Hnrp11 transcript including EST was downloaded from Ensembl mouse genome database (http://www.ensembl.org/Mus_musculus/index.html).

GAAGACCGGAAGCCACGCCCCGGCCCCATCCTTACTTTTCGCGTCCCCCCC GGGGGT GGGAA

GCCGCGAAGCTGGCGGAGGGAGAGGCTGGAGGCTGGCTGAGAGATAAAGGGAAGCGTCCC

CTCAGCCCGCGCTCCCTTCTCAGTCACGGGCTCCTGGAGCGCGCCGCGGAGGACGCCGGG

CCAGCTCAGACCCGCGCCGAGGAGGCGCAGCCGCGGGCGGGGACC GATCAGCGACATCTG

AGGCCCGTCTCCCGCGCCGTCTCCACCGCCGCCGCCATGTCCTCCTCCTCCTCCTCCTCC

CCCAAGGAGGAGACGTACGAGGAGGACCGGGAGTTCGAGAGCCAGGCCAAGCGCCTCAAG

-P--K--E--E--T--Y--E--E--D--R--E--F--E--S--Q--A--K--R--L--K-

ACCGAGGAGGGAGAGATCGTCTACTCGGCCGAGGAGAGCGAGAATCGCCAGGAAGCGACG

-T--E--E--G--E--I--V--Y--S--A--E--E--S--E--N--R--Q--E--A--T-

CCTCAGGCGGGGAGCGATAGCGACAGCGGC GGCGACGGAGGGCAGCGAGACGGAGGC

-P--Q--A--G--S--D--S--D--S--G--G--G--D--G--G--D--G--D--G--G-

AGCGGCGGCGGCGGCGACGGCGAGGAAGGCGAAGGTGGAGAAGAAGGCGACGAAGGCGAC

-S--G--G--G--G--D--G--E--E--G--E--G--G--E--E--G--D--E--G--D-

GGCGATGAGGGAGGGAGCGGCGGCGACGAAGGTGGCAGCGGCGGTGGCCCTCGGAGCATG

-G--D--E--G--G--S--G--G--D--E--G--G--S--G--G--G--P--R--S--M-

CCCCTGTCCACGAGGGGAGGTGGAAGTCATCATAAAGTTTCTGTTTCCCCTGTTGTCCAT

-P--L--S--T--E--G--G--G--S--H--H--K--V--S--V--S--P--V--V--H-

GTTTCGAGGGCTCTGTGAATCTGTTGTGGAAGCTGACCTTGTTGGAGGCACTGGAGAAATT

-V--R--G--L--C--E--S--V--V--E--A--D--L--V--E--A--L--E--K--F-

GGGACAATATGCTATGTGATGATGATGCCATTTAAACGACAGGCTCTCGTGGAGTTTGAA

-G--T--I--C--Y--V--M--M--M--P--F--K--R--Q--A--L--V--E--F--E-

AATATAGATAGTGCCAAAGAATGTGTGACATTTGCTGCAGATGTGCCTGTGTACATTGCT

-N--I--D--S--A--K--E--C--V--T--F--A--A--D--V--P--V--Y--I--A-

GGCCAGCAGGCTTTCTTCAACTATTCTACAAGTAAAAGAATCACTCGGCCAGGAAATACC
 -G--Q--Q--A--F--F--N--Y--S--T--S--K--R--I--T--R--P--G--N--T--
 626 685
 GACGATCCATCAGGGGGCAACAAAGTGCTCTTGCTCTCTATTGAGAATCCTCTTTACCCA
 -D--D--P--S--G--G--N--K--V--L--L--L--S--I--Q--N--P--L--Y--P--
 686 745
 ATTACGGTGATGTTCTGTATACTGTATGCAACCCTGTTGGAAAAGTACAGCGTATTGTT
 -I--T--V--D--V--L--Y--T--V--C--N--P--V--G--K--V--Q--R--I--V--
 746 805
 ATCTTCAAGAGAAATGGGATACAAGCAATGGTTGAGTTTGAGTCAGTCCTTTGCGCCAG
 -I--F--K--R--N--G--I--Q--A--M--V--E--F--E--S--V--L--C--A--Q--
 806 865
 AAGGCTAAAGCAGCACTCAATGGAGCTGATATATATGCCGGATGTTGCACACTAAAAATT
 -K--A--K--A--A--L--N--G--A--D--I--Y--A--G--C--C--T--L--K--I--
 866 925
 GAATATGCAAGGCCAACTCGTCTAAATGTTATTAGGAATGACAATGACAGTTGGGACTAC
 -E--Y--A--R--P--T--R--L--N--V--I--R--N--D--N--D--S--W--D--Y--
 926 985
 ACTAAACCTTATTTGGGAAGACGAGATAGAGGAAAGGGTCGCCAGAGACAAGCCATTTTG
 -T--K--P--Y--L--G--R--R--D--R--G--K--G--R--Q--R--Q--A--I--L--
 986 1045
 GGAGACCATCCTTCTTCGTTTAGACATGATGGCTATGGATCGCATGGTCCGCTACTGCCT
 -G--D--H--P--S--S--F--R--H--D--G--Y--G--S--H--G--P--L--L--P--
 1046 1105
 TTACCAAGTCGTTATAGAATGGGCTCTAGAGATACACCTGAACTTGTTGCTTATCCATTA
 -L--P--S--R--Y--R--M--G--S--R--D--T--P--E--L--V--A--Y--P--L--
 1106 1165
 CCACAAGCGTCTTCCTCTTACATGCATGGAGGCAGTCCGTCTGGCTCCGTTGTAATGGTT
 -P--Q--A--S--S--S--Y--M--H--G--G--S--P--S--G--S--V--V--M--V--
 1166 1225
 AGTGGATTACATCAGCTCAAAATGAATTGTTCAAGAGTCTTCAACCTGTTCTGCTTGTAT
 -S--G--L--H--Q--L--K--M--N--C--S--R--V--F--N--L--F--C--L--Y--
 1226 1285
 GGAAATATTGAAAAGGTAAGTTTATGAAGACCATTCTGCGACAGCACTCGTAGAAATG
 -G--N--I--E--K--V--K--F--M--K--T--I--P--G--T--A--L--V--E--M--
 1286 1345
 GGTGACGAGTACGCTGTGGAAGAGCTGTCACCCACCTTAATAATGTCAAACCTCTTTGGG
 -G--D--E--Y--A--V--E--R--A--V--T--H--L--N--N--V--K--L--F--G--
 1346 1405
 AAAAGACTTAATGTTTGTGTGTCTAAACAGCACTCAGTTGTTCCAGTCAAATATTTGAG
 -K--R--L--N--V--C--V--S--K--Q--H--S--V--V--P--S--Q--I--F--E--
 1406 1465
 CTAGAGGATGGTACGAGCAGCTACAAAGATTTTGAATGAGCAAAAATAATCGCTTTACA
 -L--E--D--G--T--S--S--Y--K--D--F--A--M--S--K--N--N--R--F--T--
 1466 1525
 AGTGTGGACAAGCATCTAAGAATATAATCCAGCCACCGTCATGTGTGCTGCATTATTAC
 -S--A--G--Q--A--S--K--N--I--I--Q--P--P--S--C--V--L--H--Y--Y--
 1526 1585
 AATGTTCCGCTGTGTGTACAGAAGAGACCTTCACAAAGTTGTGTAACGACCACGAAGTT
 -N--V--P--L--C--V--T--E--E--T--F--T--K--L--C--N--D--H--E--V--
 1586 1645
 CTTCCGTTTCATCAAAATATAAGTGTTTGACGCGAAAGCTTCCGCGAAAACCTGTCTGGG
 -L--P--F--I--K--Y--K--V--F--D--A--K--A--S--A--K--T--L--S--G--
 1646 1705
 CTATTGGAGTGGAAGTGCAAAACTGATGCCGTGGAAGCTCTCACTGCGCTCAATCACTAC
 -L--L--E--W--K--C--K--T--D--A--V--E--A--L--T--A--L--N--H--Y--
 1706 1765
 CAGATACGAGTCCCGAATGGATCCAACCCCTATACGTTGAAGCTTTGCTTTTCTACATCA
 -Q--I--R--V--P--N--G--S--N--P--Y--T--L--K--L--C--F--S--T--S--
 1766 1825
 TCCCATTTATAAGAAGAGAAGAGCATGTTAGAATTTATGTTTCATCTTTATTGCAGTTTTA
 -S--H--L--*--.....
 AAGCTATACTTCATCCATAATAATCTAAAATGGTTGGTCTAATGTTGCCTTGCTTACTAT

 AAGATCCTGTTCTGTAATAAACATATTTTGCCTC

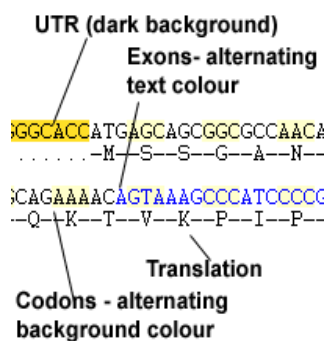


Figure 2.5 Mouse Hnrpll transcript and translation

In order to choose appropriate endonucleases for cloning, Hnrpll EST was analysed for restriction enzyme recognition by testing the sequence in the online restriction map program (<http://rna.lundberg.gu.se/cutter2/>). XhoI and EcoRI which are among the polylinkers of the pIRES-KMV vector do not cut Hnrpll EST sequence.

Primers were designed by inputting the transcript sequence to the online primer design resource (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to amplify Hnrpll EST. The 5' end primer GCCGCCATGTCCTCCAC right start 6 bp in front of transcription initial site, while the 3' end primer TAAATGGGATGATGTAGAAAAGCA site just before the stop codon TAA. The second pair of primers adopted XhoI target sequence CTCGAG to the 5' end of forward primer whereas EcoRI recognised sequence GAATTC was added to the 3' end of reverse primer with poly A tag to both primers, maintaining the sequence in reading frame.

Hnrpll cDNA was amplified by PCR:

ddH ₂ O	32 ul
5x buffer B	10 ul
dNTPs (10 mM)	1 ul
HnrpllXhoIF primer (Forward) (10 uM)	2 ul

HnrpllEcoRIR primer (Reverse) (10 uM)	2 ul
<i>Elongase</i> DNA polymerase	2 ul
cDNA	1 ul

Programs: 94°C, 3 minutes, 1 cycle.

94°C, 30 seconds,

55°C, 60 seconds,

68°C, 2 minutes, 36 cycles.

72°C, 5 minutes, 1 cycle.

Amplified Hnrpll cDNA were verified by electrophoresis and sequencing, double digested with XhoI and EcoRI restriction enzymes:

NE buffer for EcoRI	2ul
XhoI	0.9ul
EcoRI	0.9ul
Hnrpll cDNA/pIRES-KMV vector	500ng
DDH2O	up to 20ul

Samples were mixed and incubated at 37°C for one hour and the cDNA was gel purified by running samples by gel electrophoresis. The cDNA was excised from the gel and purified using Qiaquick PCR purification kit (Qiagen: 28104). The cDNA concentration was quantitated and the linearized vector and cDNA were mixed at 1:3~1:10 ratio for connection.

pIRES-KMV	4 ul
Hnrpll cDNA	4 ul
Roche 10× ligation buffer	1ul
Roche T4 DNA ligase	1ul

Samples were incubated at 16°C over night. The ligated plasmid was used to transform competent *E. coli*. by heat shock by mixing 2ul of plasmid with 20ul of

competent *E. coli* and cells were incubated on ice for 5-30 minutes. The samples were transferred to 42°C for 30 seconds without shaking, and immediately incubated on ice for 10 minutes. 200ul of pre-warmed SOC media (at room temperature) was added to the cells and these were, shaken at 200rpm at 37°C for 1 hour, and spread to pre-warmed selective plates. After 10-12 hours growth, ampicillin resistant colonies were picked and inoculated into 5ml LB media with 0.1% ampicillin and rotate at 200rpm overnight. The following day plasmids were extracted and purified from *E. coli* using the PureLink™ Quick Plasmid DNA Miniprep Kits (Invitrogen K2100-11). The constructors were verified by double digestion with XhoI and EcoRI and checking the size of insertion and vector.

2.5.3 Retrovirus package

Colonies with appropriate pIRES-KMV-HnrpII plasmid were grown in a 2L flask so that the plasmid can be yield and extracted in large scale. The Pheonix packaging cell line was grown in T75 flasks to about 50-60% confluence in a 10mL volume, add 5ul of 50mM chloroquine, 155ul of 2M CaCl₂ solution to 40ug plasmid in 1095ul volume. 60 minutes after adding chloroquine, add 1250ul of pH7.35 2×HBS was added to the cells by bubbling, immediately add the mixture to the cells, and agitate the culture gently to ensure uniform distribution. After 9 hours incubation in 37°C, the culture media was replaced with fresh full media and to the cells were incubated at 32°C. 48 hours post-transfection, the retroviral supernatant was collected. A second round of transfection was obtained by adding 10 ml of fresh media to the Pheonix cells and returns the flask to 32°C incubator and 48h later the supernatant was collected.

2.5.4 Calcium Phosphate gene transfection of primary T lymphocytes

Spleen CD4⁺ T cells purified from C57BL/6J mice or *thi/thi* mice by magnetic sorting were added into 6 well culture plates with 3 million cells per well, stimulated with anti-CD3 and anti-CD28 for 24h and then added in 3 ml retroviral supernatants (1ml/1million cells) with 3 μ l of 1000 $\times$ polybrene (4mg/ml) to each well. Spin the plates 3000rpm at 30°C for 60-90 minutes, incubate the plates at 30°C for 30 minutes, then remove the viral supernatants and add in 3 ml of fresh complete media. Culture the cells in 37°C for 2 days, check CD45 isoforms by FACs.

Media and solutions:

2 $\times$ HBS: NaCl	2g
HEPES	6.5g
Na ₂ HPO ₄	10ml (5.25g Na ₂ HPO ₄ dibasic in 500 ml H ₂ O, pH 7.35)

Growth Medium for Phoenix cell:

DMEM	440ml
Hi FCS	50ml
Penicillin/Strep and L-glutamine	10ml

Complete RPMI for splenocytes:

RPMI	440ml
Hi FCS	50ml
Pen/Strep and L-glutamine	10ml
β -mercaptoethanol (50 μ M)	500 μ l
HEPES (1M)	5ml
100 $\times$ nonessential A.A. (0.1mM)	5ml
100 $\times$ Na pyruvate	5ml

2.6 *In vitro* culture

Flat bottom 24 well plates were coated with anti-CD3 in PBS over night at serial dilutions of anti-CD3 starting from 10ug/ml. Single cell suspensions of lymphocytes were prepared in ice-cold RPMI using cell strainer from fresh tissues of spleen and lymph nodes. Cells were sorted through MACS beads by deleting red blood cells, B cells, granulocytes, macrophages, NK cells and dendritic cells. Cells were resuspended at 5.0×10^7 /ml in warm RPMI 1640 and incubated for 10 min at 37°C with 5µM CFSE (Carboxyfluorescein succinamide ester, Molecular Probes). Labelling was quenched with three washes of ice-cold RPMI/10% FCS. Cells were resuspended in warm RPMI+10% FCS at 1×10^6 cell/ml with soluble anti-CD28 (1ug/ml), added 1 ml to each well, cultured at 37°C for 3-4 days, stained with appropriate antibodies including 7AAD, and analysed by Flowcytometry.

2.7 Adoptive cell transfer

Thymocytes and splenocytes from wild type CD45.1 and *thi/thi* (CD45.2) mice were prepared from 6-8 week old mice and cell suspensions were prepared and washed, either labelled with CFSE or not. The labelled lymphocyte populations were mixed at a 50:50 ratio and 2×10^7 cells were injected into the tail vein per recipient mouse including RAG^{-/-}, irradiated TAP1^{-/-} and C57BL/6 mice, in different experiments. Mice were bled at different time points after the transfer from the retro-orbital plexus. The peripheral blood leukocytes were lysed of red blood cells, stained with antibodies to CD4, CD8, CD45.1, CD45.2 etc and the samples were analysed by FACS. Recipient mice used in these experiments were at 6-8 weeks of age. The irradiation dosage in the experiments was a single 1.5 Gy dose.

2.8 All exon array

2.8.1 Cell sorting and RNA isolation

3-6 female mice at 6-8 weeks of age, either wildtype or *thu/thu*, were sacrificed and lymphocytes from the spleen and lymph nodes were pooled to two groups in according to the genotype. The cells were stained with anti-CD4, CD8, and CD44 antibodies and were sorted to 8 samples in sterile ice-cold RPMI + 10% FCS from FACS Vantage DIVA option and FACS Aria (Becton Dickinson), wt naïve CD4 (CD44^{lo}), wt memory CD4 (CD44^{hi}), wt naïve CD8 (CD44^{lo}), wt memory CD8 (CD44^{hi}) and their *thu/thu* mutant counterparties. 2- 5 millions cells were collected for each sample. Total RNA was immediately extracted after cell sorting using RNeasy plus Mini kit (Qiagen #74134) to eliminate genomic DNA contamination. At least 1.5ug total RNA of each sample was prepared for exon array experiments with good quality that was tested with Agilent Bioanalyzer Chips in Biomedical Research Facility in John Curtin School of Medical Research. Each RNA sample has 3 biological triplicates for statistic purpose. RNA from CD4 and CD8 single positive thymocytes were also prepared in the same method.

2.8.2 Chips, hybridization, and data analysis

Exon arrays are powerful tools for the discovery and study of mRNA transcript isoforms derived from pre-mRNA alternative splicing. Affymetrix GeneChip® Mouse Exon 1.0 ST (Sense Target) Array is a single array with more than 4.5 million unique 25-mer oligonucleotide features constituting approximately 1.2 million probe sets that can be grouped into 1 million exon clusters and over 80, 000

transcript clusters. With about four probes for each exon and average 40 probes per gene, this array provides detection of both the whole transcript expression and the differential expression of each exon within a gene. Exon array chips were provided by Dr. Alan Aderem and were performed by Mr. Bruz Marzolf and Ms. Katherine Kennedy in the System Biology Institute in Seattle, WA, USA.

A total of 1ug RNA was in vitro transcribed to cRNA. 10 ug cRNA was amplified in the second round used. A hybridization cocktail including 5.5 ug of fragmented end labelled ssDNA was applied to Affymetrix Mouse Exon 1.0 ST Array chips. Hybridization was performed using F450-001 fluidics wash and stain script using the Affymetrix Gene Chip Fluidics station 450. The arrays were then scanned using Affymetrix GCS3000 7G and Gene chip operating software to produce CEL files. Detection of each exon in the mRNA enables examination of individual exon in the transcript isoforms.

Array data were analysed with Partek Genomics Suite 6.3 using the core probe sets defined by Affymetrix. A two way ANOVA of genotypes/cell subsets versus probeset ID was calculated in a given mutant cell type in comparison to their wildtype counterpart or memory T cells in comparison to the naive cells. Alternative splicing was evaluated by the p values of different probeset intensities between different genotypes or cell subsets. Approximately 15,000 genes were analysed and the candidate gene views were created and linked to known transcript sequences in the USSC Bioinformatics database for validation.

~Chapter 3~

*Immunological Characterization of the
thunder trait:*

*Loss of naïve T cells and aberrant pre-
mRNA alternative splicing*

Introduction

Mature T cells are generated in thymus. The recent thymic emigrants will circulate through the peripheral blood and secondary lymphoid tissues where they encounter APCs presenting a specific peptide/MHC complex for the clonally restricted TCR. T cells in the peripheral circulation are considered naïve before they encounter their specific antigen and display a characteristic expression of a range of cell surface markers including CD44^{lo}, CD25⁻, CD43^{lo}, CD62L^{hi}, and CD127^{hi} (Surh, C. and Sprent, J. 2003). The life span of naïve T cells in the peripheral circulation is around 6 weeks if they fail to encounter their specific antigen. Once they die they will be replaced by other mature cells arising from the thymus that expand to fill the empty niche (Surh, C. and Sprent, J. 2003). T cell homeostasis refers to the status of relatively stable cell numbers through the life and death cycle of T cells. Each subset of T cells has independent niche and different factors contributing to their homeostasis. The immune system maintains the peripheral T cell repertoire at a relatively constant size throughout the lifetime of an individual despite varying thymic production of T cells and the response of T cells to environmental antigens.

The homeostatic mechanisms that are used by the immune system to maintain the numbers of peripheral T cells is slowly being understood. These include extrinsic factors that influence naïve and memory T cell homeostasis (e.g. peptide/MHC recognition and the cytokines IL-7 and IL-15) (Takeda, S. et al. 1996; Tanchot, C. et al. 1997; Zhang, X. et al. 1998; Boursalian, T.E. and Bottomly, 1999), our knowledge of how these are integrated to control T cell repertoire size and composition is still rudimentary. Some intrinsic factors like Bim, Fas/FasL, APO2L/TRAIL, c-FLIP, and

FOXO3a etc were also reported playing important roles in memory T cell homeostasis in mouse or human (Bosque, A. et al. 2005; Sabbagh, L. et al. 2006; Wojciechowski, S. et al. 2007; Riou, C. et al. 2007; Weant, A.E. et al. 2008; Williams, M.A. et al. 2008).

Upon Ag stimulation, naive T cells quickly expand and differentiate to become effector cells which play an important role to control the spread of pathogens. A subset of primed T cells is selected from the expanding repertoire to differentiate as memory T cells of the same specificity as the effector population. The memory cells are long lived and display a more rapid and heightened response following secondary antigen stimulation. Naïve and memory T cells have distinct cellular niches and these are controlled by different homeostatic processes. One of the most widely used markers defining memory T cell differentiation in human arises from a regulated shift in the alternative splicing of *Ptprc* mRNA encoding CD45, such that memory T cells silence the inclusion of three exons encoding the CD45RA, RB and RC isoforms present on unprimed naïve T cells, and hence become CD45RO (Hermiston, M.L. et al. 2003). While this memory-associated change in splicing is a highly conserved marker across the animal kingdom, its functional significance is unclear and the factors that bring it about have remained obscure. Moreover, it is not known whether or not the change in CD45 splicing is part of a more global reprogramming of mRNA splicing in memory T cells that might change the longevity and responsiveness of memory T cells.

Primary mRNA alternative splicing is a ubiquitous phenomenon of post-transcriptional modification that helps to increase protein diversity and complexity

from the same gene (Modrek, B. and Lee, C. 2003). Splicing variants normally have different extent activity or even antagonistic effects that are important in regulating protein functions (Rothrock, C. et al. 2003). Yet little is known about the mechanisms of how pre-mRNA alternative splicing is regulated and how differential splicing influences immunological functions.

In this chapter, we report a mouse strain, *thunder*, carrying an ENU induced mutation that was identified through a screen for mice with defective T cell homeostasis. *Thunder* mice were identified by flow cytometric analysis with a reduction in peripheral CD4⁺ and CD8⁺ T cells. As we report in this chapter we identified the mutation was in a novel splicing silencer protein that leads to a defect in pre-mRNA alternative splicing.

3.1 Screening and identification of the *thunder* strain

A male C57BL/6S (B6) mouse was injected with standard dose of ENU and bred with a wild-type female B6 mouse. Subsequent breeding followed a typical 3 generation screening strategy for recessive traits (Nelms & Goodnow, C.G. 2002). The founder G1 male mouse (#19) of the ENU 126 pedigree was backcrossed to a wild-type B6 female to generate G2 offspring. Since there was only 50% chance of any G2 female carrying the *thunder* mutation, the original G1 male (#19) was rotated past four separate G2 daughters to generate a total of 19 G3 mice from 4 litters (Figure 3.1). It is expected that the G3 generation is the first time a recessive mutation would come to homozygosity. For a recessive trait we would expect 25% of G3 offspring to show a phenotypic trait. G3 offspring were bled around 8 weeks of age.

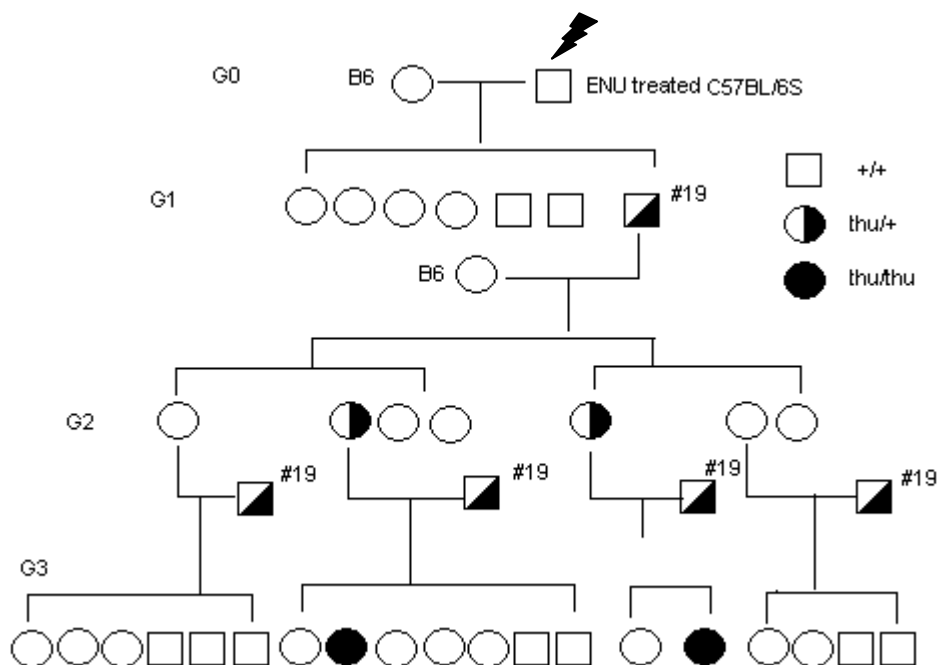


Figure 3.1 Pedigree of the ENU126 'thunder' strain

4 G3 breeder pairs produced by breeding a G2 daughter-G1 father breeders from ENU treated C57BL/6S offspring produced 4 litters. Two G3 female mice had a significant reduction in peripheral blood T cells.

Male mice = squares, female mice = circles.

Affected mice filled symbols, unaffected mice = open symbols, carriers=half filled

Peripheral blood leukocytes were analysed by multicolour flow cytometry for changes in T and B cell proportions. Two G3 female mice from the ENU126 strain were identified with reduced CD3⁺ T cell percentages (Fig. 3.1 and 3.2.1 a). A significant reduction in both CD4⁺ and CD8⁺ T cells was observed and many of the cells expressed high levels of the CD44 marker indicative of a memory cell phenotype (Fig. 3.2.1 b, c, and d). The strain was named “*thunder*” for “T helper cells under represented”.

Homozygous mutant mice on the B6 background were either out-crossed to the mapping strain of CBA to increase genetic polymorphisms to facilitate mapping of the ENU-induced mutation. F1 hybrid carriers were intercrossed to produce F2 progeny with a quarter of the offspring being homozygous mutants that could be used to map the mutant locus. *Thunder* homozygous mutant mice were also maintained on a C57BL/6 background to facilitate further biological studies. Most studies of this project were performed on the C57BL/6 background.

3.2 *Thunder* mutation encodes a recessive trait that involves the loss of naive T cells in the peripheral lymphoid tissues

3.2.1 T cell lymphopenia with hyperactivation by bleed screening

An identical cellular phenotype was observed with the *thunder* homozygous mutant on the mixed B6/CBA F2 background compared to the mutants on C57BL/6 background. Homozygous *thu/thu* mice have significantly lower percentages of CD3⁺ T cells in the blood that affect both the CD4⁺ and CD8⁺ subsets (Fig. 3.2.1a and b).

Figure 3.2.1 Naive T cell lymphopaenia

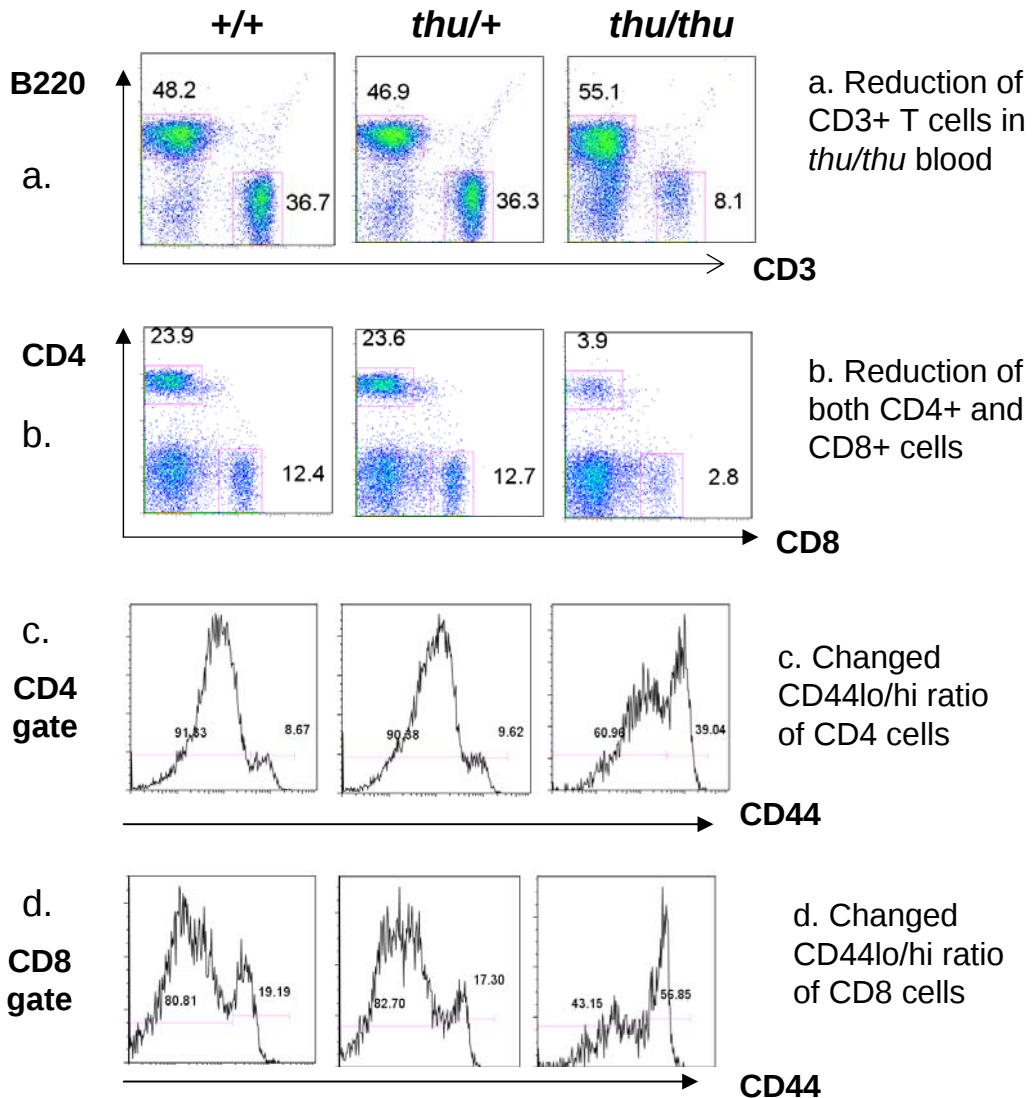


Figure 3.2.1 *Thunder* mutation leads to a loss of peripheral T cells.

Representative FACS dot plots of (a) CD3 versus B220 expression and (b) CD4 versus CD8 on peripheral blood leukocytes for individual +/+, *thu/+* and *thu/thu* mice. Representative histograms of CD44 expression on (c) CD4⁺ and (d) CD8⁺ T cells.

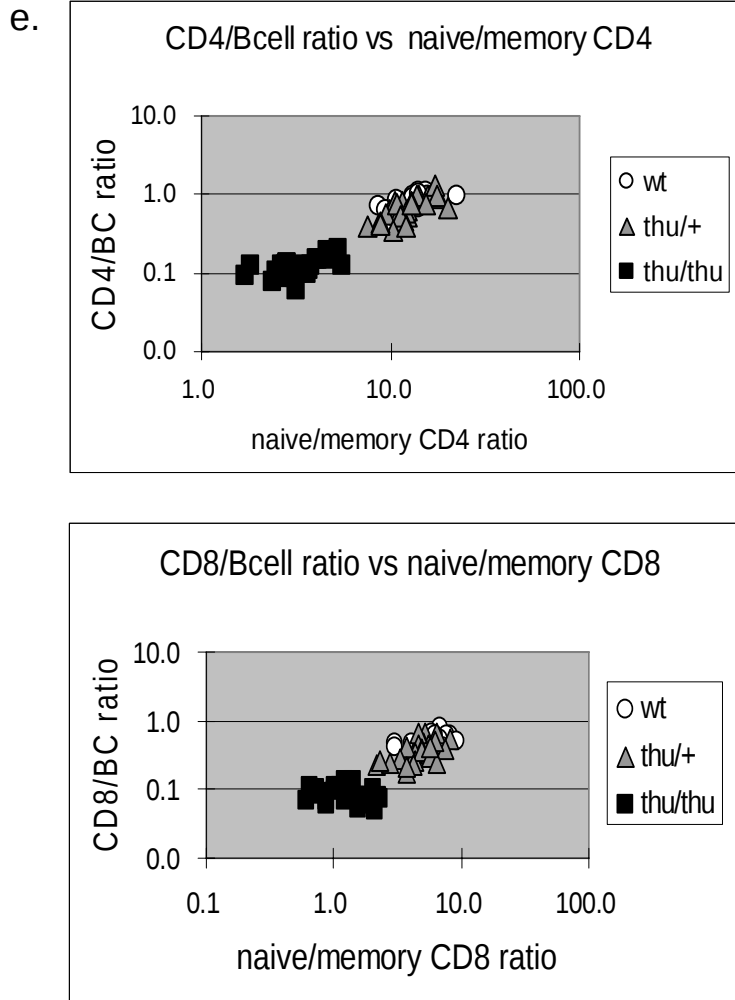


Figure 3.2.1 *Thunder* mutation leads to a loss of peripheral T cells.

(e). Accumulative data showing the frequency of T cells versus B cells and naïve versus memory T cells in CD4+ and CD8+ cell compartments as determined by flow cytometry. Each symbol represents a single animal.

Mutant T cells show an increased CD44^{hi} memory T cell frequency while CD44^{lo-mid} naïve percentages are decreased (Fig. 3.2.1 c and d). Dot plots comparing the T cell/B cell ratio vs naïve/memory T cell ratio generated from flow cytometric analysis of peripheral blood samples from individual B6/CBAF2 mice show a clear segregation of *thu/thu* homozygotes from wild-type and heterozygotes (Figure 3.2.1 e). This indicates that *thu/thu* mice have a reduced frequency of T cells in the peripheral blood and there is a significant reduction in naïve T cells compared to memory CD4⁺ and CD8⁺ cell populations.

3.2.2 Normal T cell development in the thymus

T cells originate from common lymphoid progenitor cells that arise from the bone marrow and migrate into the thymus and follow a well characterized pattern of cellular differentiation. We sought to determine whether the *thunder* mutation influences thymic development or whether it only affects the survival of T cells in the periphery. Wild-type, heterozygous and homozygous mutant mice were taken down at 8 to 12 weeks of age. The numbers of T cells present in the thymus, spleen, and lymph nodes were enumerated using flow cytometry.

Figure 3.2.2 shows that the *thunder* mutation has no effect on T cell differentiation in the thymus with normal percentages of immature double negative, double positive and mature single positive cells being present (Figure 3.2.2 a). Also there is no change in the absolute cell numbers of mature CD4 and CD8 single positive cells in wild-type or *thu/thu* mice at any stage of postnatal development ranging from 2 weeks to 18 weeks of age (Figure 3.2.2 d).

Figure 3.2.2 Normal T cell development

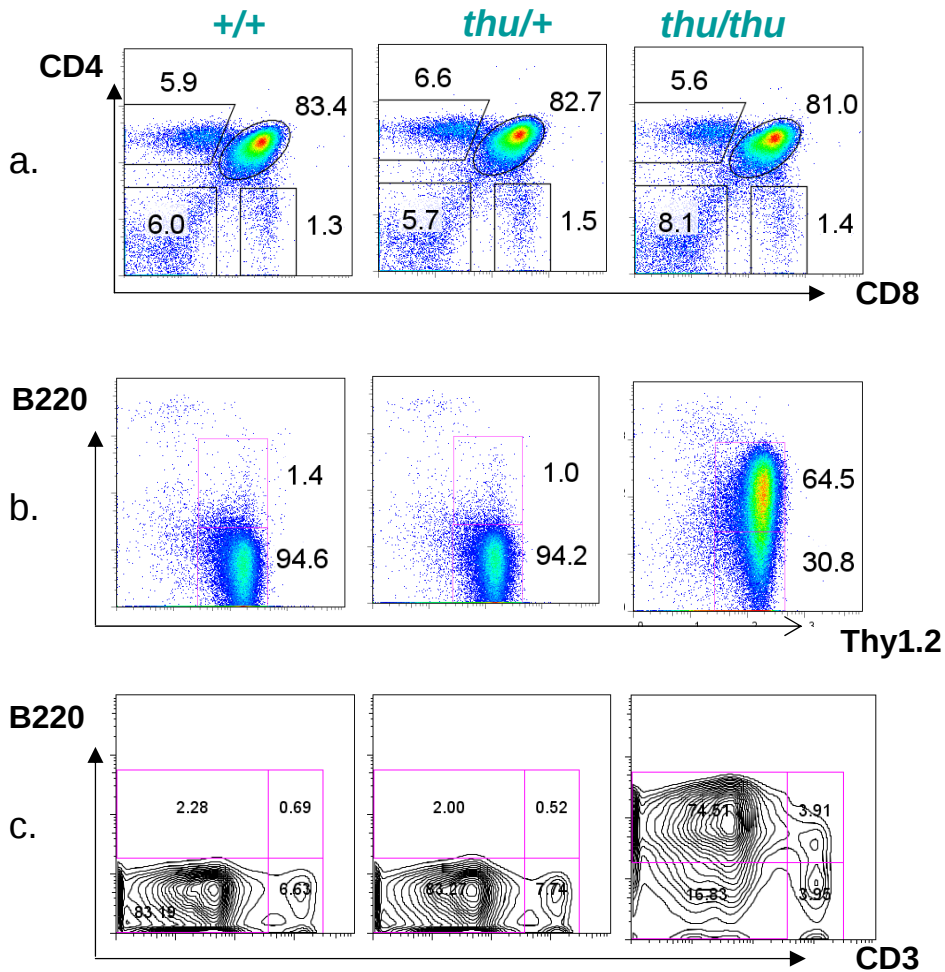


Figure 3.2.2 The *thunder* mutation does not affect T cell development in the thymus.

Representative FACS dot plots of thymocytes from *+/+*, *thu/+* and *thu/thu* mice showing (a) CD4 versus CD8, (b) B220 v Thy1.2 (CD90) and (c) contour FACS plots of thymocytes showing B220 versus CD3 expression.

d.

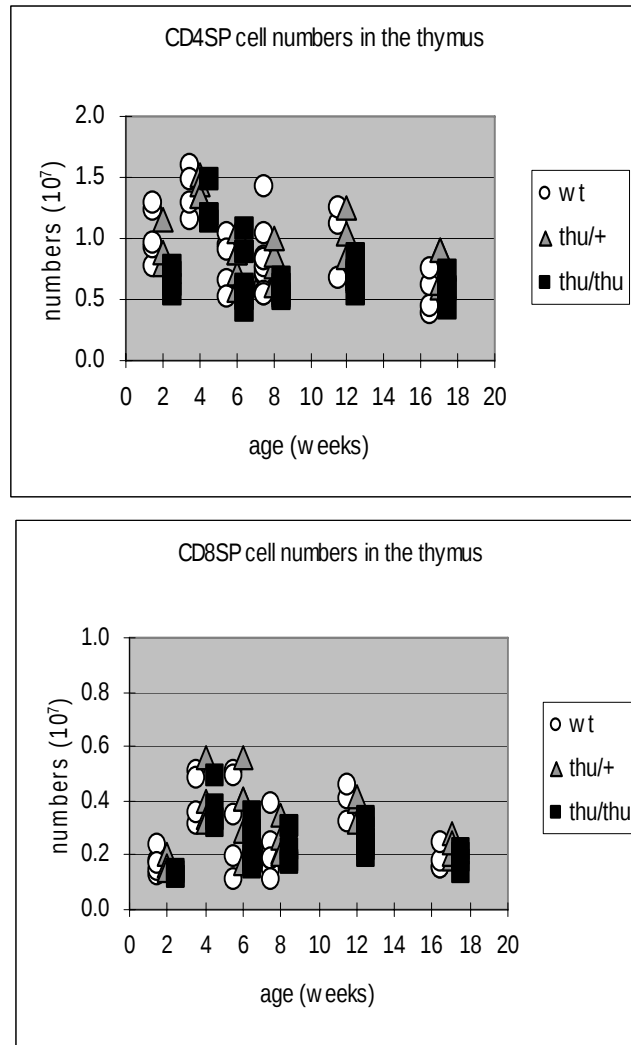


Figure 3.2.2 The *thunder* mutation does not affect T cell development in the thymus.

d. The absolute numbers of mature CD4⁺ and CD8⁺ single positive thymocytes were enumerated from individual mice. Each point on the graphs represents an individual mouse

However, most *thu/thu* thymocytes show constitutive high level of expression of the CD45R isotype (B220) at the cell surface (Figure 3.2.2 b). Both CD3^{hi} (mature thymocytes) and CD3^{lo} (immature thymocytes) cells display elevated levels of the B220 marker (Figure 3.2.2 c), although it appeared to be extinguished in most mature SP thymocytes and in peripheral T cells. This may reflect changes in glycosylation, since B220 epitope is carbohydrate dependent as well as requires the CD45RABC backbone. The constitutive expression of the B220 marker on *thunder* thymocytes suggests the mutation must affect the alternative splicing of the CD45 mRNA during T cell development.

3.2.3 Loss of naïve T cell in the peripheral lymphoid tissues

As T cell numbers and maturation were normal in thymus of *thunder* mutant mice, we examined the numbers of T and B cells in the spleen and lymph nodes (LN). Spleen cells from 8 week old mice were analysed by flow cytometry. As shown in Figure 3.2.3 a, there is a clear reduction in the frequency of CD3⁺ T cells in the spleen of *thu/thu* mice compared to wild-type and heterozygous mice and this corresponded with a decrease in both CD4⁺ and CD8⁺ T cells (Fig. 3.2.3 b).

The T cell populations were further subdivided into naïve (CD44^{lo}) and memory (CD44^{hi}) cells based on FACS staining. The numbers of naïve and memory subsets were enumerated at different ages after birth. The numbers of naïve T cells (CD44^{lo-int}) in wild-type mice increased in the spleen after birth until about 8 weeks of age due to increased thymic output of mature T cells to help fill the T cell compartment. The

Figure 3.2.3 Loss of *thu/thu* naïve T cells in the periphery

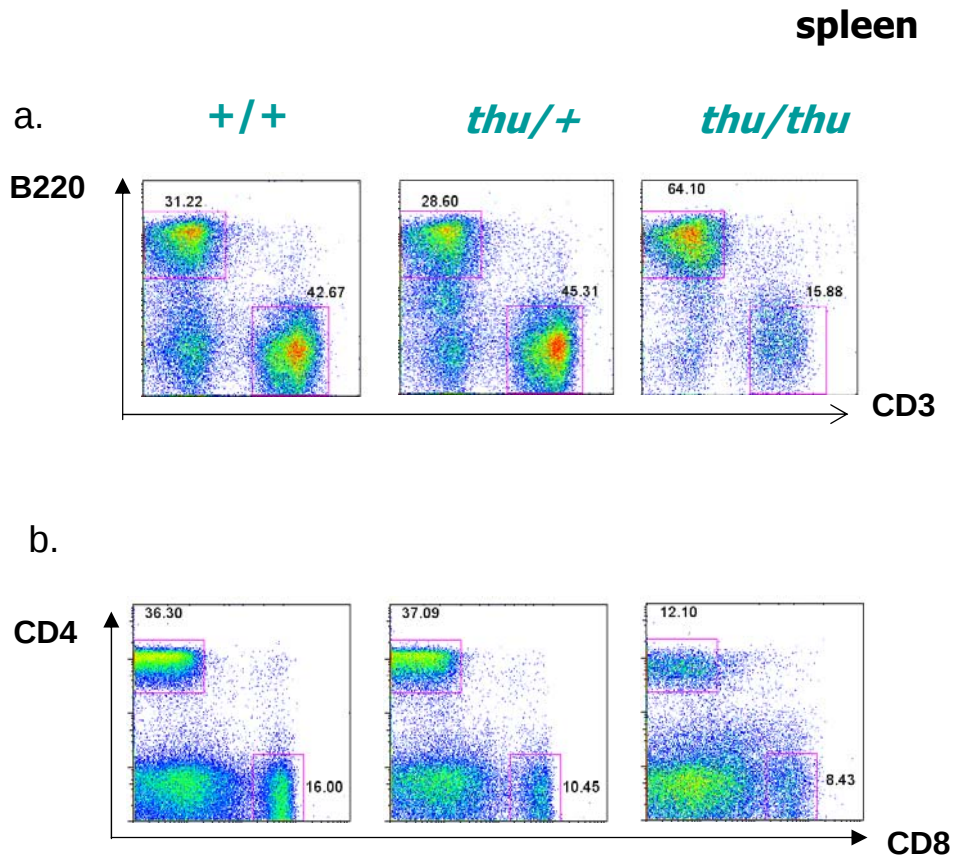
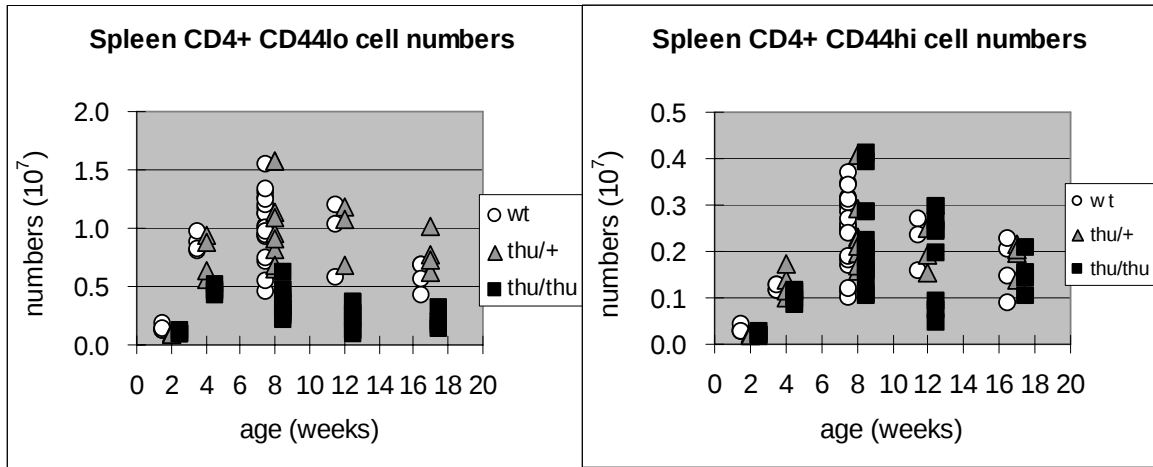


Figure 3.2.3 The *thunder* mutation results in a loss of naïve T cells in the periphery.

Representative FACS dot plots of splenocytes from *+/+*, *thu/+* and *thu/thu* mice showing (a) B220 versus CD3, (b) CD4 v CD8 staining.

C.



d.

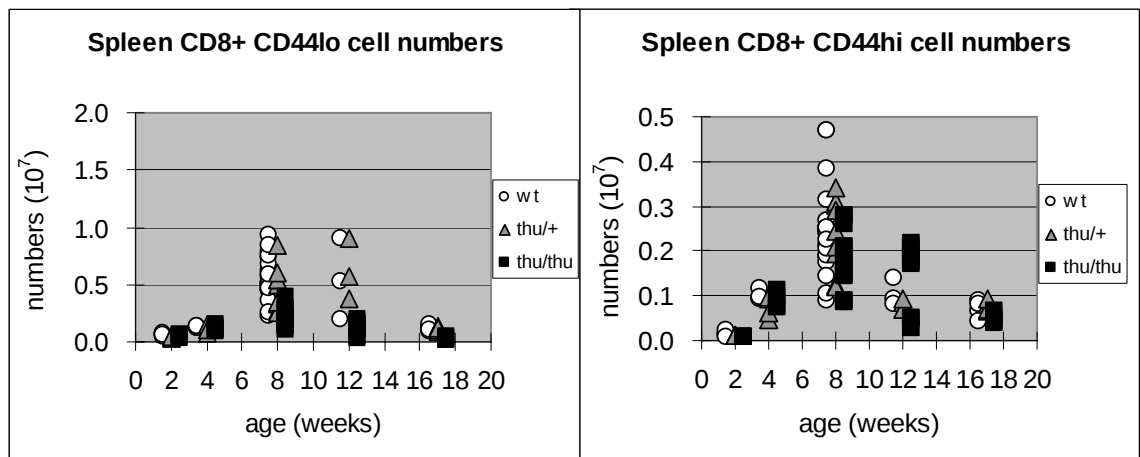


Figure 3.2.3 The *thunder* mutation results in a loss of naïve T cells in the periphery.

(c) CD4⁺ and (d) CD8⁺ T cells were enumerated from the spleen. Naïve (CD44^{lo}) and memory (CD44^{hi}) subsets were enumerated separately. Each point on the graphs represents an individual mouse.

Figure 3.2.3 Loss of *thu/thu* naïve T cells in the periphery

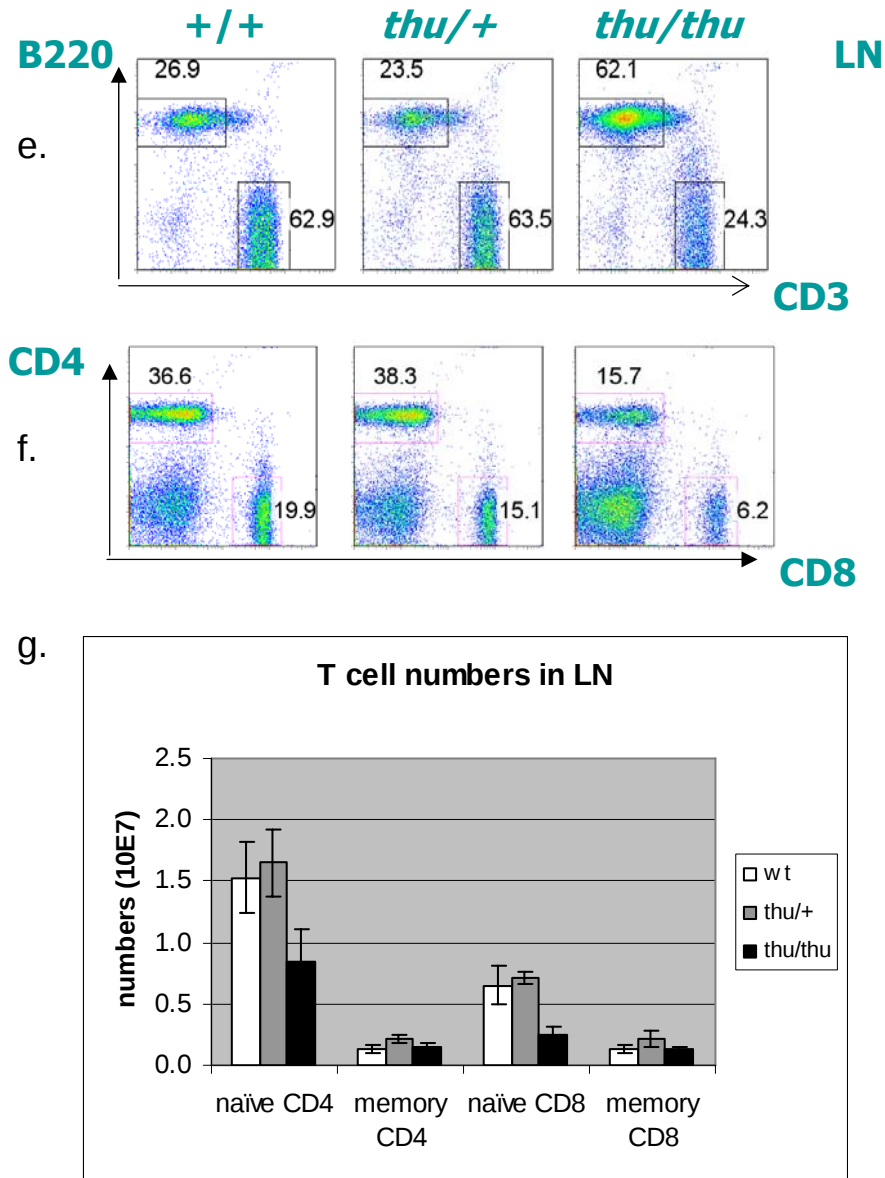


Figure 3.2.3 The *thunder* mutation results in a loss of naïve T cells in the periphery.

(e,f). Representative FACS dot plots of lymph node cells from *+/+*, *thu/+* and *thu/thu* mice showing (e) B220 versus CD3 and (f) CD4 v CD8 expression.

(g) Absolute numbers of naïve and memory CD4+ and CD8+ T cells were enumerated from lymph nodes including cervical, anterior, and superficial inguinal nodes. The data showing the mean and standard deviation were collected from 6 mice/group.

numbers of peripheral T cells in wild-type mice then remain at a relatively constant level (Fig 3.2.3.c and d). In *thu/thu* mice there was no significant difference in the numbers of naïve and memory T cells at 2 weeks of age, but, by 4 weeks of age, CD4⁺ T cells were decreased by 3 fold and there was a 2 fold reduction in naïve CD8⁺ T cells by 8 weeks of age (Figure 3.2.3 c and d). In contrast, the numbers of memory phenotype T cell (CD44^{hi}) numbers were indistinguishable between wild-type and *thu/thu* mice at any of the time points (Figure 3.2.3 c and d). There was also a significant reduction in the number of naïve CD4⁺ and CD8⁺ T cells in the lymph nodes (LN), whereas there was no difference in the number of memory phenotype T cells between wild-type and *thu/thu* mice (Figure 3.2.3 e-g).

3.3 Aberrant CD45 pre-mRNA alternative splicing

3.3.1 Multiple CD45 transcript variants in *thu/thu* T cells

As mentioned in chapter 3.2.2, *thu/thu* thymocytes constitutively express the B220 (CD45RABC) receptor, which is recognized by a monoclonal antibody (RA3-6B2) that binds an epitope on the extracellular domain of the CD45 glycoprotein (Coffman, R.L. & Weissman I.L. 1981; Asensi, V. et al. 1989). The binding of this antibody to the T cell surface is dependent on the expression of exon 4, 5, 6 and specific carbohydrate residues, indicating higher expression of variable exons in *thu/thu* mice, the expression of which is normally silenced during T cell development and upon T cell activation in the periphery.

Figure 3.3 CD45 pre-mRNA alternative splicing

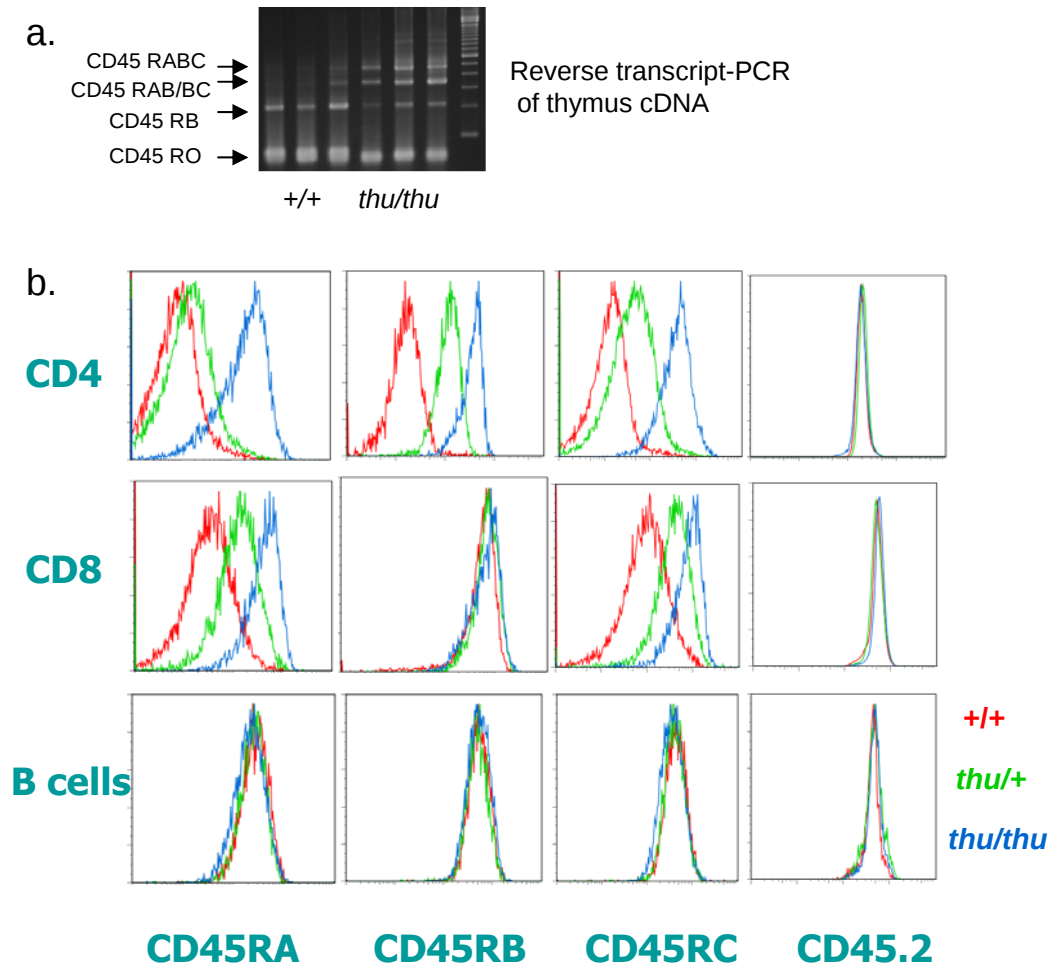


Figure 3.3 The *thunder* mutation leads to disruption of alternative splicing of CD45 in multiple cell types.

- (a) RT-PCR of thymocyte RNA purified from thymocytes of wild type and *thu/thu* mice. Arrows on the left indicate the predicted sizes of the different CD45 isoforms. Data shows representative results from 3 mice of each genotype (predicted size: RO 71bp; RB 218bp; RAB 347bp; RBC 359bp; RABC 488bp).
- (b) Representative histograms of CD4⁺, CD8⁺ T cells and B cells in the spleen of wild type (red line), *thu/+* (green line), and *thu/thu* (blue line) mice stained with CD45R segment-specific antibodies and a pan-CD45.2 antibody.

To demonstrate that *thu/thu* thymocytes undergo aberrant CD45 pre-mRNA alternative splicing, total RNA was isolated from thymocytes of wild-type and *thu/thu* mutant mice and reverse transcribed to cDNA using oligo-dT primer. CD45 transcript variants were amplified by RT-PCR using a pair of primers complementary to exon 3 and exon 7. Thymocytes from wild-type mice predominantly express the CD45RO isoform and some CD45RB, in contrast with thymocytes from *thu/thu* mice which exhibit expression of multiple transcript variants including CD45RO, RB, RAB, and RBC, suggesting defective CD45 pre-mRNA alternative splicing during thymocytes development in *thunder* mice (Figure 3.3 a).

3.3.2 T cells from *thunder* mice cannot silence the variable exons of CD45

A panel of monoclonal antibodies specific to epitopes in the CD45RA, B, and C segments was used to examine the cell surface expression of various CD45 isoforms by T and B cells using flow cytometry. T and B cells from different lymphoid tissues including thymus, spleen, LN, and blood were examined from adult wild-type, heterozygous and homozygous mutant mice. The data shown in Figure 3.3 b and c shows that B cells cannot silence the variable exons 4, 5 or 6 of CD45 and therefore constitutively express high levels of CD45RA, RB and RC epitopes. In contrast, wild-type CD4 T cells have low expression of CD45RA and RC but maintain an intermediate level of CD45RB isoform expression. The levels of the different CD45 isoforms increases 2-4 fold after CD4⁺ cells leave the thymus and enter the periphery (Fig 3.3c). Also there is a 10 fold increase in the expression of CD45RA and RC segments after CD8⁺ cells migrate from the thymus to the periphery (Fig. 3.3c). CD8⁺ T cells in the spleen of wild-type mice have approximately 10 fold higher

Figure 3.3 CD45 pre-mRNA alternative splicing

C.

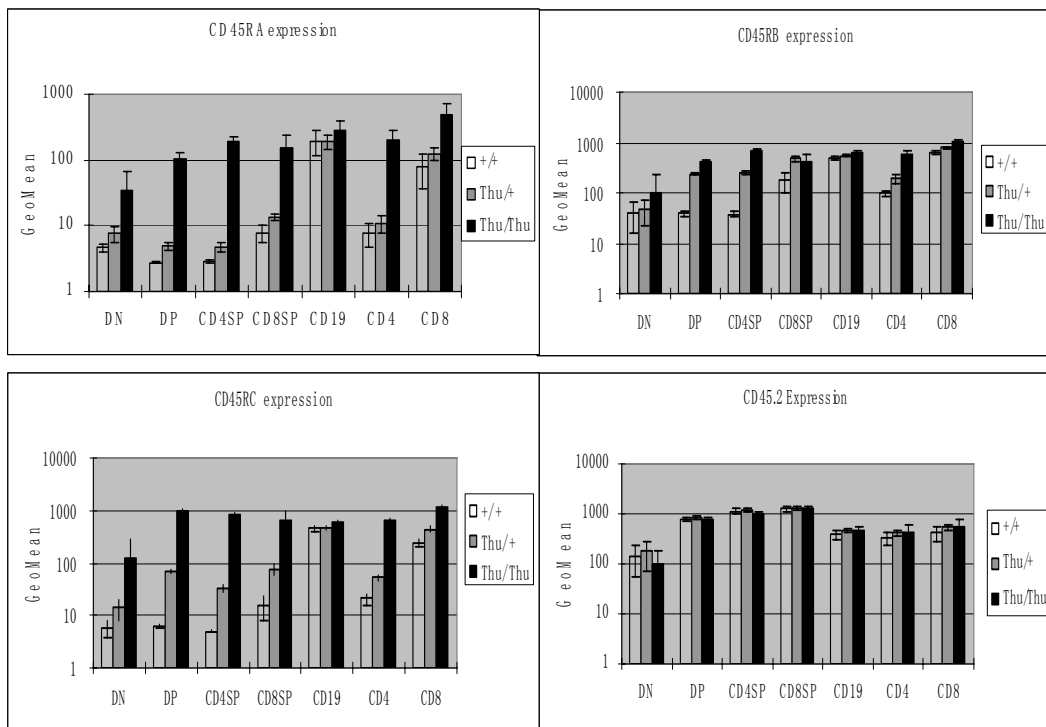


Figure 3.3 The *thunder* mutation leads to disruption of alternative splicing of CD45 in multiple cell types.

(c). Bar graphs representing the geometric mean fluorescence of different CD45R epitopes and total CD45 on the surface of DN, DP CD4SP and CD8SP thymocytes, as well as CD4+, CD8+ and CD19+ spleen cells. Data showing the mean and standard deviation of data were collected from n=6 mice/group. Wild type mice (open bars), *thu/+* (hatched bars), *thu/thu* (filled bars).

levels of CD45RA, RB and RC compared to CD4⁺ T cells (Fig. 3.3 b and c). Thus in wild-type mice the expression of CD45 isoforms changes with cell types and T cell development and maturation state of the cells.

When we examined the expression of the CD45R epitopes on thymocytes and spleen cells from *thunder* homozygous mutant mice, the *thu/thu* T cells display constitutive high level expression of all 3 segments, equivalent to the levels expressed by B cells (Fig. 3.3 b and c). In heterozygous mice the expression of all three CD45R epitopes in CD4⁺ cells (and CD45RA & RC in CD8⁺ cells) was intermediate to that observed in homozygous mutant mice (Fig. 3.3 b and c) suggesting a dose-dependent effect of the mutation on CD45 alternative splicing. The only exception was the CD45RB isoform on CD8⁺ T cells which showed little effect with the *thunder* mutation mainly because this exon is not normally silenced in CD8⁺ cells. However, quantitating the levels of expression of CD45 RB on multiple animals indicated that there was still a 1.5-2 fold increase in CD45RB levels on *thunder* homozygote mutant CD8⁺ cells compared to wild-type CD8⁺ cells (Fig. 3.3 c). These results indicate that the *thunder* mutation has an essential role in CD45 pre-mRNA alternative splicing in T cells, disruption of *thunder* gene causes failure in silencing CD45 variable exons 4, 5, and 6, and leading to the constitutive expression of high molecular weight isoforms of CD45 at the surface of T cells regardless of status of development and activation.

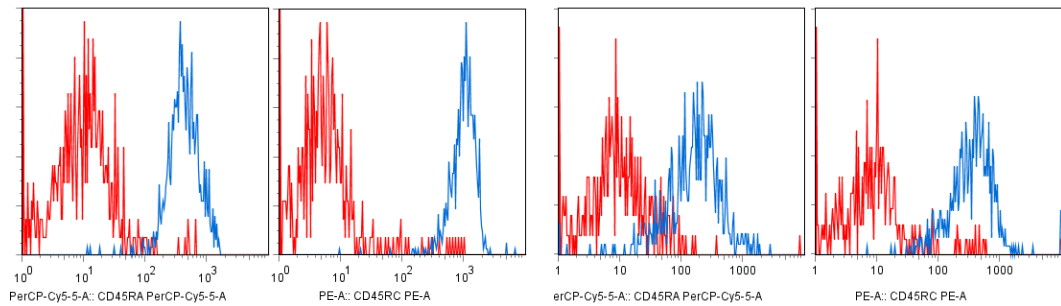
3.3.3 Aberrant CD45 alternative splicing in other hematopoietic cells

We were interested to know if the defects in CD45 alternative splicing were restricted to just T cells so we examined the expression of the different CD45

d.

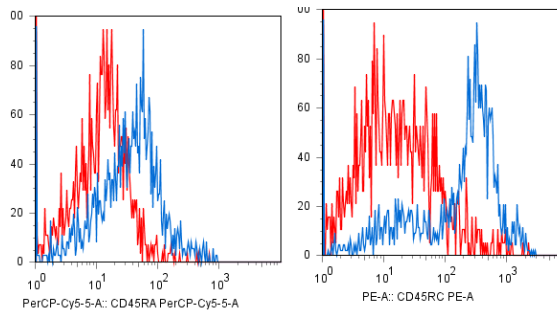
NK cells:

TCR $\gamma\delta$ cells:



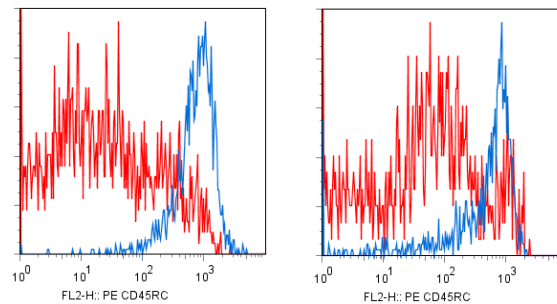
e.

Gr



CD11c DC

CD11b M ϕ



 **thu/thu**
+/+

Figure 3.3 The *thunder* mutation leads to disruption of alternative splicing of CD45 in multiple cell types.

Representative histograms comparing CD45RA and CD45RC expression on (d) NK and TCR $\gamma\delta$ + cells in the thymus and (e) Gr-1+ cells, CD11c+ DCs and CD11b+ macrophages in the spleen. (red lines = +/+ cells, blue line=*thu/thu* cells)

isoforms expression on a range of other hematopoietic cells in the thymus and spleen including NK cells, $\gamma\delta$ T cells, dendritic cells, macrophages, and granulocytes. As shown in Figure 3.3 d and e, dysregulation of CD45 splicing was observed in all leukocyte populations as all the cell types tested constitutively expressed the high MW isoforms of CD45 containing the CD45RA and/or CD45RC epitopes. This suggests *thunder* mutation has a broad role in regulating CD45 pre-mRNA alternative splicing in all nucleated haematopoietic cells.

3.4 Discussion

Homozygous *thunder* mice display normal T cell development in thymus in terms of both cell percentages and absolute numbers but there is a significant reduction in total CD4⁺ and CD8⁺ T cell numbers in the periphery only after 4-6 weeks of age. Detailed phenotypic analysis of thymocytes at different stages of development failed to reveal any significant differences between wild-type and *thu/thu* thymocytes, despite the fact that the mutant cells constitutively express the high MW isoforms of CD45 which are thought to be the most active protein tyrosine phosphatase in leukocytes. The CD45 protein is essential in T cell development because mice and humans that lack CD45 develop severe combined immune deficiency (SCID) (Tchilian, E.Z. et al. 2001). CD45 deficiency causes a significant arrest of T cell development at the DP stage leading to a severe reduction in mature single positive thymocytes and peripheral CD4⁺ and CD8⁺ T cells (Kishihara, K. et al. 1993). In the case of B cells, loss of CD45 does not affect the development of B cells in the bone marrow but they do display an arrest in development in transitional stages in the spleen (Kishihara, K. et al. 1993).

The CD45 protein is thought to be recruited to the immunological synapse to help initiate TCR signalling and to control the threshold of TCR signalling (Zhang, M. et al. 2005). Thus in the absence of CD45 the threshold becomes very high resulting in a failure of positive selection of thymocytes. The high molecular weight isoforms of CD45 (i.e. RA, RB, RC) are believed to have the highest enzymatic activity (Xu, Z. & Weiss, A. 2002). The most important substrate for CD45 is the Src tyrosine kinase p56Lck in T cells, and p59Fyn can also act as a target. CD45 plays a critical role in dephosphorylating two key tyrosine residues of Lck, tyrosine 394 and tyrosine 505 (i.e. Y394, Y505) as discussed in section 5 of the introductory chapter 1 (Zamoyska, R. 2007). However there is still no clear understanding of the roles of the different CD45 isoforms in the regulation of TCR signalling or the functional maturation of T cells.

A study by Weiss and colleagues showed that a point mutation in the assumed wedge domain of CD45 which maintains the continuous phosphatase activity of the CD45 can disrupt negative selection in the thymus and mice are predisposed to developing autoimmunity (Majeti, R. et al. 2000). Despite the established roles for CD45 in regulating T cell development the studies presented here can find no significant effect of CD45 differential splicing on T cell development. As will be discussed later (Chapter 6) similar findings are also observed when the *thunder* mutation is bred onto a TCR transgenic background. Thus despite the obvious changes in CD45 isoform expression the *thunder* thymocytes somehow are able to control potential changes in TCR signalling strength during positive and negative selection and this leads to a normal T cell maturation in the thymus.

At birth the peripheral immune system is devoid of mature T and B cells and therefore the immune system relies on the production of mature T and B cells from the thymus and bone marrow respectively. Analysis of mice at different ages during postnatal development identified that the production of mature T cells in the thymus of *thunder* mice is normal throughout postnatal life. During the first 4 weeks of life thymic output of mature T cells reaches a maximum. Because the peripheral immune system is initially devoid of T cells, the recent thymic emigrants will undergo homeostatic and Ag-induced proliferation to fill the memory compartment. It is during this period that the naïve T cells rely on extrinsic signals from self-peptide MHC and cytokine signals from IL-7/IL-7R signals that promote their expansion. The naïve T cells still require self-peptide MHC and cytokine signals from IL-7/IL-7R signals for their long term survival and persistence in the periphery in order to maintain a diverse T cell repertoire (Surh, C.D & Sprent, J. 2005).

The data presented in this chapter clearly identifies that the *thunder* mutation does not affect T cell development. Instead the *thunder* mutation severely disrupts the ability of mature naïve T cells to accumulate in the periphery. These results highlight that the *thunder* mutation has a critical role in the persistence of mature T cells in the peripheral circulation and therefore has an important role in the regulation of T cell homeostasis *in vivo*. Despite the development of T cell lymphopaenia the *thunder* mice remain healthy and have a normal lifespan. This is intriguing considering that there is evidence from different mouse models that the development of T cell lymphopaenia can predispose to autoimmunity (King, C. et al. 2004).

Despite a significant loss of the naïve cell compartment, memory T cells in *thunder* homozygous mutant mice appear not to be affected significantly. This indicates that there is a specific defect in the ability of naïve T cells to persist in the peripheral circulation and in secondary lymphoid tissues. The *thunder* strain was identified on the basis of low numbers of T cells in the peripheral blood and most of the cells were of a CD44^{hi} (memory) phenotype. The phenotypic analysis of the *thunder* strain presented in this chapter begins to explain why there is an accumulation of memory cells in these mice. The greatest loss of T cells affects the naïve compartment. It is easy to see these changes because in young wild type-mice (e.g. 4-16 weeks of age) the number of naïve T cells should outnumber memory phenotype cells by almost 2:1. However the reverse ratio is observed in the *thunder* mice. We hypothesize that the increased frequency of memory phenotype T cells in *thunder* mice is caused by the constant homeostatic proliferation of T cells in response to the lymphopaenic environment resulting from constant attrition of mature T cells.

Although memory T cells can form in *thunder* mice it would appear that the long term survival of both naïve and memory T cells in *thunder* mice are affected because the number of mature T cells (i.e. both naïve & memory) in the secondary lymphoid tissues never reaches the same level as that achieved in wild-type mice with increasing age. This could be because the size of the memory compartment is much smaller than the naïve compartment. Once naïve cells differentiate and adopt a memory phenotype they cannot re-enter the naïve cell niche because of changes in the pattern of cell surface integrins, selectins, chemokine receptors etc. Alternatively the memory cells may persist for a slightly longer half life in the circulation but

eventually they are destined to die and to be replaced by a new population of memory cells.

A defining feature of the *thunder* mouse strain is that T cells fail to silence the three variable exons of CD45. The failure in exon silencing was identified both at the level of mRNA in thymocytes as well as at the level of protein expression using a panel of well characterised isoform specific monoclonal antibodies. CD45 is the most abundant membrane tyrosine protein phosphatase in T cells but still remains one of the most enigmatic proteins expressed by leukocytes (Hermiston, M.L. et al. 2003). It has been known for almost 30 years that CD45 protein undergoes alternative splicing and this feature is conserved throughout the animal kingdom. CD45 is an important component of TCR signalling cascade with a role in initiation of TCR signal transduction upon Ag stimulation. However there is still no clear understanding of the role of the different CD45 isoforms in the regulation of TCR signalling or the functional maturation of T cells.

Loss of naïve T cells and aberrant CD45 pre-mRNA alternative splicing are key features of *thunder* strain, however, they show different heritability. The loss of naïve T cells is strictly a recessive trait as heterozygotes share the same phenotype as wild-type with normal numbers of naïve CD4⁺ and CD8⁺ T cells in the thymus and periphery. In comparison the CD45 variable exon expression shows evidence of a gene dosage dependent semi-dominant effect such that heterozygote mice express an intermediate level of CD45RA, RB & RC in CD4⁺ and CD8⁺ T cells. These results raise the possibility that the changes in CD45 isoform expression may not account for

the defect in homeostasis of naïve T cells. However this line of work will be followed up in more detail in chapter 5.

3.5 Chapter Summary

We have identified a novel mouse strain generated by ENU mutagenesis which is characterised by the selective reduction of naïve CD4⁺ and CD8⁺ T cells in the peripheral lymphoid tissues and aberrant CD45 pre-mRNA alternative splicing causing constitutive expression of CD45RA, RB and RC isoforms in T cells. The association between expression of the high molecular weight CD45 isoforms and naïve T cell homeostasis is unclear and will be a focus of later chapters in this thesis. This novel ENU variant mouse strain provides an ideal model and a unique entry point to investigate the role of pre-mRNA alternative splicing and some important aspects of naïve T cell biology, such as homeostasis, survival, activation, and immune memory cell transition.

~Chapter 4~

*Identification of thunder mutation
in a novel gene Hnrpll*

Introduction

Protein encoding genes are transcribed to messenger RNA with genetic information and then translated into proteins to perform biological functions. Nascent mRNAs, also called primary mRNA (pre-mRNA), undergo splicing to remove introns so that only continuous sequence of codons exists in mature mRNAs. There are specific sequences near exon-intron boundaries called exonic/intronic splicing enhancer/silencer (ESE/ISE or ESS/ISS) that respectively bind either splicing factors (SR proteins) or heterogeneous nuclear ribonuclear proteins (hnRNP) to initiate or suppress exon recognition by the core splicing machinery (spliceosome). About 30 hnRNPs have been identified playing multiple roles in RNA processing. hnRNP L has been suggested to repress the inclusion of CD45 variable exons via regulated ESS (Rothrock CR, et al. 2005), but the complete mechanism of CD45 pre-mRNA alternative splicing is still obscure. In this chapter, we reveal the identification of the *thunder* mutation and its essential role in regulating CD45 variable exon splicing.

4.1 Genome mapping and sequencing *thunder* mutation

4.1.1 Genome screening for chromosome linkage

Several generic mapping approaches have been successfully used in the Immunogenomics laboratory to efficiently localise ENU mutations to a particular chromosomal region by cosegregation of the mutation with genetic markers throughout the genome. A homozygous *thu/thu* mouse (C57BL/6S background), which was confirmed by flow cytometry to have low numbers of peripheral T cells and constitutive expression of the CD45 isoforms by flow cytometry, was outcrossed

to an inbred mapping strain, CBA, to produce heterozygous F1 progeny with one set of B6 and CBA chromosomes each. Each F1 mouse will carry one mutant allele and one wild-type allele of the gene responsible for the low T cell trait in *thunder* mice. During a meiosis in the F1 animals meiotic recombination shuffles the parental chromosomes. Intercrossing F1 carrier mice by brother-sister pairings generated F2 progeny of which 25% are homozygous for the recessive *thunder* mutation. Comparing a group of homozygous mutants with wildtype or heterozygous mice using B6/CBA polymorphic genetic markers across all chromosomes distinguishes the chromosomal regions closely linked to the mutation, as the affected mice will all bear homozygous B6 alleles in the mutant locus whereas unaffected mice will have CBA or heterozygous alleles (Figure 4.1 a). Once the linkage region is narrowed down to a reasonable size (usually < 2Mbp), candidate genes can be sequenced to verify the mutation (Figure 4.1 b) (Nelms KS, Goodnow CC. 2001).

To map the *thunder* mutation F2 mice were screened by flow cytometry to identify mice with low numbers of peripheral T cells and those which show constitutive expression of CD45 RA, RB and RC on peripheral T cells. Genomic DNA was prepared from a cohort of 15 affected F2 mice and 4 unaffected F2 mice and these were tested individually with 50 independent microsatellite markers spaced at about 30-40 cM intervals across the genome. Genome scanning showed that the *thunder* mutation was linked to the distal end of chromosome 17 near the microsatellite marker D17Mit38 at about 74.1 Mb, as 11/15 individual affected mice showed a homozygous B6 allele plus 3/15 had heterozygous B6/CBA allele in contrast with $\frac{3}{4}$ unaffected siblings showing homozygous CBA allele at the same region. Chi square test showed that $\chi^2=7.34$ ($\chi^2_{0.05(1)}=3.84$), indicating $P<0.05$ and significant statistic difference between the two groups (Figure 4.2).

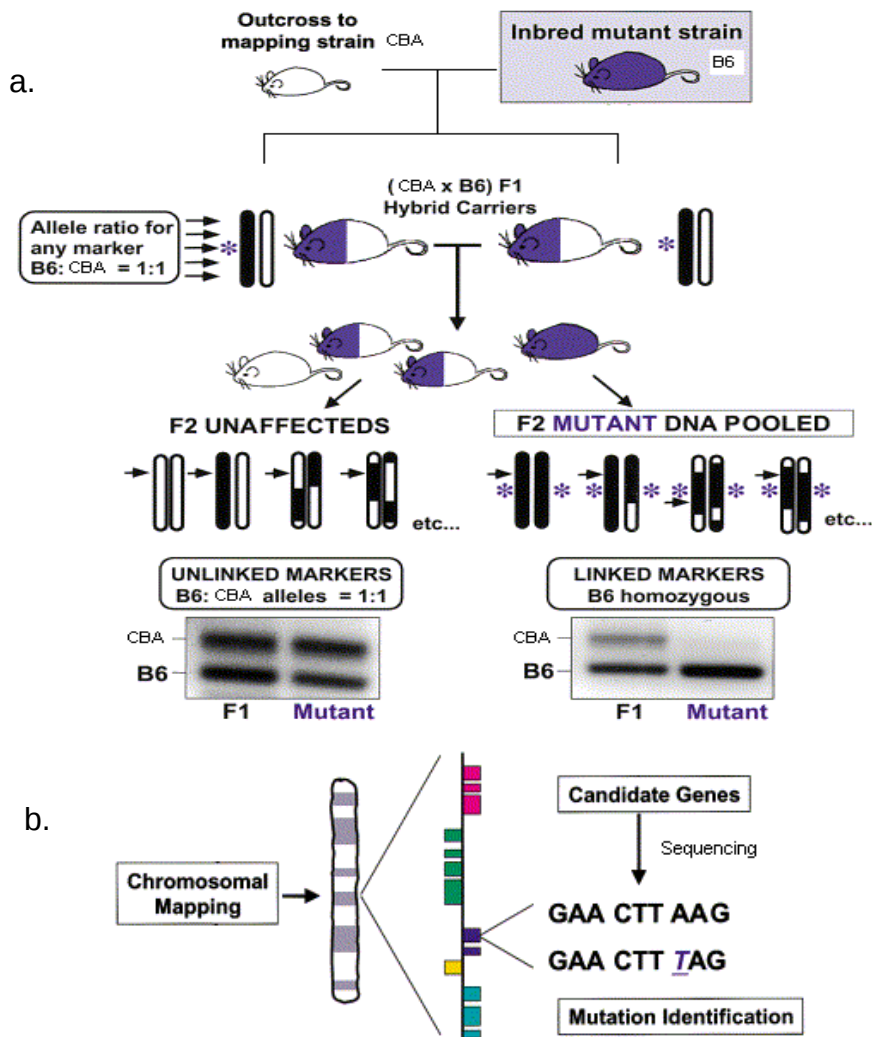
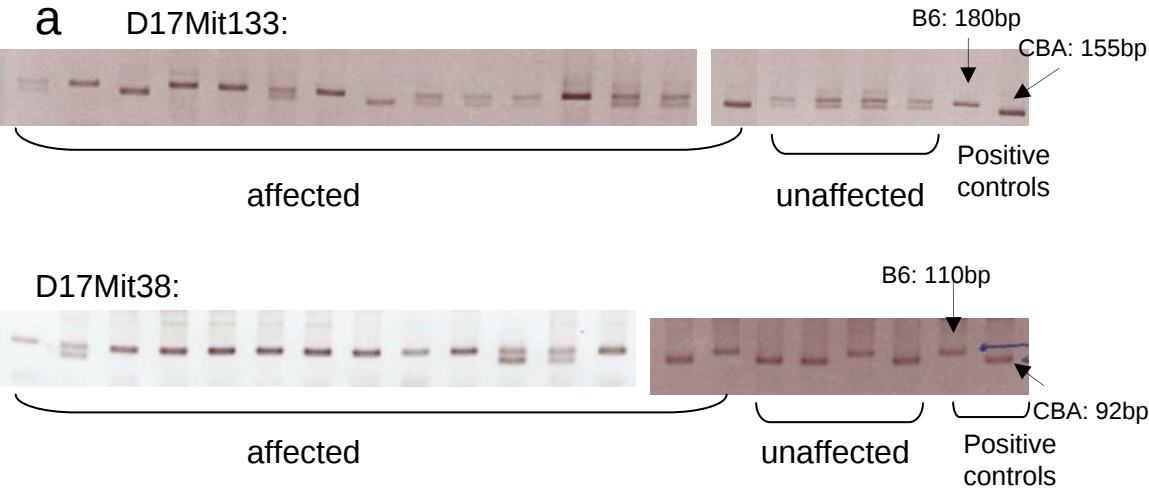


Figure 4.1 Genome mapping and sequencing to identify recessive thunder mutation.

(a) Intercross breeding strategy used to map recessive mutation by genetic linkage analysis. Mutant C57BL/6 mice are backcrossed to a mapping strain (CBA) to produce F1 hybrid mice that carry the mutation and these are intercrossed to produce F2 mice where 25% of offspring should be homozygous mutant for the mutation. DNA from the affected F2 progeny can be pooled and analyzed by microsatellite markers. The B6 and CBA alleles can be separated by size of PCR fragments in gel electrophoresis. Markers closely linked to the mutation will yield on B6 homozygous alleles, whereas unlinked markers will show both B6 and CBA alleles in F2 mutant mice. DNA from the F1 progeny should give both B6 and CBA alleles. (b) If the linked region is <2Mb then all in the interval are resequenced and the mutation in the coding region can be identified.

Figure 4.2 Genome scan for linkage to distal end of chr 17



b

B6CBA F2 mouse	markers	D17Mit133	D17Mit38
	chr location	23.5 Mbp	74.1 Mbp
12	Affected	B6//CBA	B6
14		B6	B6//CBA
15		CBA	B6
22		B6	B6
44		B6	B6
45		B6//CBA	B6
88		B6	B6
116		CBA	B6
119		B6//CBA	B6
140		B6//CBA	B6
162		B6/CBA	B6//CBA
163		B6	B6//CBA
164		B6//CBA	B6
168		B6//CBA	CBA
197		CBA	B6

129	unaffected	B6//CBA	CBA
137		B6//CBA	CBA
146		B6//CBA	B6
145		B6//CBA	CBA

Figure 4.2 Linkage of thunder mutation with microsatellite markers to the distal end of chromosome 17. (a) Representative RT-PCR results for 2 markers used to define the linked region on chromosome 17. The allelic differences between B6 and CBA are indicated on the right of each gel. (b) Results of linkage analysis using DNA from 15 affected F2 mice and 4 non affected F2 mice using the same two markers as shown in (a).

4.1.2 Fine mapping

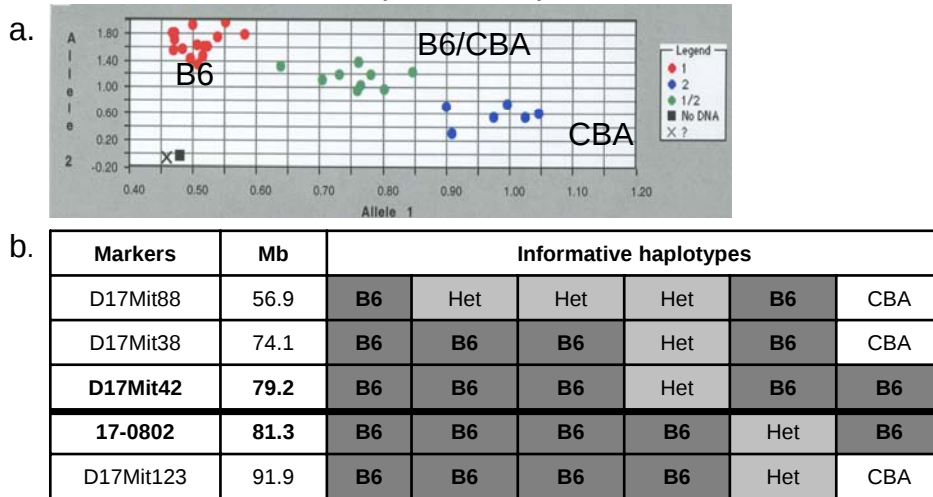
In order to confirm and delimit the linkage region and accurately define the location of the mutation, more genomic samples were tested to find affected individuals that might have an informative recombination event that would help define more closely the region containing the ENU variant gene responsible for the *thunder* phenotype. This involved the use of additional markers within the linked region using DNA from 60 affected F2 mice to help in fine mapping. The linked interval was further reduced in size down to a 2.1 Mb region defined by the two microsatellite markers D17Mit42 and a SNP marker at the position of 17-080241937, which are localised at 79.2 and 81.3 Mb of chromosome 17 (Figure 4.3 a-b).

According to the Ensembl mouse genome database, there are 21 transcription units within the linked interval as listed in Figure 4.3 c. Among them there are several genes particularly involved in RNA processing based upon protein sequence motifs, including *Hnrpll* belongs to hnRNP family, *sfrs7* is related to known SR protein splicing factors, *Arl6ip2* has homologs to nucleotide binding and *AW494914* has motifs featured in RNA helicases.

4.1.3 Sequencing *thunder* mutation: 407T→A transition in *Hnrpll*

cDNAs were amplified from the thymus mRNA of both unaffected and affected mice and were sequenced in priority based on a candidate gene approach, but eventually all the coding region of genes in the linkage interval was resequenced.

Marker 17-080241937 (rs3676709)



c.

	Gene ID	CDS (bp)	Description
1	Arl6ip2	2842	ADP-ribosylation factor-like 6 interacting protein 2 (nucleotide binding)
2	UNKNOWN		
3	UNKNOWN		
4	Hnrpl	3002	heterogeneous nuclear ribonucleoprotein L-like
5	Galm	2245	galactose mutarotase
6	Sfrs7	2272	splicing factor, arginine/serine-rich 7
7	4930560E09Rik		PREDICTED: hypothetical protein LOC67737
8	Gemin6	1086	gem (nuclear organelle) associated protein 6
9	AW494914	4101	expressed sequence AW494914 (ATP dependent RNA helicase)
10	Mopt	647	protein containing single MORN motif in testis
11	Gm941	1207	gene model 941, (NCBI)
12	Q8BSD7_MOUSE	324	12 days embryo embryonic body between diaphragm region and neck cDNA,
13	Sos1	4747	Son of sevenless homolog 1 (Drosophila)
14	XP_355031.3	984	PREDICTED: similar to cyclin-dependent kinase-like 4
15	Map4k3	3382	mitogen-activated protein kinase kinase kinase kinase 3
16	UNKNOWN		
17	UNKNOWN	248	
18	2810417M05Rik	1690	RIKEN cDNA 2810417M05 gene
19	Thumpd2	1848	THUMP domain containing 2
20	NM_178927.2		expressed sequence AV344025 (AV344025), mRNA
21	Slc8a1	2823	solute carrier family 8 (sodium/calcium exchanger), member 1

Figure 4.3 Fine mapping of the Thunder mutation to a 2.1Mb interval.

(a) Fine mapping on independent cohort of 60 F2 mice was performed using a number of microsatellite markers using the amplifluor technology. (b) An informative recombination was identified between 79.2 and 81.3 Mb on chr 17. (c) The table shows the list of 21 genes contained within the 2.1 Mb linked interval.

The only identified mutation was a 407T→A transition in the transcript of *Hnrpll*, resulting in a valine to aspartic acid substitution in the protein (Figure 4.4 a). The mutation was confirmed by resequencing genomic DNA samples of other affected mice.

Hnrpll belongs to the hnRNP family which consist of a class of diverse RNA binding proteins associated with nascent mRNA processing and was named heterogenous nuclear ribonuclear protein L-like as it shares 58% homolog to hnRNP L. HnRNPs are highly conserved proteins and hnRNP LL has more than 97% residue identity between mouse and human (Black, D.L. 2003). A common feature of hnRNP proteins is that they contain at least one RNA recognition motif (RRM) also called RNA binding domain (RBD). So far there are more than 30 hnRNP proteins identified. hnRNP A/B can block splicing factor binding to splicing enhancer to promote exon silencing during alternative mRNA splicing, and hnRNP I (PTB) can bind to the 3' splicing site of pyrimidine-rich sequence (Domsic JK, et al. 2003; Lou, H. et al. 1999; Hall-Pogar T, et al. 2007).

Comparison of the primary structure of hnRNP LL and hnRNP L shows the similarity of the two proteins (Figure 4.4 b). They both have 3 RRM domains but hnRNP LL has an additional coiled-coil domain at the N-terminus and the related hnRNP L protein has a proline-rich region between the RRM2 and 3 domains. The mutation in the *thunder* strain is located within the RRM1 domain (Figure 4.4 b).

The RRM motif is the major domain of many eukaryotic RNA binding proteins binding to single-stranded RNA and consists of about 90 amino acids (Black, D.L.

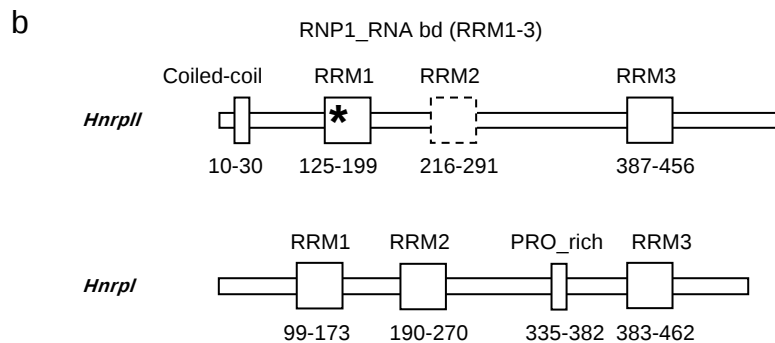
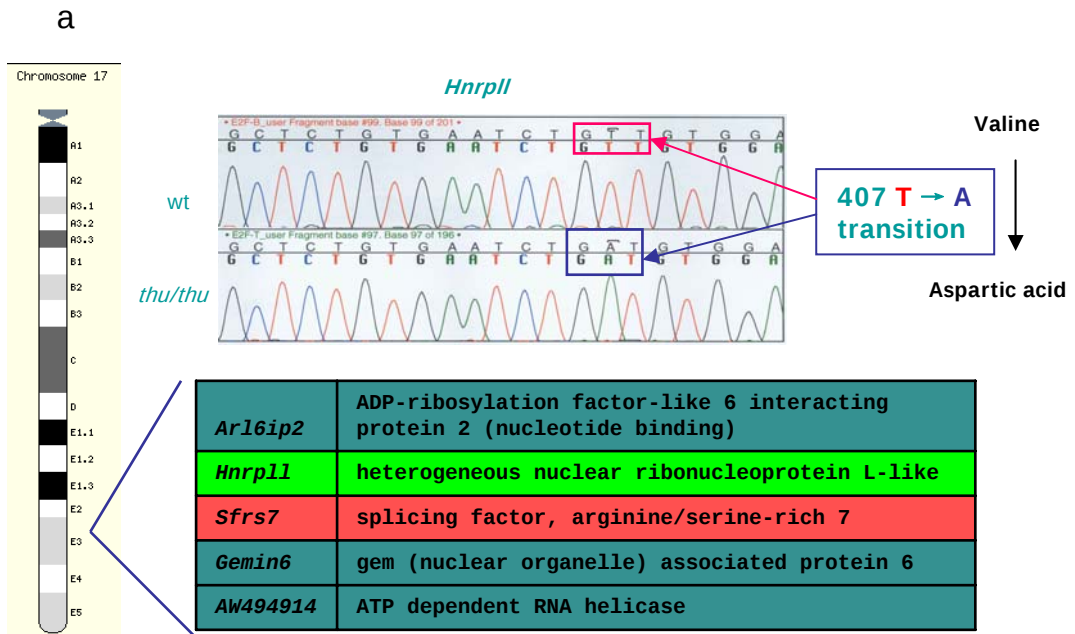
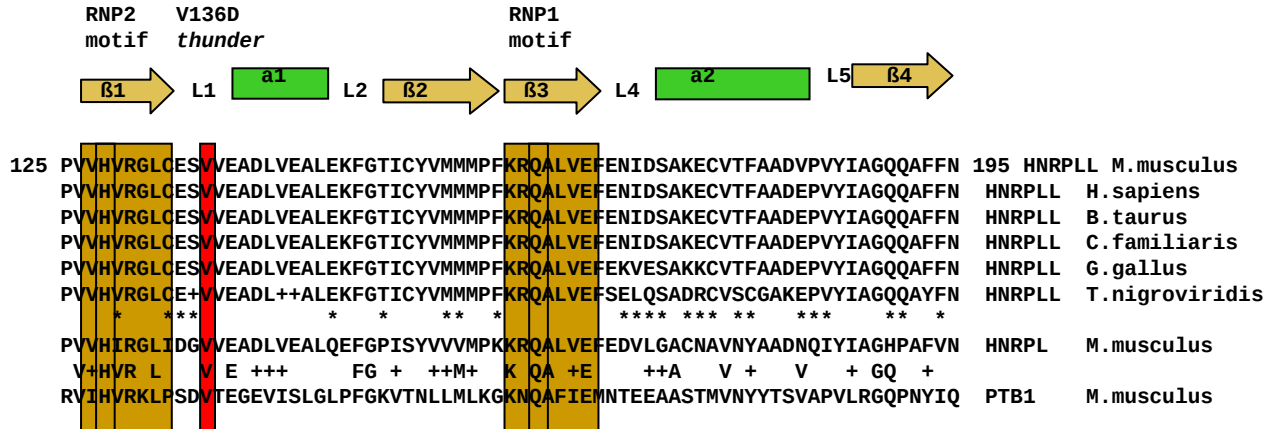


Figure 4.4 sequencing *thunder* mutation.

- (a) Genes involved in RNA processing in the linkage region of chr 17 and T→A transition in *Hnrp11* base 407.
- (b) Primary structure of *Hnrp11* and *Hnrpl*. The numbers indicate the location of the domains within the primary amino acid sequence for each gene.

C.



d.

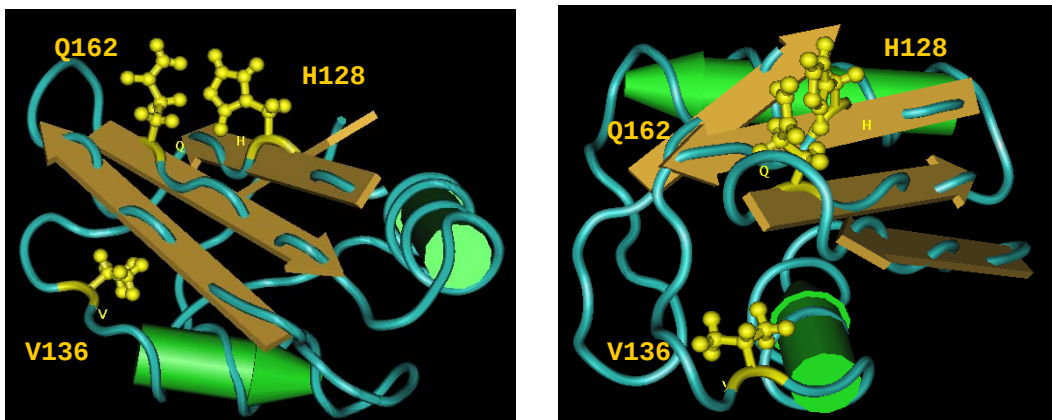


Figure 4.4 sequencing thunder mutation.

(C) Conservation of Val 136 residue in the hnRNPLL RRM1 domain across several species. The mutated valine in *thunder* mice is indicated in red and as can be seen this residue is completely conserved across the species and is also conserved in related hnRNP proteins such as hnRNPL and PTB. Above the sequence alignment are the locations of the residues relative to the secondary protein structure. The brown arrows indicate β -pleated sheets, whereas the green boxes indicate the α -helices. The brown boxes indicated on the sequence alignment identify the RNP1 and RNP2 motifs, and within these domains the key conserved H and Q residues are highlighted in the square.

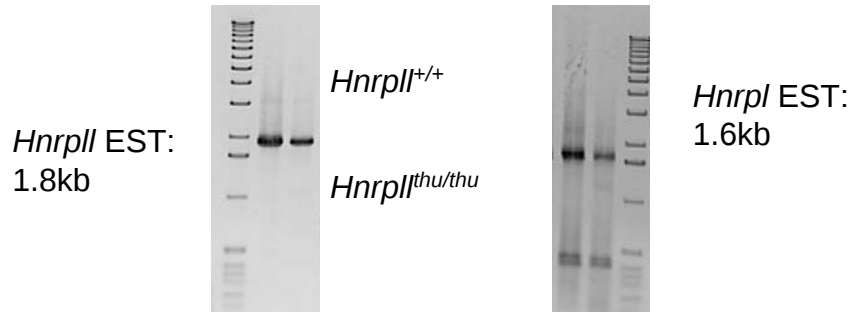
(d) NMR structure of the wild type hnRNPLL RRM1 domain. The mutated valine 136 residue lies beneath the RNP1 and RNP2 motifs and depicted on the diagram are the two key RNA contact points H128 and Q162 (NMR model from RIKEN database).

2003). RRM s are found in a variety of RNA binding proteins, including heterogeneous nuclear ribonucleoproteins (hnRNPs) and protein components of small nuclear ribonucleoproteins (snRNPs). The RRM domain contains two consensus sequences known as the RNP1 and RNP2 motifs which are highly conserved octapeptide and hexapeptide sequences respectively. The RNP1 and RNP2 motifs form the core of the binding site for a target RNA. In particular there are two highly conserved amino acids, a glutamine within the RNP1 motif and a histidine in the RNP2 motif, that provide essential contact points to the RNA target (Dreyfuss G. et al. 1993). The X-ray or NMR structures of several hnRNP proteins has been solved and these have helped to define a general RRM structure that consists of four β strands and two α helices arranged in an alpha/beta sandwich. Recently the RRM1 domain of hnRNP LL was solved in collaboration with Prof Gottfried and colleagues at the Research School of Chemistry at the ANU. The conserved valine residue mutated in *thunder* mice is located within loop1 between the β 1 sheet (RNP2) and the α 1 helix highlighted in red in Figure 4.4 c. The conserved RNA contact residues are highlighted on the structure as Glutamine (Q162) within the RNP1 domain and histidine (H128) in the RNP2 domain. These residues are boxed in the amino acid alignment of the RRM1 domain (Figure 4.4 c) and the conserved valine (V136) is indicated in red. NMR structural model of RRM1 domain is shown in Figure 4.4 c with H128, V136, and Q162 labelled.

4.2 *Hnrp11* is a novel pre-mRNA alternative splicing regulator

Retrovirus based gene transfer: over-expression of *Hnrp11* can down-regulate CD45 exon 6 expression in *Hnrp11*^{thu/thu} CD4 T cells

a. RT-PCR amplify *HnrpII* and *HnrpI* cDNA



b. Recombinant vectors digested with restriction enzyme *XhoI* and *EcoRI*

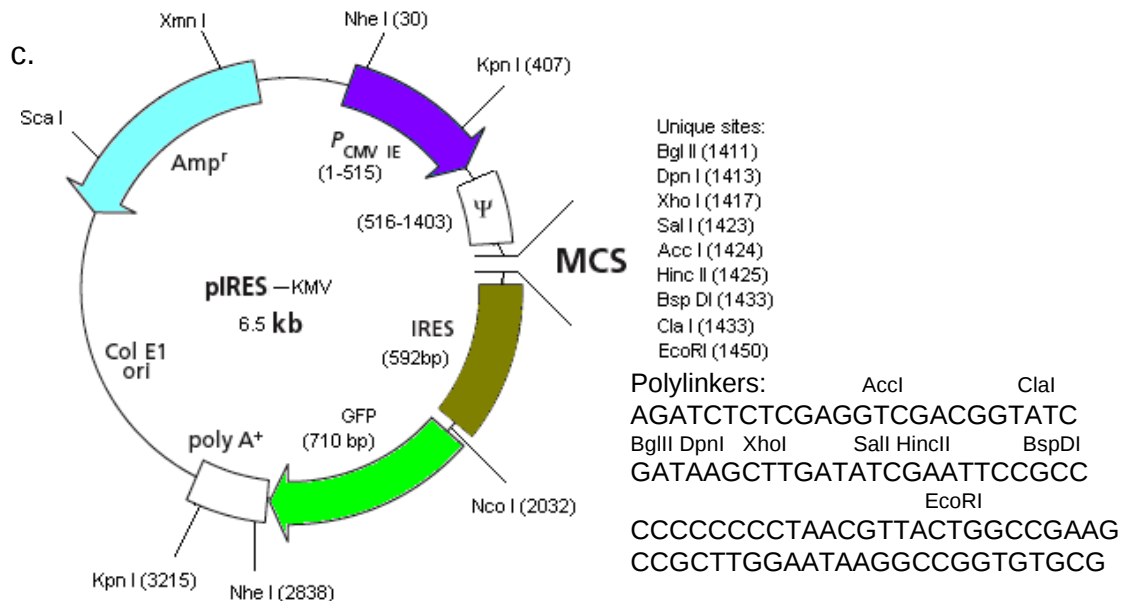
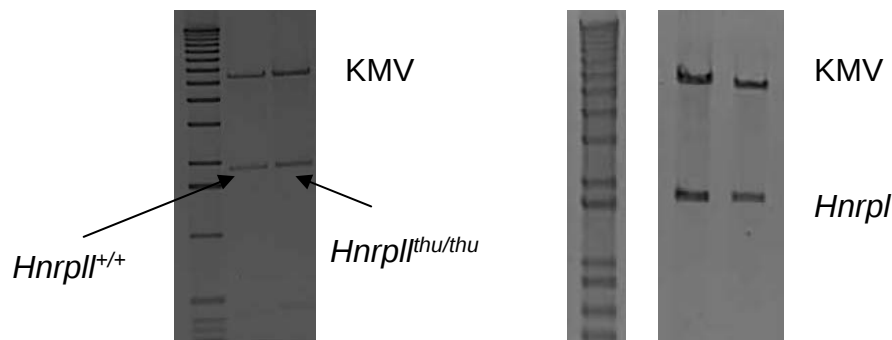


Figure 4.5 Retrovirus based gene transfer rescue of CD45 alternative splicing by expression of wild type *HnrpII*. (a) RT-PCR amplification of wild type and mutant *HnrpII* and wild type *HnrpI* cDNA. (b) Restriction enzyme digestion to confirm the cloning of the relevant cDNAs. (c) Plasmid map of the KMV retroviral vector used to over express the wild type cDNA in mutant T cells.

To prove that the mutation in *Hnrpll* was responsible for the defect in CD45 pre-mRNA alternative splicing, a retrovirus based gene transfer experiment was carried out. In theory, if the 407T→A *Hnrpll* mutation causes CD45 pre-mRNA splicing defects in *Hnrpll*^{th^u/th^u} T cells, expression of wild-type *Hnrpll* cDNA should restore silencing of CD45R exons and epitopes.

Both wildtype *Hnrpll* and *Hnrpll*^{th^u/th^u} cDNA were amplified (Figure 4.5 a), confirmed by sequencing, and cloned into a bicistronic retroviral vector (p-IRES-KMV) (Figure 4.5 a, b and c). Retroviral vector p-IRES-KMV is modified from pIRES bicistronic expression vector from Clontech laboratories, Inc. It contains the internal ribosome entry site (IRES) of encephalomyocarditis virus (ECMV) allowing translation of two open reading frames from a single mRNA. Ribosomes can enter the bicistronic mRNA either at the 5' end of the inserted gene of interest or at the ECMV IRES to translate the reporter gene GFP. The single cassette expresses both the cDNA of interest and GFP so all transduced cells co-express GFP and the gene of interest (Figure 4.5 c). Successful constructs were cloned with the correct size of the vector and the correct expected sizes of the *Hnrpll*, *Hnrpll*^{th^u/th^u}, or *Hnrpl* cDNAs (Figure 4.5 b, c). Successful constructs were then amplified by large scale culture of the positive colonies. Plasmids were extracted and transfected into a phoenix packaging cells expressing ectopic env gag virus coating proteins. The supernatants containing infective retroviral particles were used to transduce mouse primary T cells activated with anti-CD3 and anti-CD28 and cultured for 3 days in vitro. At the end of the culture the cells were harvested, stained with CD45RC antibody and analysed by flow cytometry to compare CD45RC expression on GFP+ and GFP- cells.

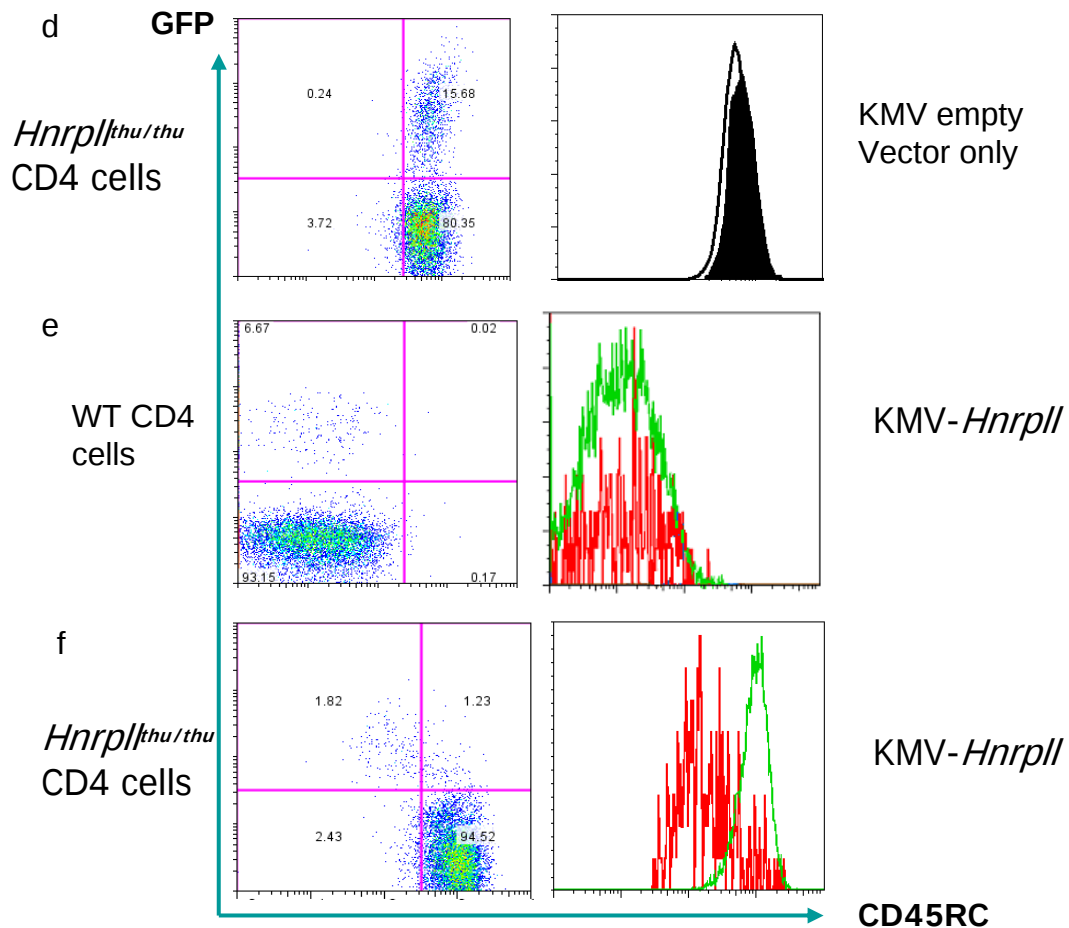


Figure 4.5 Retrovirus based gene transfer rescue of CD45 alternative splicing by expression of wild type *HnrpII*.

(d) *Thunder* CD4⁺ T cells were transduced with an empty vector retrovirus and stained for CD45 RC expression. The GFP⁻ and GFP⁺ cells show constitutive expression of the CD45RC epitope at the cell surface. (e) Wild type CD4⁺ T cells were transduced with a *HnrpII* expressing retrovirus and the cells were stained with CD45RC and analyzed by FACS. Data shows that wild type T cells normally have low expression of CD45RC and there is no difference in CD45RC expression in GFP⁺ cells (red line) expressing the wild type *HnrpII* gene and non-transduced cells (green line). The blue line shown on the graph represents the level of CD45RC expression on *thunder* CD4⁺ T cells (f) *Thunder* CD4⁺ T cells were transduced by a retrovirus encoding the wild type *HnrpII* gene. The data show that *thunder* T cells forced to express the wild type *HnrpII* gene (GFP⁺ cells, red line) downregulate CD45RC expression as a result of alternative splicing. In contrast non transduced *thunder* T cells that fail to silence CD45RC expression (GFP⁻, Green line).

The results show that when *thunder* CD4⁺ T cells are transduced with an empty vector, these GFP⁺ cells retain constitutive expression of the CD45RC protein at the cell surface that is indistinguishable from non-transduced GFP⁻ cells in the same culture (Figure 4.5 d). By contrast when the vector also expresses wild type *Hnrpll* cDNA there is a marked decrease in CD45RC expression selectively on the GFP⁺ cells (Figure 4.5 f). As an internal control, the non-transduced *thunder* T cells in the culture fail to silence CD45RC expression and maintain high levels of CD45RC on the cell surface (Figure 4.5 f). Wildtype CD4 T cells transfected with p-IRES-KMV-*Hnrpll* virus did not change CD45 splicing as CD45 variable exons have been silenced with endogenous *Hnrpll* (Figure 4.5 d). This gene complementation experiment establishes that the mutation in *Hnrpll* is responsible for the loss of CD45 exon silencing in *thunder* mice.

4.3 *Thunder* mutation destabilizes RRM1 domain of hnRNP LL protein

Structural studies were carried out in collaboration with Prof. Gottfried Otting and his PhD student Xinying Jia in the Research School of Chemistry ANU to understand how the mutation affects the RRM1 domain of hnRNP LL. NMR solution structures of bacterially expressed wild-type and mutant RRM1 revealed that both adopted a classic RRM fold, however, the non-polar valine mutated to a positive charged aspartic acid in the hydrophobic core of the protein destabilized the domain above 25 °C (Figure 4.6 a). The protein is coloured from blue (N-terminus) to red (C-terminus). The surface with the most positive charge potential is on the top representing RNA binding site. Below 15 °C the resonance assignment of the 15N-

Figure 4.6 *Thunder* mutation destabilizes RRM structure of hnRNP LL protein

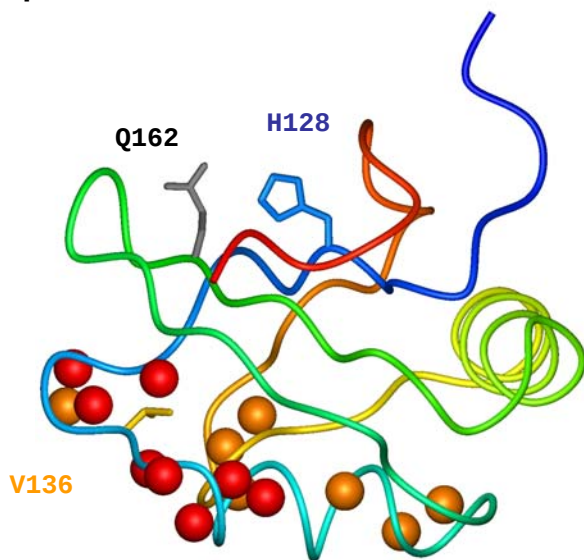


Fig.4.6 a. NMR structure of RRM1 combined with resonance assignment of ^{15}N -HSQC spectrum. The protein is colored from N-terminus in blue to C-terminus in red. Q162 and H128 residues are marked in grey and light blue representing RNA binding site. V136 is marked in yellow underneath and the mutation to D136 results in the missing of some amide protons shown in red spheres and significantly shifted peaks shown in orange. (Data from Mr. Xinyin Jia and Dr. Gottfried Otting).

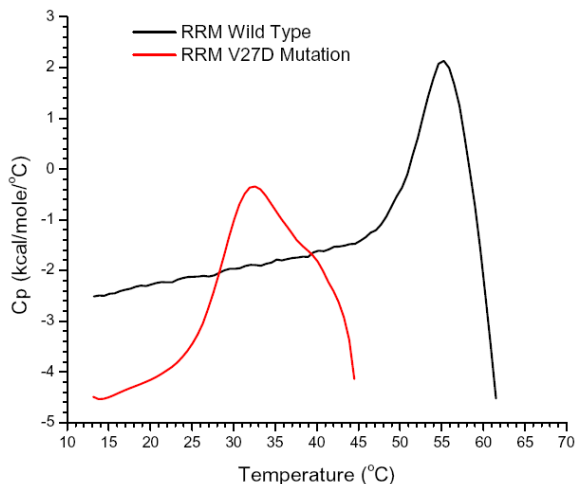


Fig.4.6 b. DSC thermograms of RRM1 wild type (—) and RRM1 V136D mutation (—). Heating rate is $1.5^\circ\text{C}/\text{min}$. Prescan thermostat is 5 min. Postscan thermostat is 0 min. RRM wild type was analyzed at concentration of 0.0725 mM and RRM V27D mutation was analyzed at concentration of 0.126 mM (Data from Mr. Xinying Jia and Dr. Gottfried Otting).

HSQC spectrum of the wild-type and the mutant RRM1 domain showed that wild-type peaks are selectively altered as missing from the spectrum of the $\alpha 1$ helix (Figure 4.6 a). The respective amide protons in the structure have been highlighted as spheres. Red spheres mean missing peaks; orange spheres mean significantly shifted peaks. The side chain of Val136 is shown in yellow underneath the RNA binding surface composed of RNP1 and RNP2 domains marked by Q162 in grey and H128 in light blue..

The stabilities of the wild-type and mutant RRM1 domains were assessed by differential scanning calorimetry. The mutation resulted in a much lower melting temperature and a significant degree of unfolding above 25 °C of the mutant RRM1 domain, whereas the structure of the wild-type domain was stable until 45 °C (Fig. 4.6 b). The combined NMR and DSC data indicate that the V136D mutation is likely to interfere with RNA binding indirectly by producing a thermolabile structure which is mostly unfolded at 37 °C.

4.4 *Hnrpl* expression in lymphocyte subsets

To determine *Hnrpl* expression in lymphocytes, CD4SP and CD8SP from thymus and CD4+ T cells, CD8+ T cells, and CD19+ B cells from spleen were sorted by flow cytometry. Total RNA was isolated and quantified to use equal amount of RNA for realtime-PCR. The results show that *Hnrpl* is highly expressed in both CD4+ and CD8+ T cells but lowly expressed in B cells, in contrast with *Hnrpl* is expressed in all lymphocytes. This is consistent with the observation that mouse B cells cannot silence the 3 variable exons of CD45 (exon 4, 5 and 6) and provides further evidence for the

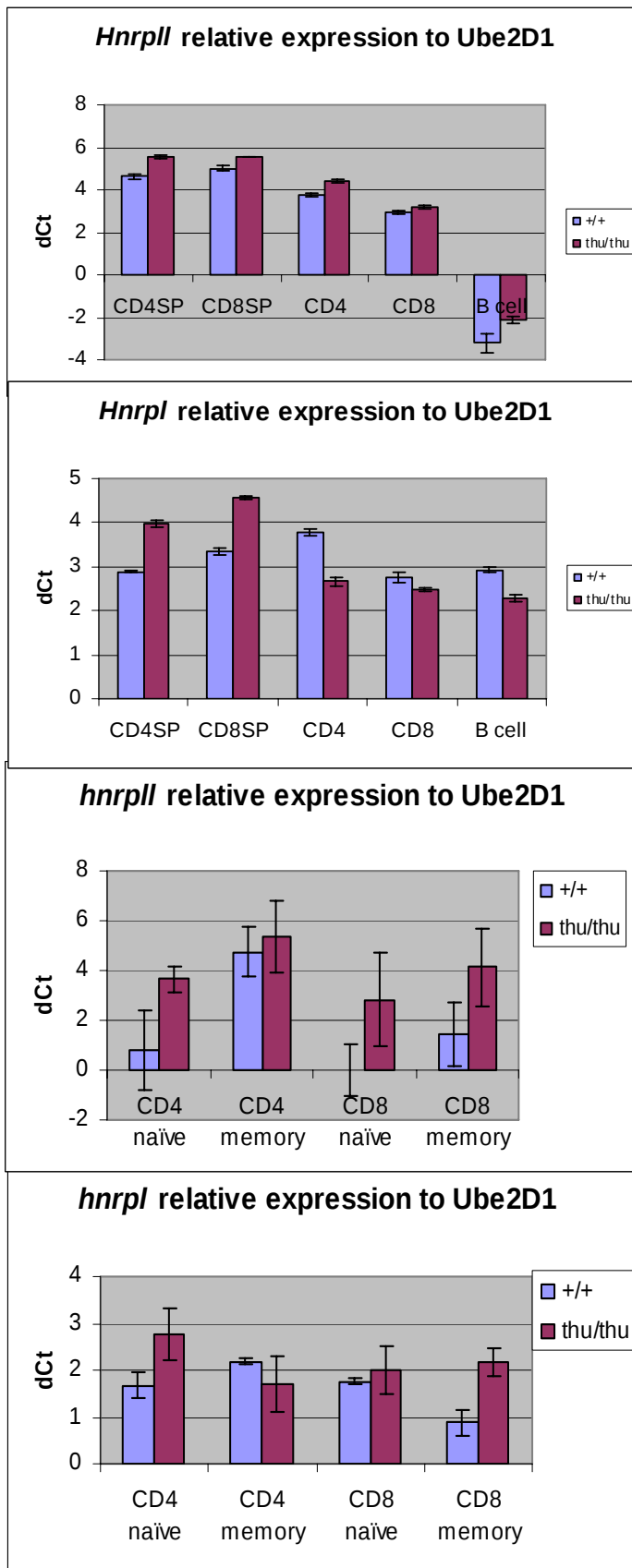


Figure 4.7
Hnrpl and *Hnrpl*
expression
relative to Ube2d1
in different
lymphocyte
subsets

non-redundant role for the *Hnrpll* gene to allow T cells to silence the three variable CD45 exons. Furthermore, *Hnrpll* is more highly expressed in memory T cells than naïve T cells, suggesting *Hnrpll* is developmentally regulated and is up-regulated as T cells differentiate to become memory cells. It is also well established that memory T cells predominantly express CD45RO isoform due to activation induced silencing of the 3 variable exons in CD45 molecule (Bell, E.B., Sparshott, S.M., 1990; Beverley, P. 1991). *Hnrpll*^{thu/thu} naïve T cells have more *Hnrpll* transcripts than wildtype counterparts. This may be due to loss of *Hnrpll* function in *Hnrpll*^{thu/thu} mutants and the compensation of relative higher expression (Figure 4.7).

4.5 Discussion

HnRNP proteins are important regulators of pre-mRNA alternative splicing. Several members of the hnRNP protein family such as hnRNP A, PTB (hnRNP I) have been demonstrated to play multiple roles in pre-mRNA processing (Wang, Z. et al. 2004; Oberstrass, F.C. et al. 2005; Paradis, C. et al. 2007). Here we have identified a mutation in a previously uncharacterized member of the hnRNP protein family hnRNP LL. Like other members of this diverse protein family. Based on analysis of the *thunder* mutation and complementation with wild-type protein expressed from a retroviral vector, hnRNP LL plays an important role in regulating alternative splicing of at least one known target gene in CD4⁺ and CD8⁺ T cells and that is the *Ptpnc* gene which encodes the tyrosine phosphatase CD45.

Although the detailed mechanism of pre-mRNA splicing is still poorly understood, it is generally accepted that the major function of hnRNP proteins is to bind to

ESS/ISS sequences (Black, D.L. 2003). In this way, they repress recognition of the consensus cis-acting exon splicing enhancer sequences by splicing factors of the Serine –arginine rich (SR) protein family that promotes recruitment of the spliceosome to the intron/exon boundary and to promote exon inclusion on the mRNA target. When hnRNP proteins are bound to the ESS/ISS cis regulatory sequences of exons this thereby prevents the recruitment of the spliceosome and leads to exon silencing/skipping (Black, D.L. 2003).

Although alternative splicing of the *Ptprc* gene has been known for more than 20 years the critical *trans*-acting factor(s) that mediates exon silencing during alternative splicing have remained elusive. In recent years there has been considerable effort placed into understanding the molecular regulation of CD45 splicing. Although the three variable exons of CD45 exons 4, 5 and 6 are variably included in the CD45 mRNA depending on the state of maturation and activation, it has been shown that exons 4 and 6 are most tightly regulated (Lynch, K. 2004). Exon 5 usage appears more stochastic or leaky. Regulation of alternative splicing of CD45 is an important issue because it is a key marker of memory T cells. Dysregulation of CD45 splicing may predispose susceptible individuals to autoimmune diseases such as multiple sclerosis and to infections (Lynch, K.W. & Weiss, A. 2001; Boxall, S. et al. 2004). The *cis*- acting regulatory sites in these variable exons that have been implicated in disease etiology are the same that govern the isoform switch in activated T cells.

Several splicing factors of the SR family have been implicated in CD45 alternative splicing. Many of these studies have been performed at a biochemical

level because gene knockouts of the ubiquitously expressed SR proteins lead to embryonic lethality (Wang, H.Y. et al. 2001).

Previous studies by Lynch and colleagues have focused on the *cis*-acting ARS elements that regulate exon 4 silencing of CD45, since this is regulated in an activation dependent manner (Rothrock, C., et al. 2003; Rothrock, C. R., A. E. House, and K. W. Lynch. 2005; House, A. E., and K. W. Lynch. 2006; Melton, A.A. et al. 2007). They performed RNA affinity chromatography to elute the proteins bound to a RNA oligonucleotide probe containing the ESS1 *cis*-regulatory sequence of variable exon 4 that mediates partial exon repression in resting cells and increased exon skipping upon activation. They identified that multiple hnRNP proteins could associate with the ESS1 sequence including PTB, hnRNP E2 and hnRNP L (Rothrock, C. et al. 2005; Melton, A.A. et al. 2007). hnRNP L shares 58% homology with hnRNP LL. Within the RRM1 domain hnRNP L shares 66% identity with the hnRNP LL protein and the key conserved residues such as Val136, His128 and Q162 are completely conserved (Figure 4.4).

T cells from *thunder* mice have normal expression of the *Hnrpl* gene but the mutant cells still cannot splice CD45 correctly. We conclude from this finding that hnRNP LL must play a critical and non-redundant role in the regulation of CD45 alternative splicing in mouse T cells. Although hnRNP LL was not identified in the complex purified in human Jurkat cells these experiments are technically demanding and may not necessarily pull down all the relevant proteins *in vitro*. Another possibility is that the requirements for hnRNP L and hnRNP LL are reversed and so

the hnRNP L assumes a more dominant role in CD45 splicing in human T cells. Further studies should hopefully provide an answer to this question.

The NMR structure of the RRM1 domain of hnRNP LL shows that this previously uncharacterized protein shares many of the structural properties of other hnRNP proteins. The RRM1 domain of the hnRNP LL protein is conserved across the species, as are the location and structure of the RNP1 and RNP2 motifs. The valine mutated in *thunder* cells highlights the important roles of this residue for the first time that it plays in maintaining the stability and function of the RRM1 domain. Previous studies have identified the critical role that the conserved His and Glu residues play within the RNP1 & 2 motifs to contact the RNA template. The NMR structural studies performed on the wild type and mutant RRM1 domain of hnRNP LL revealed that introducing a non conserved amino acid renders folding of the whole domain temperature-sensitive.

Although hnRNP LL has three RRM domains it is clear from the phenotype of the *thunder* mouse and the disruption caused to CD45 splicing that the RRM1 domain of hnRNP LL must play an important and critical role in contacting the RNA template to allow exon silencing to occur. The hnRNP proteins usually have multiple RRM domains and it is not understood exactly how the multiple RRM domains facilitate in binding RNA. Binding data showed that RRM1 has micromolar affinity for ARS motif RNA (not shown, from Gottfried Otting's recent collaboration). It is possible that one RRM domain may provide a major contact site and the other domains could help stabilize the protein bound to the ESS/ISS *cis* regulatory sequences. Alternatively,

it is possible that unfolding of the RRM1 domain leads to degradation of mutant hnRNP LL.

The data shown in Chapter 3 and 4 shows that hnRNP LL has a non-redundant role in regulating alternative splicing of CD45 in T cells. Thus the related hnRNP L protein although expressed cannot substitute the functional role for hnRNP LL when it is mutated in *thunder* T cells. FACS staining showed evidence of a dose dependency in exon 4 & exon 6 silencing in both CD4⁺ and CD8⁺ T cells. These two exons are the most tightly regulated in T cell activation, whereas silencing of exon 5 is more stochastic. Although FACS staining does reveal some role for hnRNP LL in regulating efficient silencing it is likely other splicing regulators could be involved in this exon. Recently it was shown that the splicing factor U2AF26 and the Gfi1 proteins could regulate exon 5 usage in T cells (Heyd, F. et al. 2006).

The *Hnrpll* gene is developmentally regulated in T cells and appears to be low or absent in B cells. It is expressed by thymocytes and its expression is significantly upregulated as T cells differentiate from a naïve to memory cell. In contrast the *Hnrpl* gene does not show developmental regulation and is expressed equivalently between naïve and memory T cells and B cells. The differential expression of *Hnrpll* and *Hnrpl* between B cells is consistent with the pattern of CD45 splicing in B cells observed by flow cytometry. Normally B cells do not silence CD45RA, B, and C and the lack of *Hnrpll* expression in B cells may explain this. As T cells differentiate between the naïve and memory states they are believed to alter their program of gene expression that somehow changes their functional behaviour and allows for their long term persistence in the peripheral circulation without the need for persistent antigen.

A key feature of memory T cells is that they can proliferate more rapidly upon reactivation and can differentiate rapidly to become effector cells with increased secretion of cytokines. Exactly how this global change in gene expression is achieved remains poorly understood.

4.6 Chapter Summary

The data presented in this chapter identifies that the hnRNP LL protein is a novel *trans*-acting factor that regulates CD45 splicing in T cells. The phenotype of the *thunder* mouse suggests that hnRNP LL through its ability to regulate alternative splicing directly impacts on the process of T cell homeostasis. CD45 is clearly a target of the hnRNP LL protein in murine T cells but we hypothesize that this represents just the tip of the iceberg and that hnRNP LL is likely to control a much wider program of alternative splicing in T cells that may involve hundreds of genes. This is an area of work which will be expanded more on later in the thesis.

The data presented in chapters 3 and 4 of this thesis also provides further support for the important role that ENU mutagenesis programs can have in phenotype driven approaches to identify novel genes that regulate lymphocyte development and homeostasis in the immune system. Since the mutagenesis screens are non biased i.e. they make no prior assumptions for the role of genes in biological or cellular processes, this allows the identity of novel genes that regulate fundamental processes in the immune system to be discovered. Since the hnRNP proteins are ubiquitous in their expression this has made study of these proteins *in vivo*, especially in the

immune system, extremely difficult since mutations in these proteins normally result in an embryonic lethal phenotype.

Currently there are no known mutations in any of the hnRNP proteins in mice or humans as most of them have only been studied at a biochemical level. This makes the *thunder* strain an extremely valuable and unique resource that should allow us to gain important insights into: (i) how changes in alternative splicing affect the long term survival of naïve T cells and the generation of memory cells (ii) to explain how CD45 signalling influences T cell homeostasis, (iii) how changes in alternative splicing affects the generation and homeostasis of antigen-specific effector and memory T cells and (iv) generally provide new insights into the genetic regulation of T cell homeostasis.

~Chapter 5~

Defective T cell homeostasis resulting from

$Hnrp1l^{thu/thu}$ mutation

Introduction

The immune system aims to maintain a diverse repertoire of T cells in the peripheral circulation so it can fight off infections. It allows the rapid expansion and contraction of antigen-specific cells during an immune response. Some existing memory cells are destined to die and replaced by the new memory cells. Similarly naïve T cells have a defined life span and as one cell dies it is quickly replaced by a thymic emigrant that expands in number to fill the void. Studies over the last 10 years have begun to define the signals that regulate T cell homeostasis in vivo. There is ample evidence that naïve and memory CD4⁺ and naïve CD8⁺ T cells depend upon pro-survival signals mediated by IL-7/IL-7R signalling and TCR signals delivered by recognition of self-peptide/MHC interactions. In contrast, the long term survival of CD8⁺ memory cells is independent of TCR signals and relies upon IL-15 (Sprent, J. & Surh, C.D. 2002).

Memory T cells can be generated from either homeostatic proliferation (IL-7 dependent) or antigen-induced proliferation (TCR signal dependent) pathways. CD45 is an important molecule to initiate TCR signalling by modulating Lck activity (Hermiston, M.L. et al. 2003). In this chapter we explore the association between T cell homeostasis and CD45 expression and splicing in *thunder* mice. We further characterize changes in other cell surface markers that are known to influence T cell survival in the peripheral immune system, so that we can gain an insight into the homeostatic defects of *Hnrpl*^{th^u/th^u} naïve T cells.

5.1 Defective T cell homeostasis in *thunder* mice: failure in persistence

5.1.1 Normal proliferation of *Hnrp1l^{th1/th1}* T cells with anti-CD3/anti-CD28 stimulation *in vitro*

The failure of mature *Hnrp1l^{th1/th1}* naïve T cells to survive in the periphery could be due to defective TCR signalling. To test this we cultured wild-type CD45.1+ congenic T cells with *Hnrp1l^{th1/th1}* (CD45.2+) T cells *in vitro*. The T cells were prepared from the spleen and were purified by MACS separation to deplete NK cells, B cells, granulocytes, red blood cells, macrophages and dendritic cells. The T cells were labelled with the membrane dye CFSE and cultured on plates coated with various concentrations of anti-CD3 and soluble anti-CD28 in the culture medium. At the completion of the assay cells were harvested, stained with antibodies to CD4 and CD8 and 7AAD and analysed by FACS. As shown in Figure 5.1.1, there was no difference in the proliferative response of wild-type or *Hnrp1l^{th1/th1}* T cells *in vitro* as assessed by the rate of CFSE dilution to any of the concentrations of anti-CD3 used.

5.1.2 Normal homeostatic proliferation of *Hnrp1l^{th1/th1}* naïve CD4 and CD8 cells with or without MHC restricted peptide presentation in irradiated B6 and *Tap2* mutant mice

When T cells are placed into a lymphopaenic environment they can sense the empty lymphoid compartment and spontaneously proliferate in response to TCR-pMHC signals and pro-survival signals from IL-7. We wanted to determine if *thunder* T cells could undergo homeostatic proliferation when transferred to a

Figure 5.1.1 Normal T cell proliferation of thunder T cells in vitro

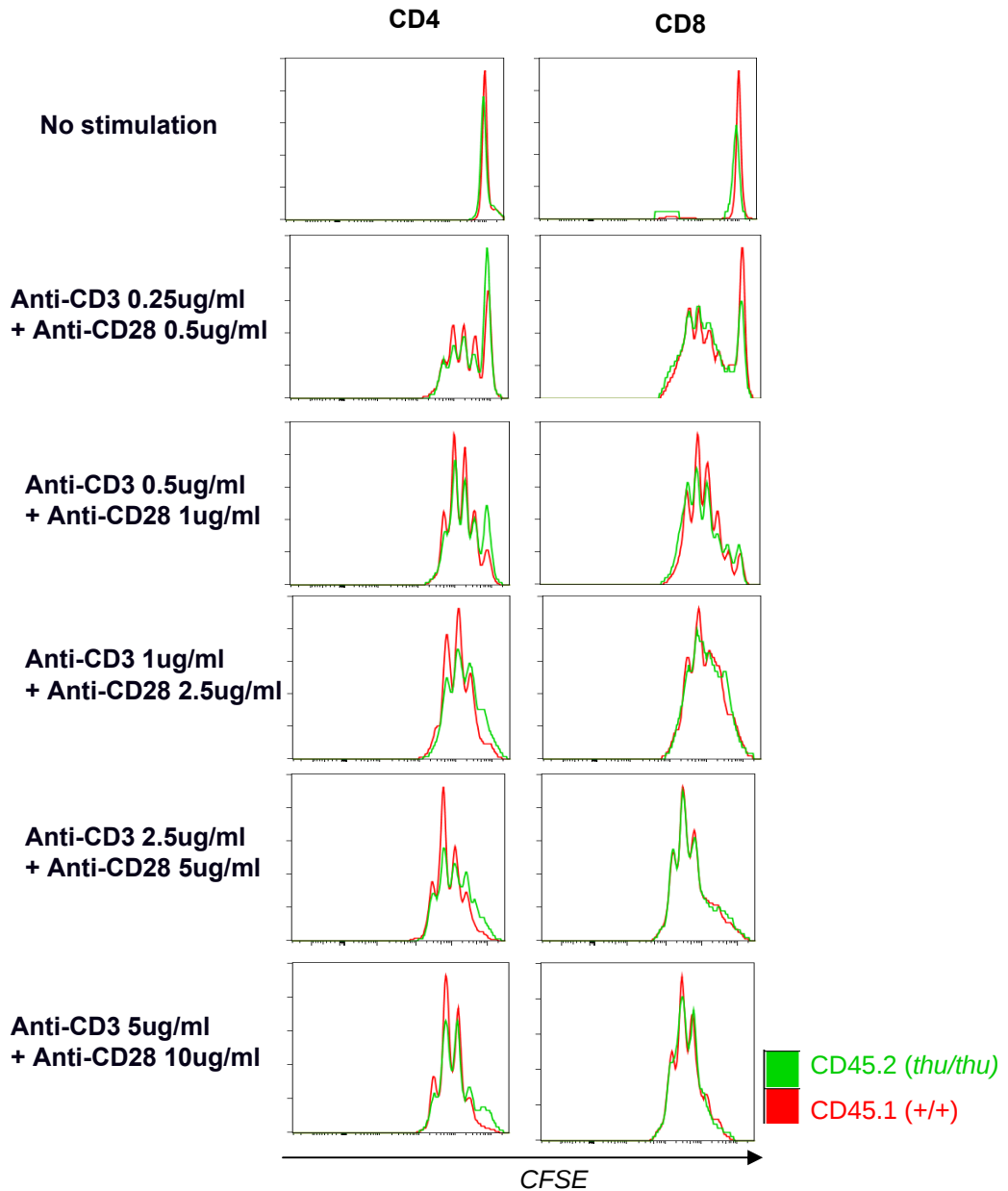


Fig. 5.1.1 Mixture of wild-type (CD45.1, red) and *thu/thu* (CD45.2, green) splenocytes were labelled with CFSE and co-cultured in medium containing different concentrations of anti-CD3 and anti-CD28. Data showed comparable proliferation of *thunder* T cells in response to anti-CD3 and anti-CD28 stimulation.

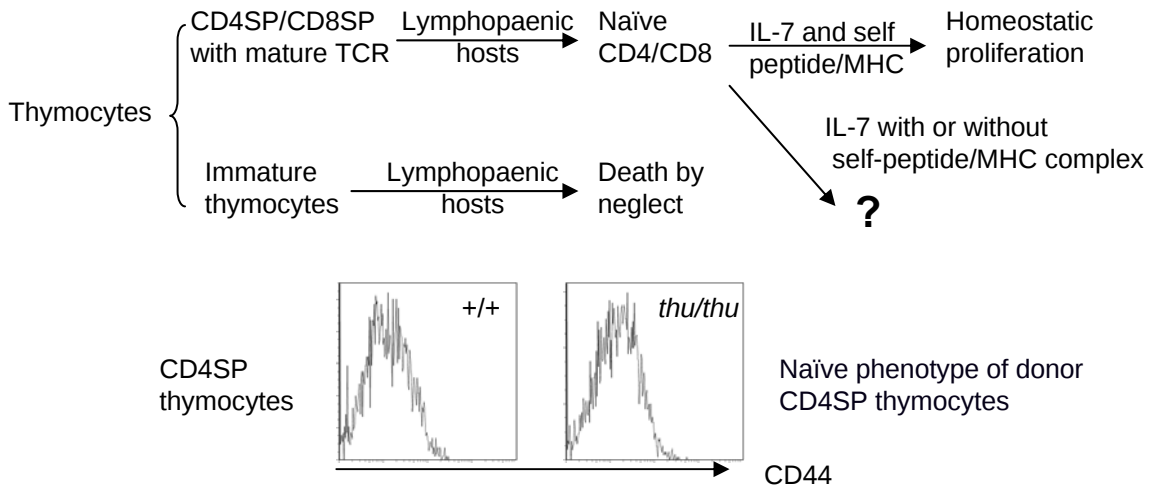
lymphopaenic host. Two different experimental models have been widely used to study homeostatic proliferation of T cells *in vivo*. One is to transfer T cells into *Rag2^{null}* mice carrying a mutation in the *Rag2* gene, in which lymphocyte precursors are unable to rearrange their antigen receptors resulting in the arrest of lymphocyte maturation and failure to make T or B cells (Mombaerts P, et al. 1992). A second experimental model involves transferring T cells into wild-type mice that have first been conditioned with a sublethal dose of γ -irradiation which kills off lymphocytes. This treatment creates “space” to allow donor T cells to homeostatically expand and fill the lymphoid compartment.

In the experiments described below we have used both types of animal model to examine the capacity of *Hnrp1^{th^u/th^u}* T cells to undergo homeostatic proliferation and to persist in the circulation once the lymphocyte compartment has been filled. Previous studies have used naïve T cells usually from the spleen or lymph node as donor cell populations but in *thunder* mice the majority of mature T cells in the periphery are of a memory phenotype (see Figure 3.2.1c). Therefore to have a starting population of naïve T cells for these experiments we chose to use thymocytes as the donor cell population since only the mature single positive cells in the thymus will survive in the recipient and these cells will initially be of a naïve T cell phenotype (Fig 5.1.2 a).

First we wanted to examine if *Hnrp1^{th^u/th^u}* naïve T cells are defective in their ability to undergo homeostatic proliferation. The thymocytes were prepared from either wild-type CD45 .1 congenic mice or *th^u/th^u* (CD45.2) mice and were labelled with CFSE to monitor their proliferation *in vivo*. Prior to the injection wild-type and

Figure 5.1.2 T cell Homeostatic proliferation in irradiated hosts

a. Investigating roles of peptide/MHC in naïve T cell homeostasis:



b. Procedure of adoptive cell transfer experiment:

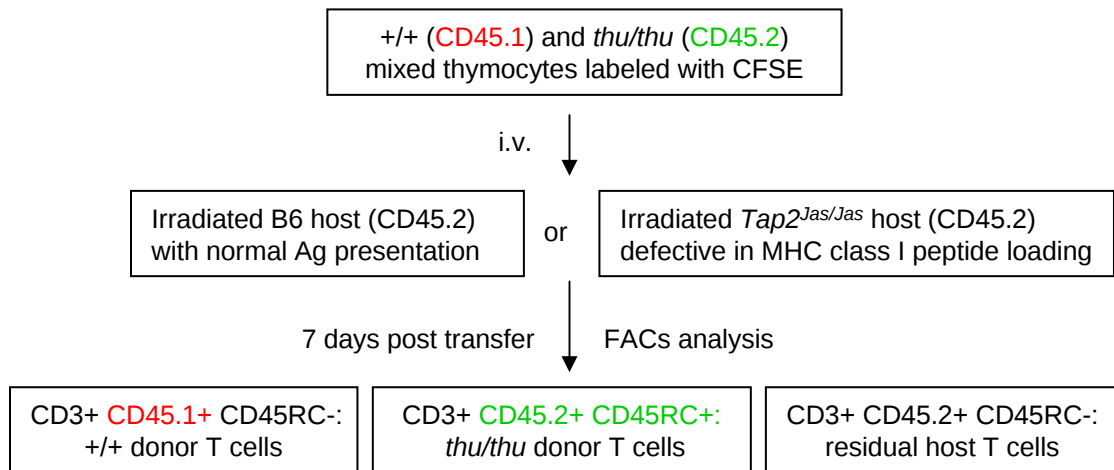


Fig. 5.1.2 T cell homeostatic proliferation in irradiated hosts.

- To study the responsiveness of *thu/thu* naïve T cell to pMHC, thymocytes were transferred to lymphodepleted recipients. CD4/CD8 SP act as naïve T cells and proliferate in the presence of IL-7 and self-peptide/MHC.
- Mixture of wild-type (CD45.1) and *thu/thu* (CD45.2) thymocytes were labelled with CFSE and injected to irradiated B6 or *Tap2* mutant mice. 7 days after injection, *thu/thu* T cells were distinguished from wild-type donor and residual host cells from CD45.2 and CD45RC staining. Cell division was analysed by CFSE dilution.

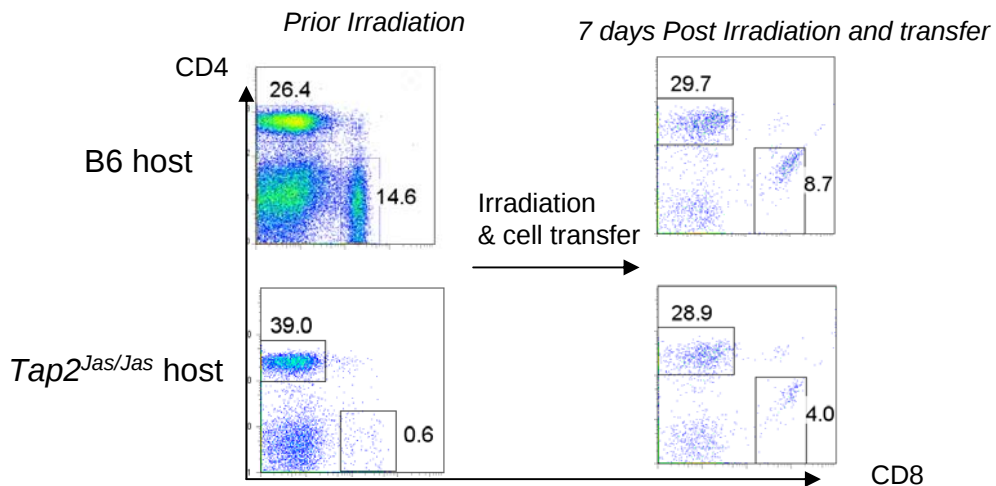
Hnrp1l^{thu/thu} cells were mixed in a 50:50 ratio. The mixture was then injected intravenously into recipient C57BL/6 mice that had been irradiated with a single dose of 6 Gy (Fig 5.1.2 a and b).

The recipient mice were taken down 7 days after the injection. The spleen and peripheral blood lymphocytes were stained with antibodies to CD4, CD8, CD45RC, CD45.1 and CD45.2 and analysed by flow cytometry. The donor wild-type and *thunder* T cells could be distinguished based on the difference in allelic expression of the CD45 markers (i.e. CD45.1 vs CD45.2), whereas the residual T cells of the irradiated host can be distinguished from donor *Hnrp1l^{thu/thu}* T cells by the constitutive expression of CD45RC in the mutant cells (experimental procedure is shown in Figure 5.1.2 b).

Also we wanted to determine if the homeostatic proliferation of wild-type or *Hnrp1l^{thu/thu}* CD8⁺ T cells may be impeded as a result of a lack of peptide/MHC I complexes *in vivo*. The recipient *jasmine* mouse strain was identified from an ENU mutagenesis screen by a lack of peripheral CD8⁺ T cells (Fahrer, A. Goodnow, C. unpublished). Through genetic mapping the mutation was identified in the *Tap2* gene, encoding an essential transporter for loading MHC class I-restricted peptides into the endoplasmic reticulum. In *Tap2* deficient mice MHC-I fail to be loaded with antigenic peptides, are unable to present the peptides, and remain unstable, thus only low levels of MHC1 is present on the cell surface (de la Salle H, et al. 1994). This abolishes the differentiation of mature CD8⁺ T cells in the thymus, and causes a lack of mature CD8⁺ T cells in the peripheral circulation.

Figure 5.1.2 T cell Homeostatic proliferation in irradiated hosts

c. Blood T cell profiles prior and post irradiation and transfer:



d. *Thunder* naïve T cells (splenocytes) show normal proliferation in irradiated hosts

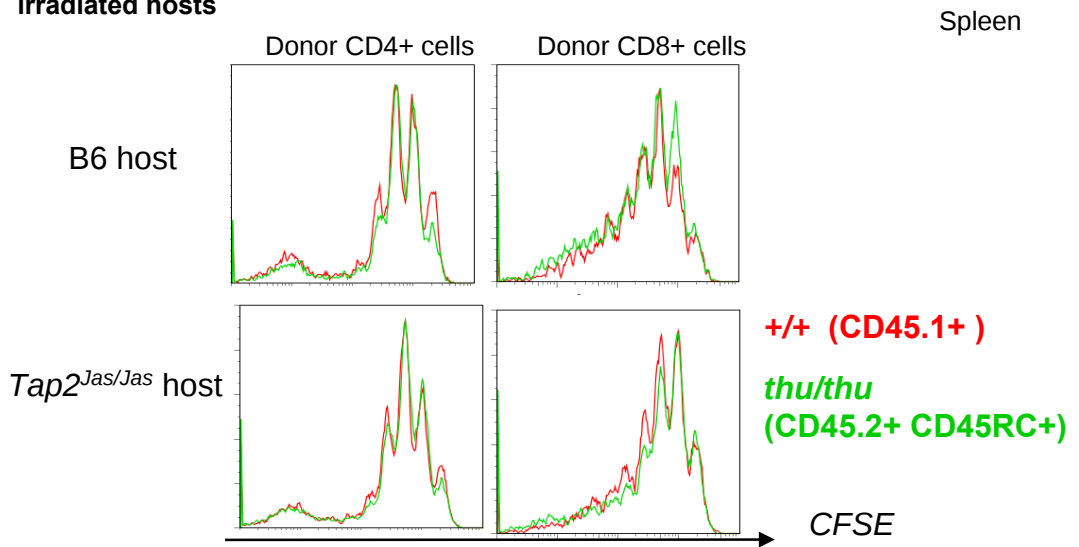


Figure 5.1.2 c.

Blood CD4 vs CD8 profile of recipient mice prior and post irradiation and mixed cell transfer showing lower % CD8+ cell in *Tap2^{Jas/Jas}* mice

d. Comparable proliferation of wild-type and *thu/thu* T cells in irradiated B6 and *Tap2^{Jas/Jas}* hosts.

Figure 5.1.2 c shows that CD4⁺ and CD8⁺ cell percentages in the host blood prior to and after irradiation and cell transfer. *Tap2^{Jas/Jas}* mice have very few mature CD8⁺ cells. One week after adoptive transfer of thymocytes, the CD8⁺ cell percentages in the blood are 50% less in the *Tap2^{Jas/Jas}* recipient mice, compared to the wild-type recipients (Fig. 5.1.2 c). However, there was no difference in the rate of proliferation as measured by CFSE dilution for either wild-type or *Hnrp11^{thu/thu}* CD4⁺ or CD8⁺ T cells in wild-type C57BL/6 recipients, as shown in Figure 5.1.2 d, and the cells convert to a memory phenotype (CD44^{hi}) at a similar rate for the wild-type and *Hnrp11^{thu/thu}* T cells (Figure 5.1.2 e).

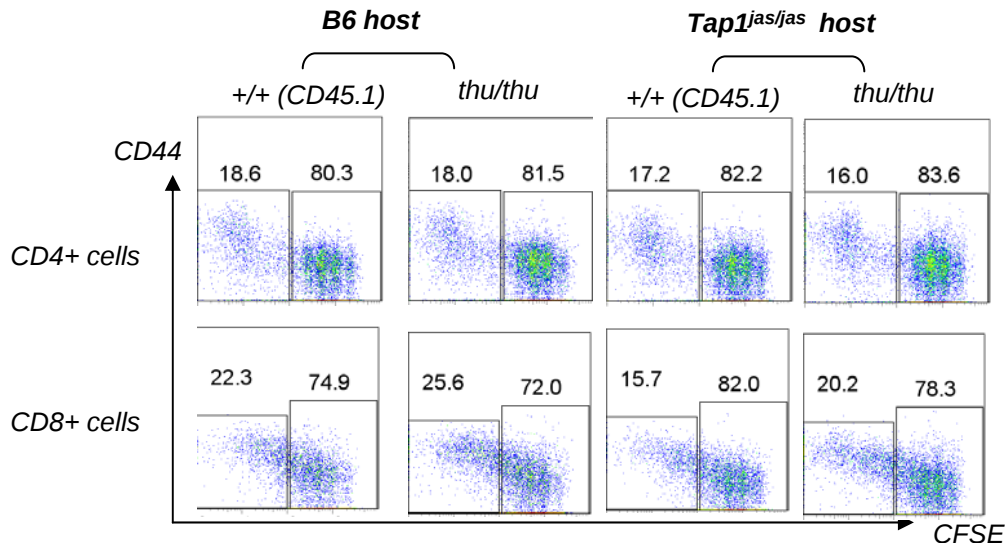
The homeostatic proliferation of wild-type and *Hnrp11^{thu/thu}* naïve CD8⁺ T cells both appeared to be reduced as a result of lack of peptide/MHC I complexes expressed on peripheral APCs in *jasmine* recipient mice. As shown in Fig. 5.1.2 f CD8⁺ T cells displayed weaker and delayed proliferation in *Tap2^{Jas/Jas}* recipient mice compared to that in B6 hosts, and again there is no difference in the proliferation rate of wild-type and *Hnrp11^{thu/thu}* naïve CD8⁺ T cells in wild-type B6 and Jasmine recipient mice respectively.

5.1.3 *Hnrp11^{thu/thu}* T cells fail to persist after adoptive transferring in *RAG-1^{null}* hosts

Next we examined the capacity of wild-type and *Hnrp11^{thu/thu}* T cells to expand in *RAG-1^{null}* hosts. Wild-type and *Hnrp11^{thu/thu}* thymocytes were mixed at a 50:50 ratio and injected intravenously into *RAG-1^{null}* recipients and bled on days 7 and 21 after the transfer (Figure 5.1.3 a). The peripheral blood samples were stained with anti-

Figure 5.1.2 T cell Homeostatic proliferation in irradiated hosts

e. Normal proliferation and naïve to memory transition of *thu/thu* T cells



f. Failure in MHC I restricted peptide presentation results in impeded CD8+ cell proliferation

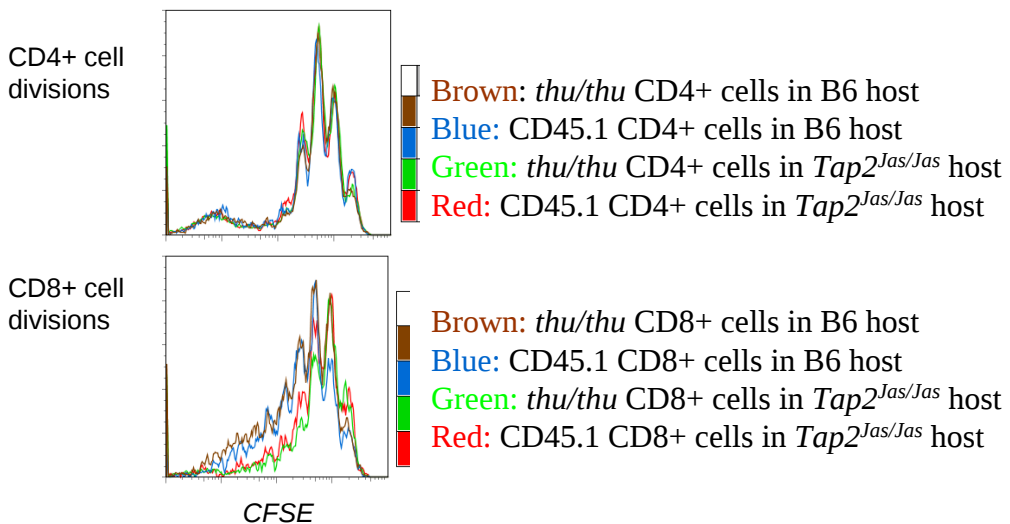


Fig. 5.1.2 e. Both *+/+* and *thu/thu* donor T cells were segregated from B6 and *Tap^{Jas/Jas}* recipients and analysed for CFSE dilution and CD44 expression.

f. Histogram overlays showing CFSE dilution in wild-type and *thu/thu* T cells in B6 and *Tap^{Jas/Jas}* recipients 7 days after transfer,

CD4, CD8, CD45.1 and CD45.2 to distinguish the two donor cell populations. As shown in Figure 5.1.3b at the start of the experiment both CD4⁺ and CD8⁺ T cell populations started at 50:50, and the T cell compartment expands rapidly during the 21 days. When we analysed the T cells to identify their origin we found by the end of the first week the wild-type CD4⁺ T cells represented 60% of the peripheral CD4⁺ T cells while *Hnrp11*^{th^u/th^u} derived CD4⁺ T cells represented 40% of the peripheral CD4⁺ population. By 3 weeks after the transfer there was a significant difference between the proportion of wild-type and *Hnrp11*^{th^u/th^u} derived T cells, where wild-type cells constituted about 80% of the CD4⁺ cells and 90-95% of the CD8⁺ cell populations, whereas *Hnrp11*^{th^u/th^u} cells represented just 20% of CD4⁺ and <10% of CD8⁺ T cells in the peripheral blood (Figure 5.1.3b).

We conclude from these data that the *Hnrp11*^{th^u/th^u} mutation does not affect the ability of T cells to undergo homeostatic proliferation as they show effective cell division compared with wild-type T cells and can expand at a similar rate to that of wild-type T cells in response to TCR stimulation in vitro and pro-survival cytokine signal in vivo. However, *Hnrp11*^{th^u/th^u} T cells fail to persist in the circulation. The most possible explanation is due to increased cell death but it does not rule out the possibility of different homing potency.

5.1.4 *Hnrp11*^{th^u/th^u} T cells fail to persist in lymphoreplete hosts

When T cells are transferred to a lymphoreplete host, the peripheral T cell compartment is already “full” and there is limited space to be occupied by donor T cells. In the case of transferring T cells to a lymphoreplete host, donor T cells cannot

Figure 5.1.3 Defect of persistence in lymphopaenic environment

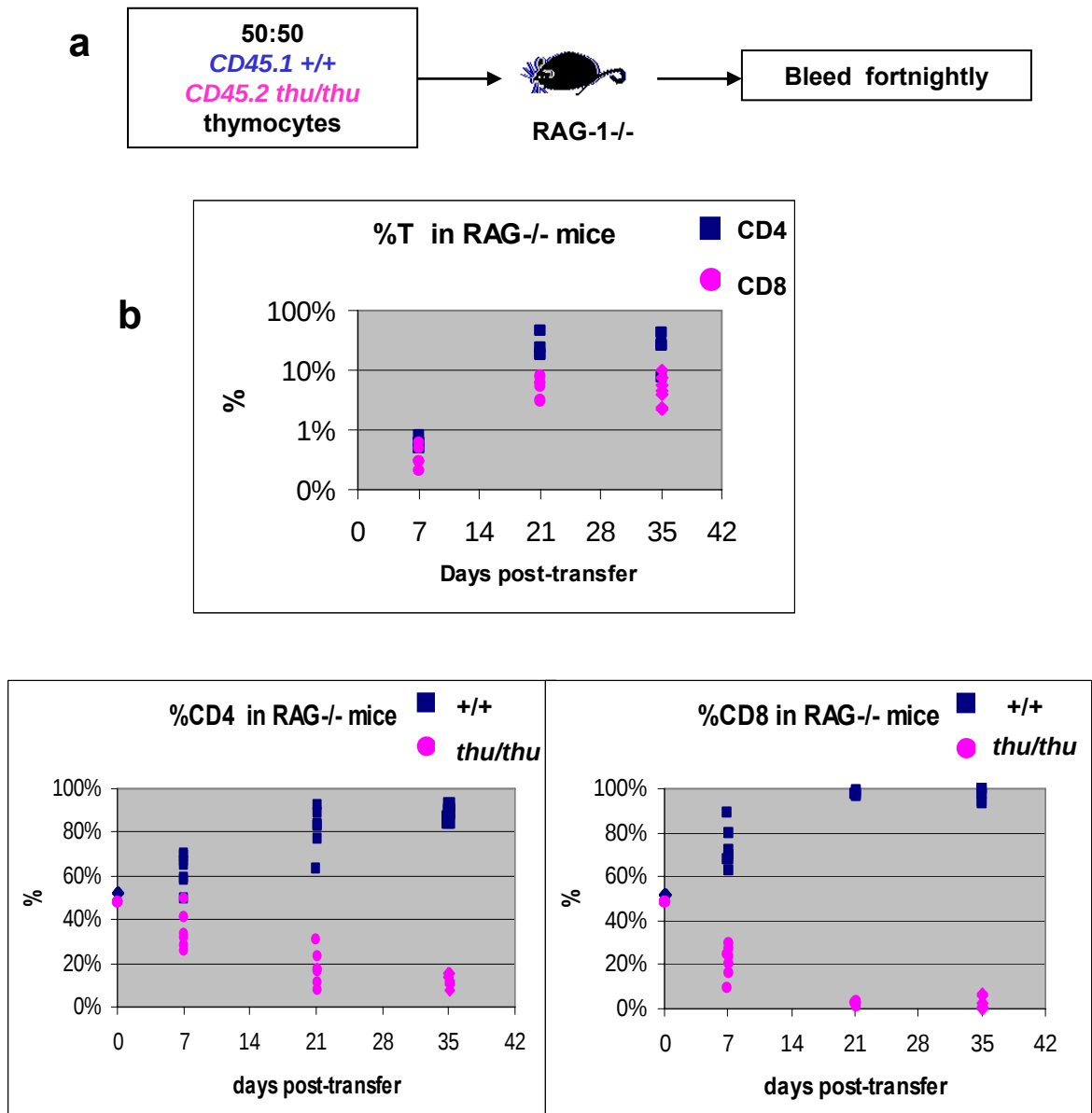


Fig. 5.1.3 T cell persistence in RAG-1 KO recipients

- a. Mixture of wild-type and *thu/thu* thymocytes were adoptively transferred to RAG1 deficient mice and bled fortnightly to monitor the persistence.
- b. Top: Blood CD4 and CD8 cell percentages in RAG1 KO recipients after transfer. Blue dots: CD4; pink dots: CD8. Bottom: proportion of wild-type (blue dots) and *thu/thu* (pink dots) in RAG1^{-/-} recipients during a 5-weeks course. Each pair of dots represents data from one recipient.

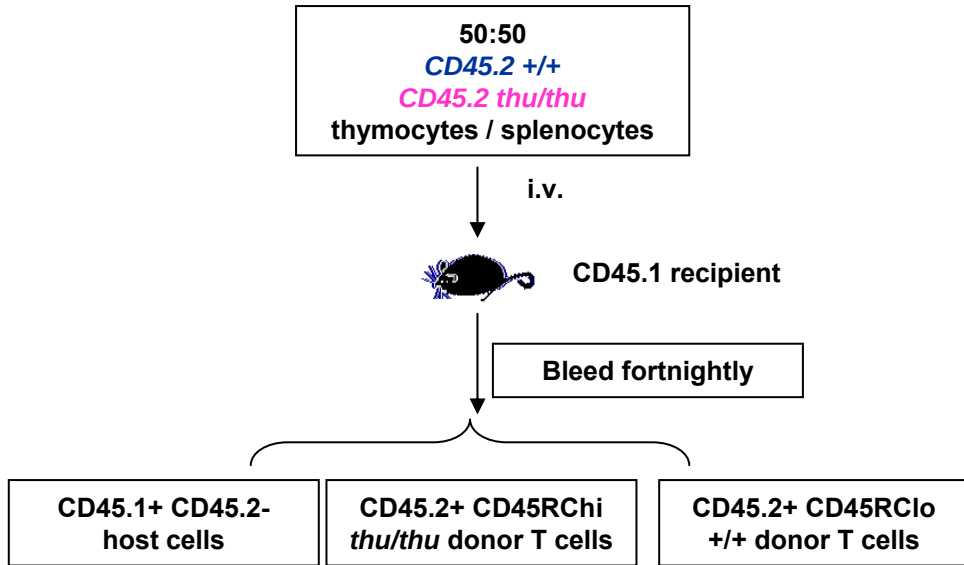
proliferate but instead should be able to persist albeit at a low frequency, since they would receive the necessary signals (i.e. p/MHC and IL-7) to promote their survival. The data from the preceding experiment indicated that *Hnrpl^{thu/thu}* mutation selectively affects the ability of *Hnrpl^{thu/thu}* T cells to persist *in vivo*. Therefore a prediction is that when *Hnrpl^{thu/thu}* T cells are transferred to a lymphoreplete host they would fail to persist and would die off rapidly.

To test this hypothesis a 50:50 mixture of wild-type CD45.2+ (CD45RClo) and *Hnrpl^{thu/thu}* CD45.2+ (CD45RChi) thymocytes or splenocytes (at 4 weeks of age so mostly naïve T cells) were injected intravenously into intact CD45.1 hosts to monitor the persistence of donor cells (Figure 5.1.4 a). The recipient mice were bled fortnightly after the injection and we analysed the proportion of CD45.2+ T cells that were retained in the mice. The data showed that donor CD4+ T cells had declined in the blood from 2 weeks to 4 weeks post transfer (Figure 5.1.4 b). The wild type and *Hnrpl^{thu/thu}* T cells could be distinguished based on the difference in CD45RC expression. As shown in Figure 5.1.4 c and d, whether thymocytes or splenocytes were used as the source of donor cells, the *Hnrpl^{thu/thu}* derived CD4+ T cells failed to persist in the peripheral blood compared to wild-type cells. This result confirms a cell autonomous effect of the *Hnrpl^{thu/thu}* mutation on T cell persistence *in vivo*.

Through a series of adoptive cell transfer experiments, we found that *Hnrpl^{thu/thu}* naïve T cells have normal proliferation rate in response to TCR stimulation either *in vitro* or *in vivo*. They can compete with wild-type T cells during the homeostatic expansion phase, in contrast to wild-type T cells the *Hnrpl^{thu/thu}* naïve T cells cannot be sustained in the peripheral circulation once the T cell compartment is filled.

Figure 5.1.4 *thu/thu* T cells cannot persist in lymphoreplete hosts

a. Experiment procedure:



b. Donor CD4⁺ CD45.2⁺ T cells:

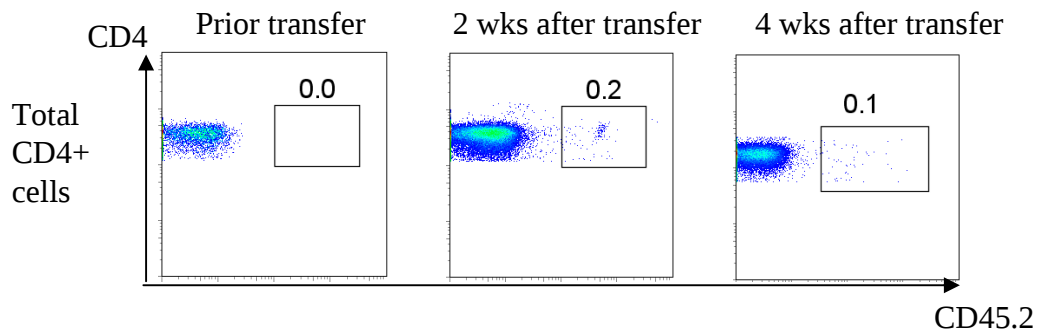
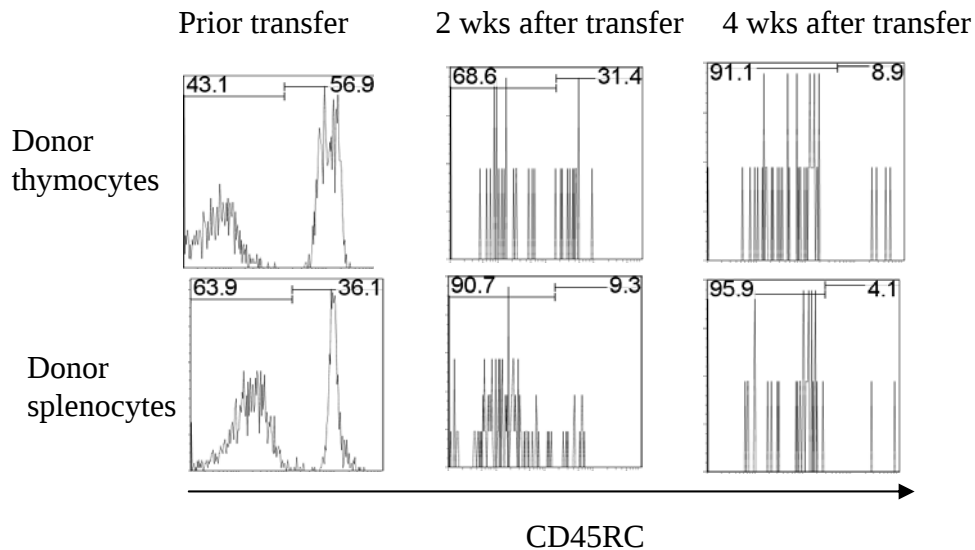


Fig. 5.1.4 a. 50:50 mixture of wild-type and *thu/thu* T cells were injected into CD45.1 intact mice to monitor their persistence.

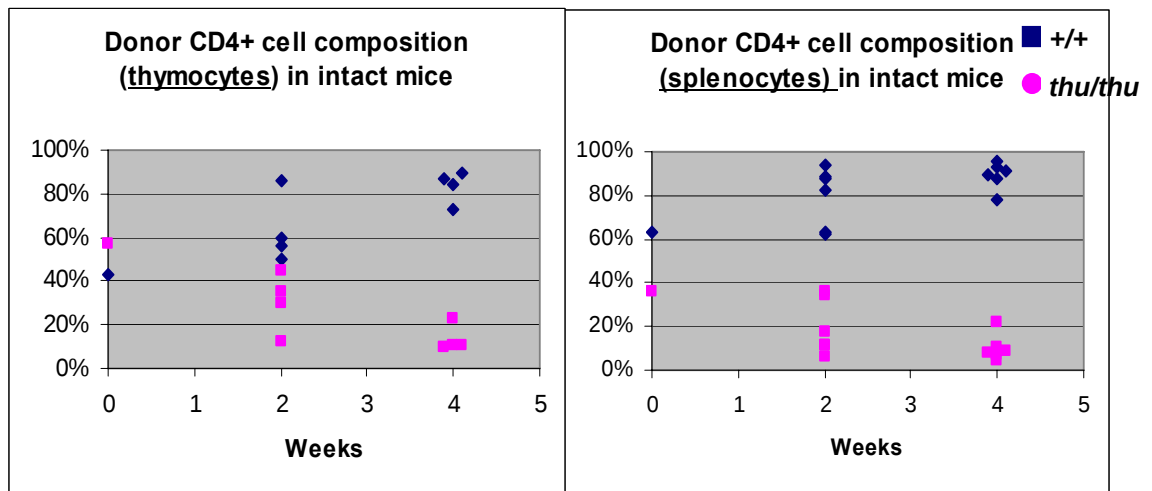
b. Gated CD45.2⁺ cells represent donor cells mixed with wild-type and *HnrpII*^{*thu/thu*} CD4⁺ T cells before and after transfer.

Figure 5.1.4 *thu/thu* T cells cannot persist in lymphoreplete hosts

c. $+/+$ (CD45RC^{lo}) vs *thu/thu* (CD45RC^{hi}) donor CD4⁺ T cells



d. $+/+$ and *thu/thu* cell proportions of donor CD4⁺ T cells in the blood



c. Representative CD45RC staining to distinguish $+/+$ (CD45RC^{lo}) and *Hnrp11*^{*thu/thu*} (CD45RC^{hi}) CD4⁺ T cells in the composition of donor cells before and after transferring.

d. Composition of donor cell showing wild-type (blue dots) and *thu/thu* (pink dots) CD4 and CD8 cell percentages in the blood of CD45.1 recipients. Each pair of dots represents data from one individual recipient.

5.2. *Hnrpll*^{th^u/th^u} naïve T cells show multiple phenotypic changes

The homeostasis of naïve T cells is dependent upon TCR signals and exposure to the cytokine IL-7 (Sprent, J. & Surh, C.D. 2002). The TCR of mature naïve T cells recognize self peptide/MHC complexes in the periphery that are thought to mimic those peptides presented in the thymus and provide a tonic signal to promote cell survival rather than allowing full activation. Exposure to IL-7 is critical for the survival of naïve T cells in the periphery (Fry, T.J. & Mackall, C.L. 2005). We hypothesized that decreased survival of *Hnrpll*^{th^u/th^u} naïve T cells could be due to a cell intrinsic problem that affected the ability of *Hnrpll*^{th^u/th^u} T cells to respond to peptide/MHC complexes or to IL-7. Lymphotropic IL-7 signaling is transduced through binding to the IL-7R in T cell surface, which is composed of a multimeric complex including the IL-7R α (i.e. CD127), IL-2R β (CD122) and the common γ chain (CD132). Monoclonal antibodies exist to all three components and their expression at the cell surface can be quantitated by flow cytometry.

5.2.1 Lower expression of IL-7R on *Hnrpll*^{th^u/th^u} naïve T cells

We compared the expression of CD127 (IL-7R α) on the surface of naïve (CD4⁺ or CD8⁺, CD44^{lo}) or memory (CD4⁺ or CD8⁺, CD44^{hi}) spleen T cells of wild-type, *Hnrpll*^{th^u/+} and *Hnrpll*^{th^u/th^u} mice by flow cytometry. The data in Figure 5.2.1a shows that the majority of *Hnrpll*^{th^u/th^u} CD44^{lo} T cells have relatively low expression of CD127 fluorescence compared to wild-type or *Hnrpll*^{th^u/+} naïve T cells. The histogram overlay of gated CD44^{lo} cells shows a clear shift to the left for IL-7R α

Figure 5.2.1 Lower IL-7R expression on *HnrpII*^{thu/thu} naïve T cells

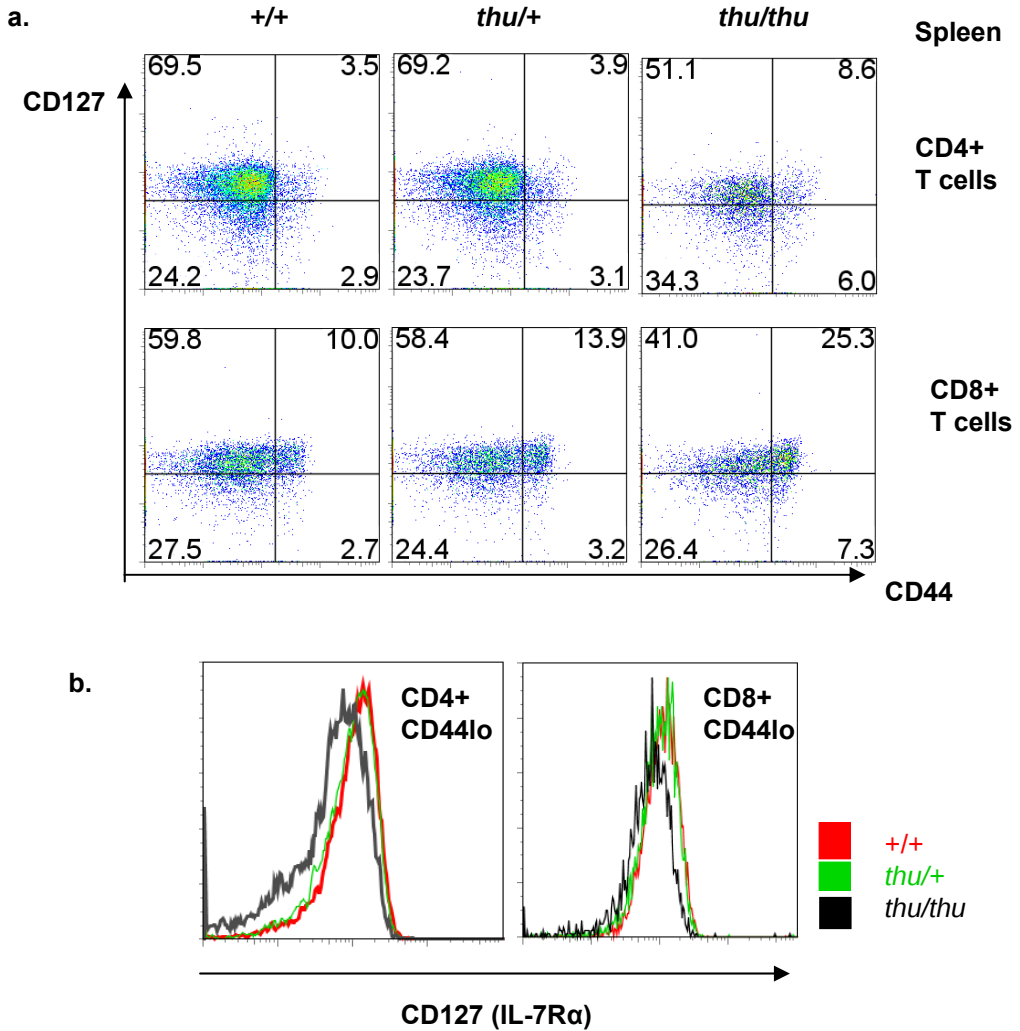


Fig. 5.2.1 a. Representative dot plots of spleen CD4 and CD8 T cells from wild-type, *thu*/+, and *thu*/*thu* mice showing CD127 (IL-7Rα) expression in CD44^{lo} naïve and CD44^{hi} memory compartments.

b. Histogram overlay of naïve T cells showing IL-7Rα expression in wild-type (red line), *thu*/+ (green line), and *thu*/*thu* (dark line) mice.

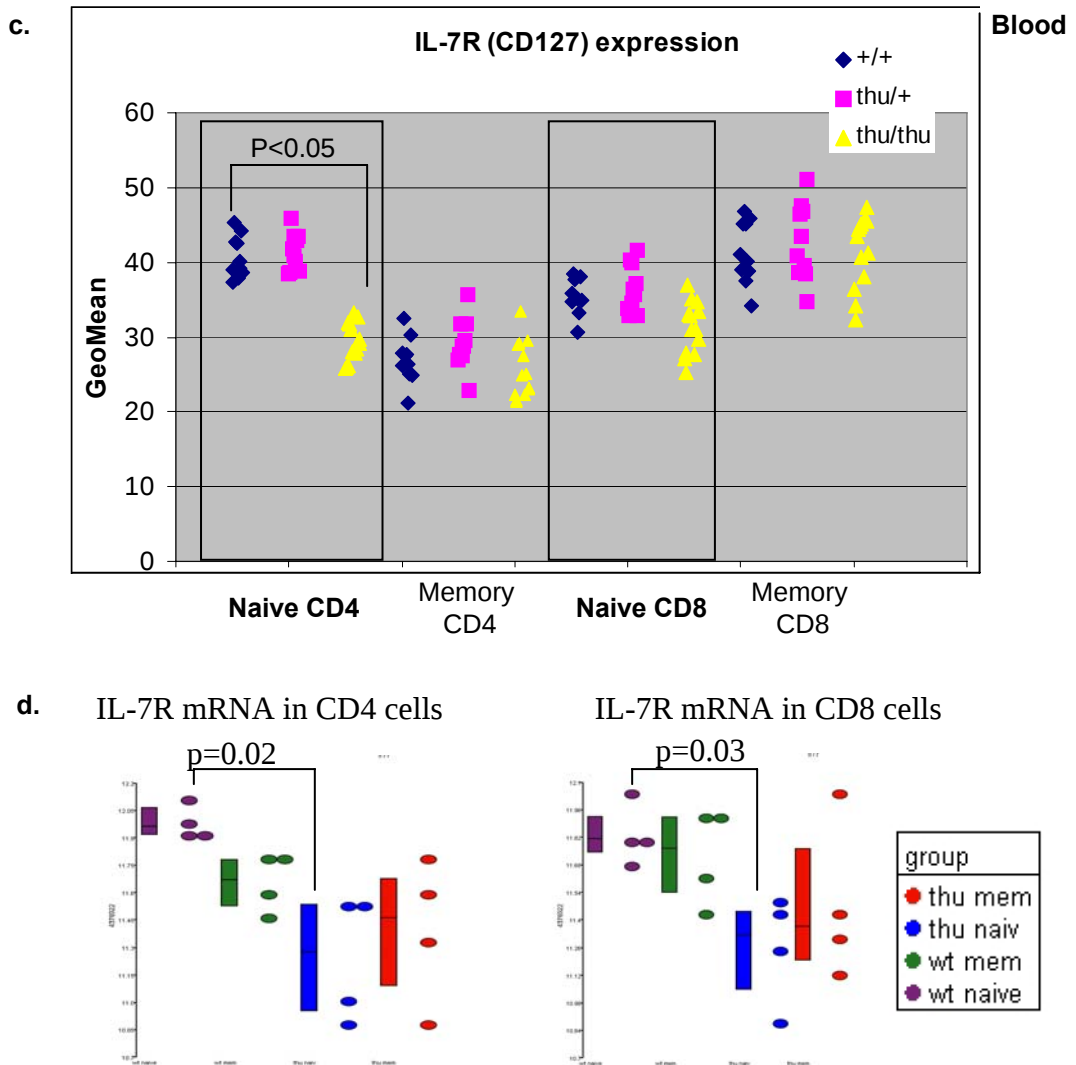
expression on naïve *Hnrp11*^{th^u/th^u} T cells, and this shift was more obvious on naïve CD4⁺ cells than CD8⁺ cells (Figure 5.2.1 b). A similar pattern of staining for IL-7R α was also observed on naïve T cells in the peripheral blood of *Hnrp11*^{th^u/th^u} mice. The data in Figure 5.2.1c shows the geometric mean expression of CD127 on CD4⁺ and CD8⁺ naïve and memory T cell subsets in the peripheral blood obtained from a cohort of wild-type, *Hnrp11*^{th^u/+} and *Hnrp11*^{th^u/th^u} mice. There is a statistically significant difference in IL-7R α expression on *Hnrp11*^{th^u/th^u} naïve CD4⁺ and CD8⁺ cells but not in the memory T cells (Figure 5.2.1 c).

We have also observed a significant decrease of IL-7R α mRNA in *Hnrp11*^{th^u/th^u} naïve CD4⁺ and CD8⁺ T cells compared to their wild-type counterparts from exon array experiments, whereas memory T cells showed no difference in IL-7R expression (Figure 5.2.1d). Thus it appears that IL-7R expression is decreased in *Hnrp11*^{th^u/th^u} naïve T cells at both the level of mRNA and protein.

5.2.2 Bone marrow chimeras show T cell autonomous defects in CD45 splicing and IL-7R expression in naïve T cells

The lower expression of IL-7R on the surface of *Hnrp11*^{th^u/th^u} naïve T cells could be due to a cell extrinsic effect caused by the lymphopaenia such as elevated IL-7. To confirm that the *Hnrp11*^{th^u/th^u} mutation affects T cell persistence in a cell autonomous manner and to examine if the decrease in IL-7R on *Hnrp11*^{th^u/th^u} T cells is due to a cell autonomous effect of the *Hnrp11*^{th^u/th^u} mutation, we generated mixed bone marrow chimeras. Two different 50:50 bone marrow chimeras were set up, a control group reconstituted with the mixture of wild-type CD45.1 and wild-type CD45.2 bone

Figure 5.2.1 Lower IL-7R expression on *HnrpII*^{thu/thu} naive T cells



c. IL-7Ra (CD127) Geomean of naive and memory T cells in the blood. Each plot represents an individual mouse. Wild-type=blue dots, *thu*/+=pink dots, *thu*/*thu*=yellow dots. P value was calculated by student T test.

d. IL-7R gene expression in naïve versus memory CD4+ and CD8+ T cells as determined by affymetrix all exon microarray analysed with Partek Genomic Suite. Each dot represents a separate sample. The bars represent the mean & standard deviation of the data and the dots are the expression levels within individual wild-type and *thunder* naïve CD4 and CD8 cells

marrow cells, and a test group with wild-type CD45.1 and *Hnrp11*^{th^u/th^u} CD45.2 donor marrow. The irradiated recipients were bled initially 7 weeks after the reconstitution and were bled every 2 weeks for a further 6 weeks to monitor the proportions of T cells in the peripheral blood and the level of IL-7R expression on T cells by flow cytometry.

At 7 weeks we could confirm that the defect in CD45 alternative splicing is T cell intrinsic effect due to *Hnrp11*^{th^u/th^u} mutation, as *Hnrp11*^{th^u/th^u} T cells maintained the CD45RChi phenotype irrespective of whether they grow in the presence of wild-type cells (Figure 5.2.2 a). Analysis of the T cell populations in the CD45.1 (+/+) and CD45.2 (+/+) chimeras revealed that by the end of the 16 week period the CD4+ and CD8+ T cells were approximately 50:50 derived from the two marrow donors (Fig. 5.2.2 b and data not shown). By contrast, in the CD45.1 (+/+) and CD45.2 (*Hnrp11*^{th^u/th^u}) chimeras, by 7 weeks after reconstitution *Hnrp11*^{th^u/th^u} derived T cells represented just 25% of the peripheral CD4+ T cell pool. Over the remaining 6 weeks of the experiment the *Hnrp11*^{th^u/th^u} T cells failed to increase in number and remained at just 20% of the peripheral CD4+ pool (Fig. 5.2.2 b).

Analysis of the IL-7R expression on T cells from the two different chimeras was also informative. Whereas IL-7R expression decreased over time on CD4+ cells in the CD45.1 (+/+) and CD45.2 (+/+) chimeras there was no significant difference in the geometric mean of CD127 for either the CD45.1+ or CD45.2+ derived cells. In contrast the *Hnrp11*^{th^u/th^u} derived T cells showed a lower level of IL-7R expression throughout the course of the experiment and this was statistically significant at $p < 0.002$ (Fig. 5.2.2 c). Therefore the results of these experiments confirm that the

Figure 5.2.2 T cell autonomous defects of *thu/thu* T cells

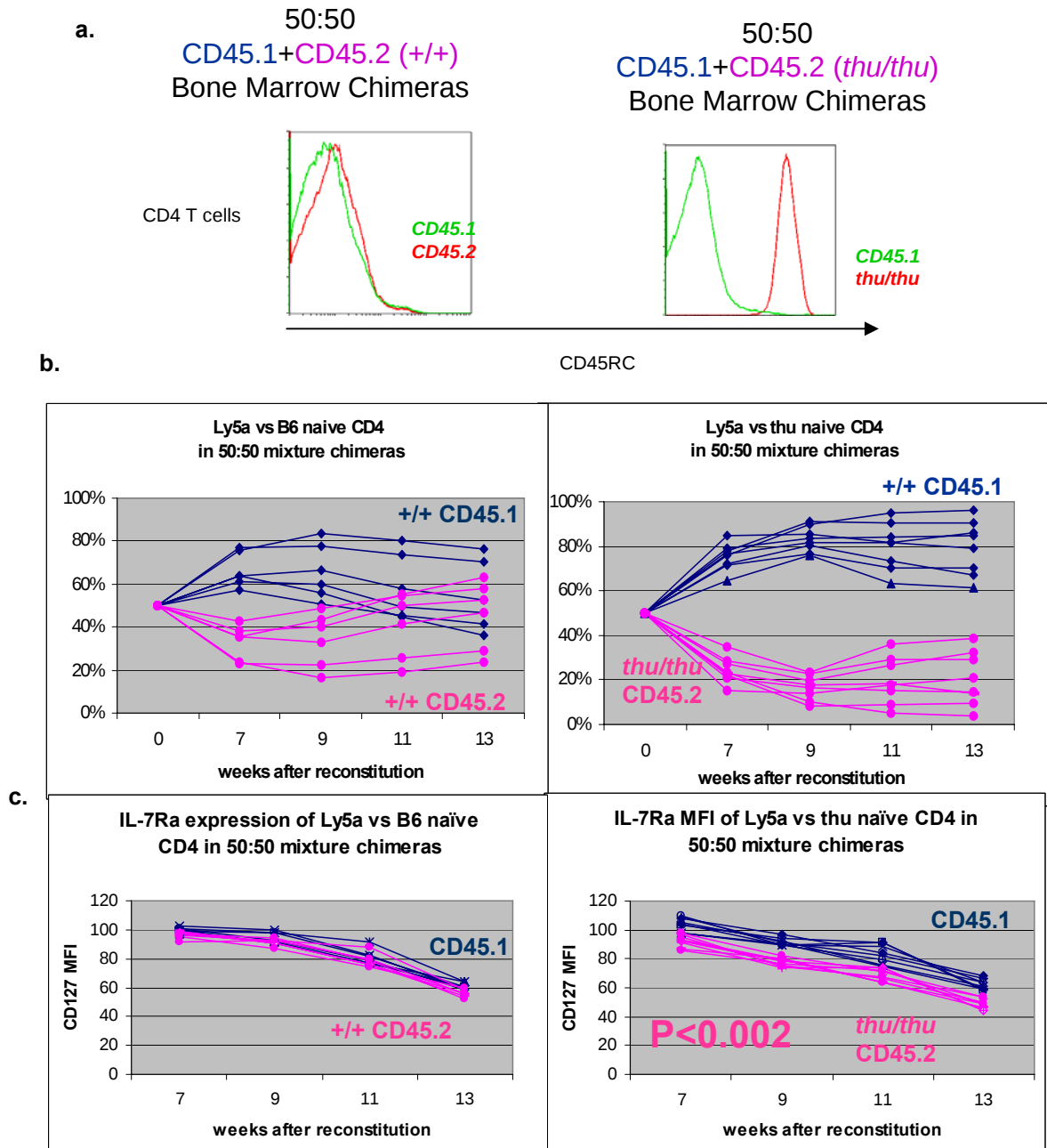


Fig. 5.2.2 a. Two groups of bone marrow chimeras were set up in irradiated CD45.1 mice. One with mixture of CD45.1 and CD45.2 wild-type cells, the other with CD45.1 wild-type and CD45.2 *thu/thu* cells. Cells derived from the first group of BM cells can be distinguished by CD45.1/CD45.2 markers whereas cells in the second group are different in CD45RC expression as well as CD45.1/CD45.2.

b. Blood CD4 cell composition of wild-type CD45.1vs CD45.2 and wild-type vs *thu/thu*.

c. IL-7Ra GeoMeans of CD4 cells in the chimeras. Each pair of lines represent data from one host.

Figure 5.2.2 T cell autonomous defects of *thu/thu* T cells

d.

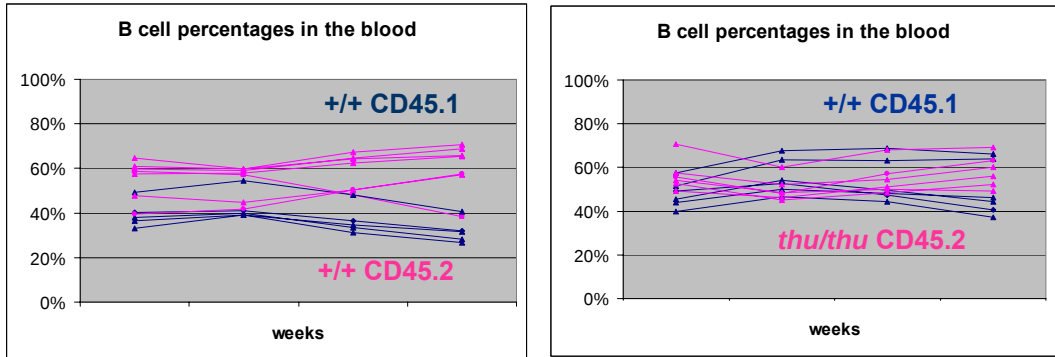


Fig. 5.2.2 d. Blood B cell composition of wild-type CD45.1 (blue) vs CD45.2 (pink) on the left and wild-type CD45.1 (blue) vs *thu/thu* CD45.2 (pink) on the right in the bone marrow chimeras.

Figure 5.2.3 Normal common γ -chain cytokine receptor expression

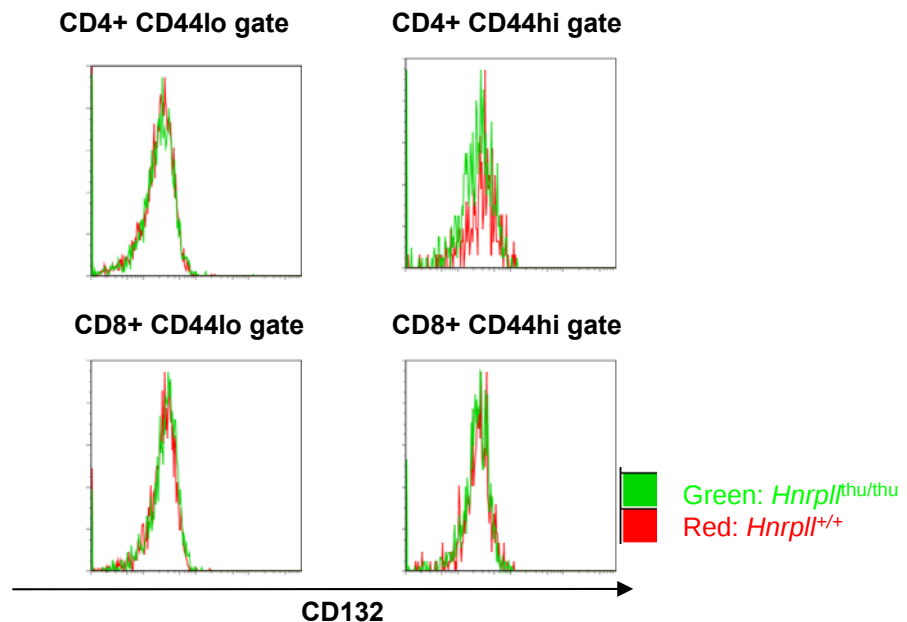


Figure 5.2.3 Common γ chain (CD132) expression in wild-type and *thu/thu* T cells in the peripheral blood of intact mice. Naïve and memory T cells were gated on the level of CD44 staining.

Hnrp11^{th^u/th^u} mutation acts in a cell autonomous manner to regulate IL-7R expression and also to influence T cell homeostasis *in vivo*.

5.2.3 Normal common γ -chain cytokine receptor

In contrast to the IL-7R expression there was no difference in expression of the common γ chain (CD132) on naïve and memory T cells from either wild-type, *Hnrp11*^{th^u/+} or *Hnrp11*^{th^u/th^u} intact mice (Figure 5.2.3).

5.2.4 Change of IL-7R expression is not due to alternative splicing

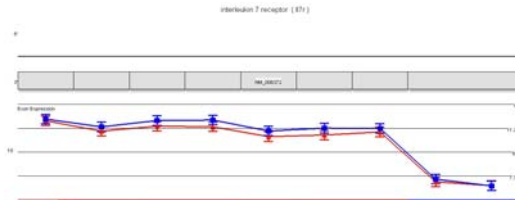
Alternative splicing of pre-mRNA is frequently observed in cytokines and cytokine receptors that generate complex regulation of cytokine signalling (Atamas, S.P. 1997). It is not surprising that receptor abnormalities or a pathological ratio of different isoforms may contribute to the defects in T cell homeostasis by circumventing normal growth factor control or altering the balance of proliferation and differentiation. IL-7R α has been reported with alternatively spliced transcripts coding truncated receptor proteins and potentially modulating IL-7 signalling in patients with leukaemia (Korte, A. et al. Cytokine. 2000). So we decided to examine if the change in IL-7R expression in *thunder* naïve T cells is caused by or accompanied with a change in alternative splicing of the receptor.

CD4⁺ and CD8⁺ single positive thymocytes and naïve and memory peripheral CD4⁺ and CD8⁺ T cells were FACS sorted to isolate total RNA. It was then hybridized to Affymetrix mouse all exon array chips to screen splicing targets of

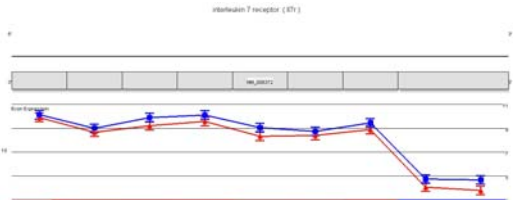
Figure 5.2.4 IL-7R is not alternative spliced in thunder T cells

a.

Exon view of IL-7R transcript in
CD4SP thymocytes



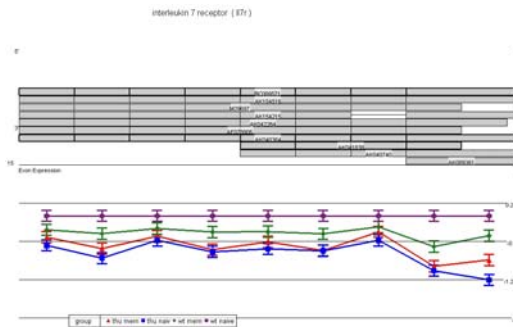
Exon view of IL-7R transcript in
CD8SP thymocytes



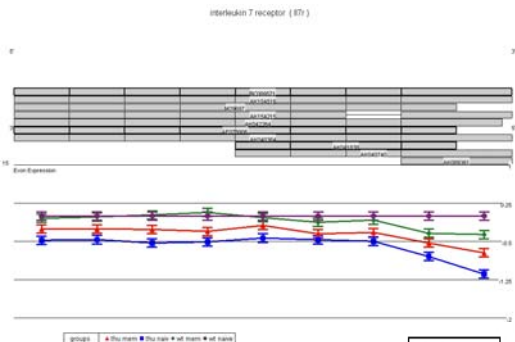
a. Exon array data from mRNA of wild-type (blue) and *thu/thu* (red) CD4 and CD8 single positive thymocytes showing exon level of IL-7R transcript. Each point represents average probe set intensity of 4 samples. Transcript cluster were from UCSC genome database.

b.

IL-7R transcript in peripheral CD4 cells



IL-7R transcript in peripheral CD8 cells



b. Exon array data from mRNA of wild-type and *thu/thu* peripheral naïve and memory CD4 and CD8 cells showing IL-7R transcripts in the four cell subsets. The exon level detection of IL-7R transcript shows no evidence of alternative splicing in the mutant cells, but the overall expression of IL-7R in *thu/thu* naïve T cells (in blue) is lower than that of wt naïve cells (in purple).



Hnrpll^{thi/thi} mutation throughout the genome (further details in chapter 7). IL-7R α (CD127) transcripts were analysed and viewed using Partek Genomic Suite software. The data showed no evidence of alternative splicing of the IL-7R in the *Hnrpll^{thi/thi}* thymocytes (Figure 5.2.4 a) or peripheral T cells compared to wild-type cells (Figure 5.2.4 b).

5.2.5 Lower expression of TCR/CD3 complex and coreceptors

In addition to changes in IL-7R expression, *Hnrpll^{thi/thi}* naïve T cells showed reproducible changes in TCR, CD3 and CD5 expression at the cell surface detected by flow cytometry. The data in Figure 5.2.5 shows a significant decrease in the geometric mean of TCR and CD3 expression in *Hnrpll^{thi/thi}* CD4⁺ and CD8⁺ T cells, compared to the wild-type and *Hnrpll^{thi/+}* litter mates (Figure 5.2.5 a and b).

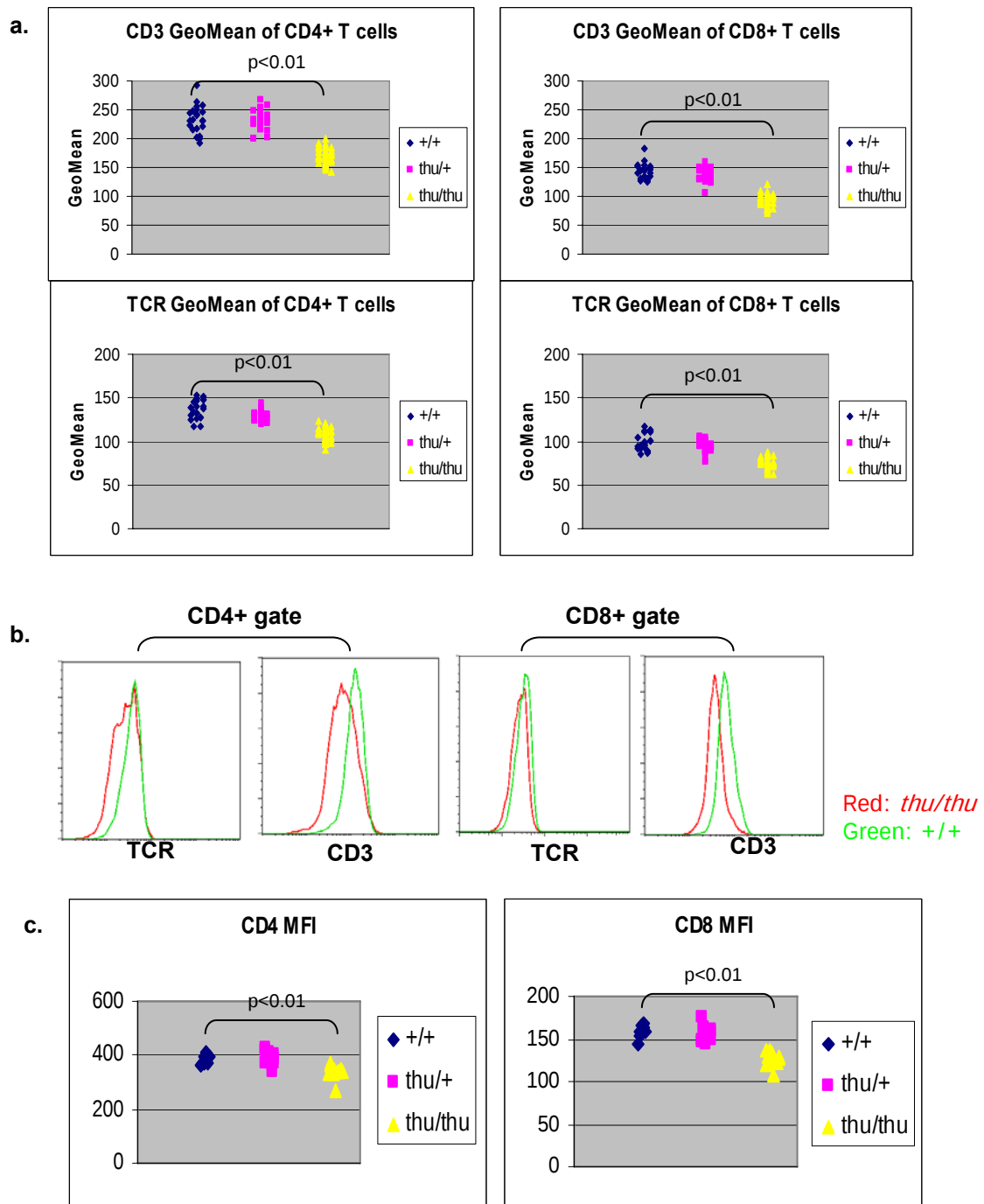
Lower expression of the coreceptors CD4 and CD8 were also observed in *Hnrpll^{thi/thi}* CD4⁺ and CD8⁺ T cells respectively (Figure 5.2.5 c).

Naïve CD4⁺ and CD8⁺ T cells in *Hnrpll^{thi/thi}* mice showed higher expression of CD5 compared to T cell subsets from wild-type or *Hnrpll^{thi/+}* mice, while there was no difference in CD5 expression on memory CD4⁺T cells from any genotype (Figure 5.2.5 d and e). In contrast both naïve and memory *Hnrpll^{thi/thi}* CD8⁺ cells have higher CD5 expression than their wild-type counterparts (Figure 5.2.5 d and e).

Collectively the phenotypic changes observed in TCR, CD3, CD5, and coreceptors CD4 and CD8 expression in *Hnrpll^{thi/thi}* T cells are equivalent to changes

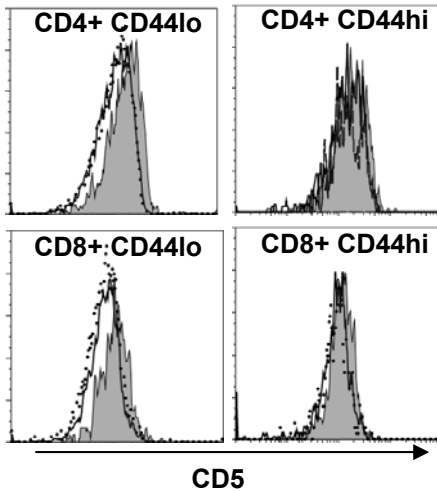
Figure 5.2.5 Lower expression of TCR/CD3 complex and coreceptors on *thu/thu* T cells

Blood



- a. CD3 and TCR GeoMean of blood CD4 and CD8 cells in *+/+*, *thu/+*, and *thu/thu* mice, each dot represents an individual mouse. P value were from a Student's t-Test.
- b. Representative histogram overlays showing TCR and CD3 expression on wild-type and *thu/thu* peripheral blood T cells. Wild-type=green, *thu/thu*=red.
- c. co-receptors CD4 and CD8 expression on wild-type and *thu/thu* CD4+ and CD8+ cells respectively in peripheral blood. Statistic p value were calculated from a Student's t-Test.

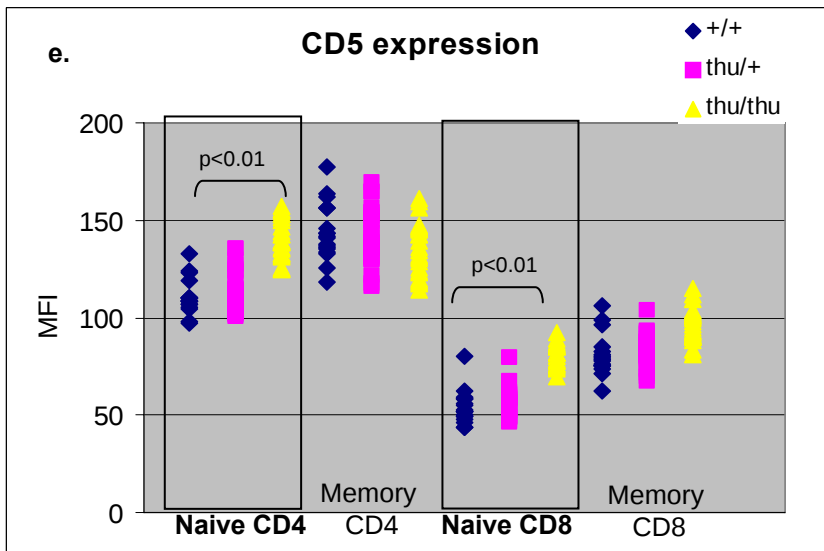
d.



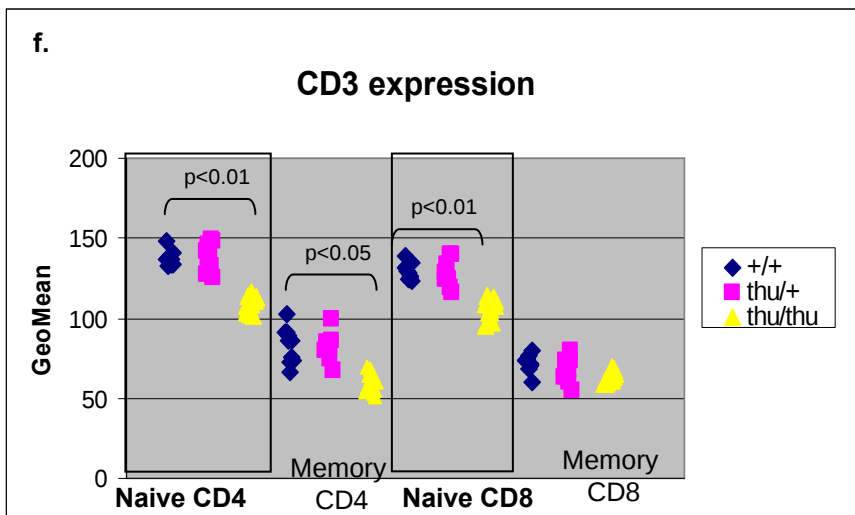
d. Representative histogram overlay showing CD5 expression on wild-type (solid line), thi/+ (dotted), and thi/thi (filled) naïve and memory T cells.

e. CD5 GeoMean of blood T cell subsets. Each dot represent an individual mouse. Student's t-Tests were used to calculate p values.

Solid line: +/+
Dotted: thi/+
Trinted: thi/thi



f.



f. CD3 GeoMean of blood T cell subsets. Each dot represent an individual mouse. Student's t-Tests were used to calculate p values.

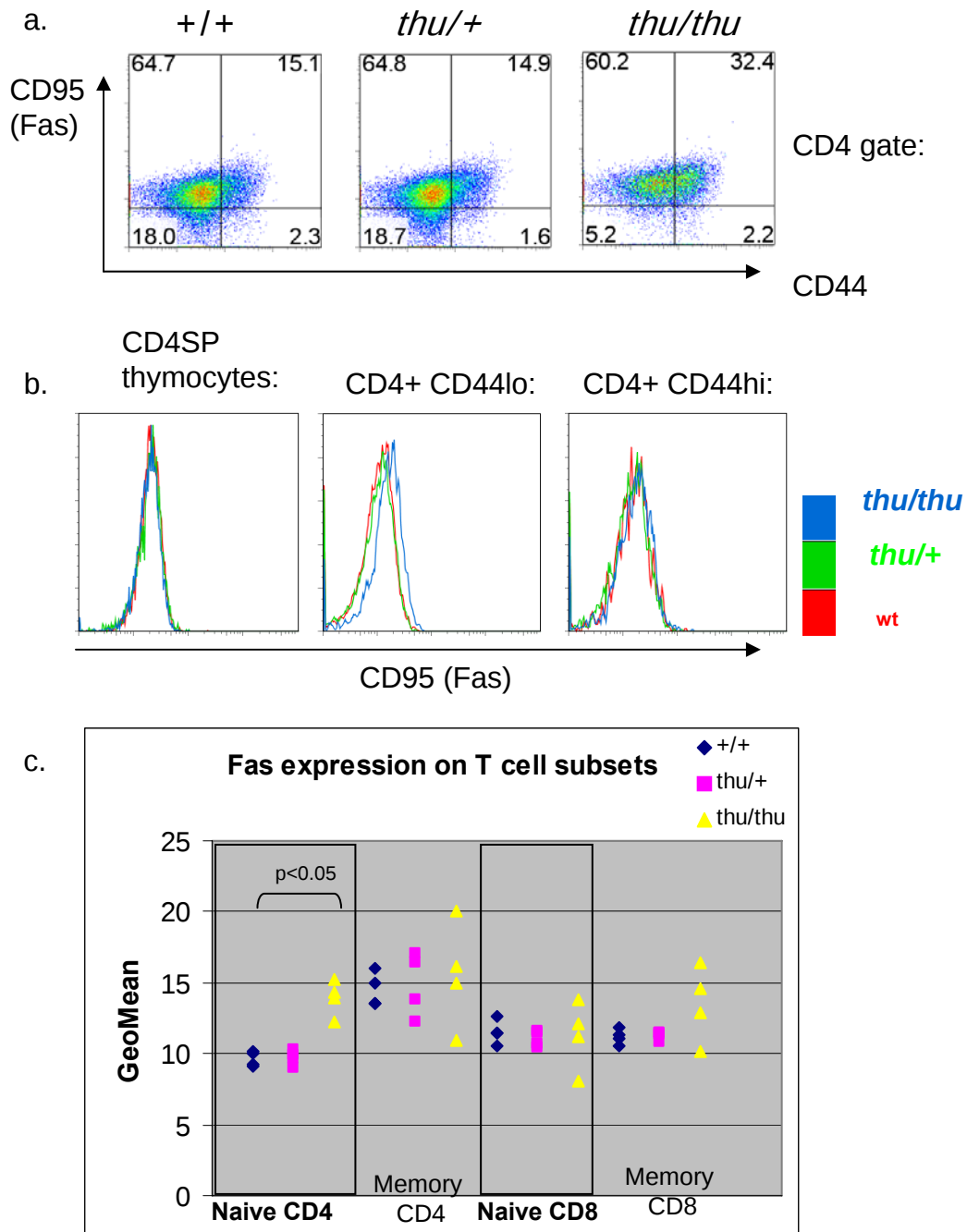
that would normally occur when T cells display enhanced levels of TCR signalling in response to chronic antigen stimulation (Berg, L. et al. 2000).

5.2.6 Increased apoptosis in *Hnrp11^{thn/thn}* naïve T cells

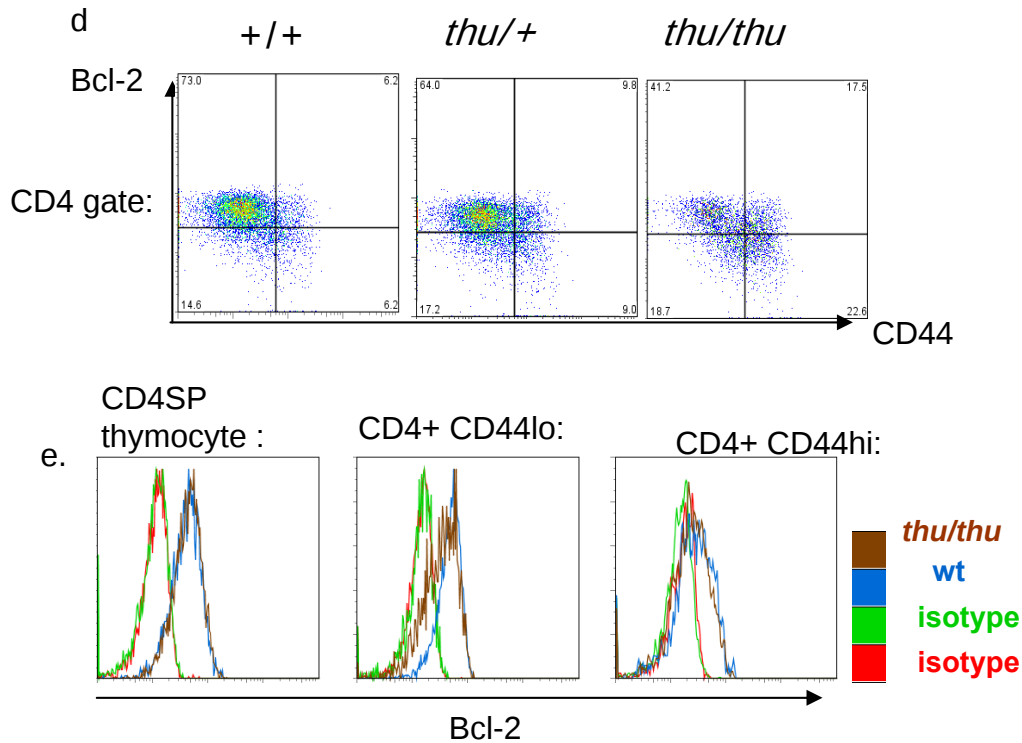
In response to chronic TCR signalling T cells can upregulate the Fas receptor (CD95) which binds to FasL. This interaction can trigger apoptosis of the Fas expressing cells. We examined the expression of Fas on the surface of wild-type, *Hnrp11^{thn/+}* and *Hnrp11^{thn/thn}* peripheral blood T cells and found that naïve CD4⁺ T cells in *thn* mice had higher levels of Fas expression compared to wild-type or *Hnrp11^{thn/+}* mice (Figure 5.2.6 a and b). There was no difference in Fas levels on memory T cells and mature single positive thymocytes (Figure 5.2.6 b & c).

Exposure to IL-7 provides a pro-survival signal mediated by AKT and PI3K and this leads to an increase in the expression of the Bcl-2 proteins that prevent apoptosis (Marrack, P. & Kappler, J. 2004). We stained naïve and memory T cells from wild-type, *Hnrp11^{thn/+}* and *Hnrp11^{thn/thn}* mice and found that naïve CD4⁺ T cells from *Hnrp11^{thn/thn}* mice have lower expression of Bcl-2 protein as detected by intracellular staining by flow cytometry. In comparison there was no difference in Bcl-2 expression in memory T cells or in thymocytes (Figure 5.2.6 d, e and f).

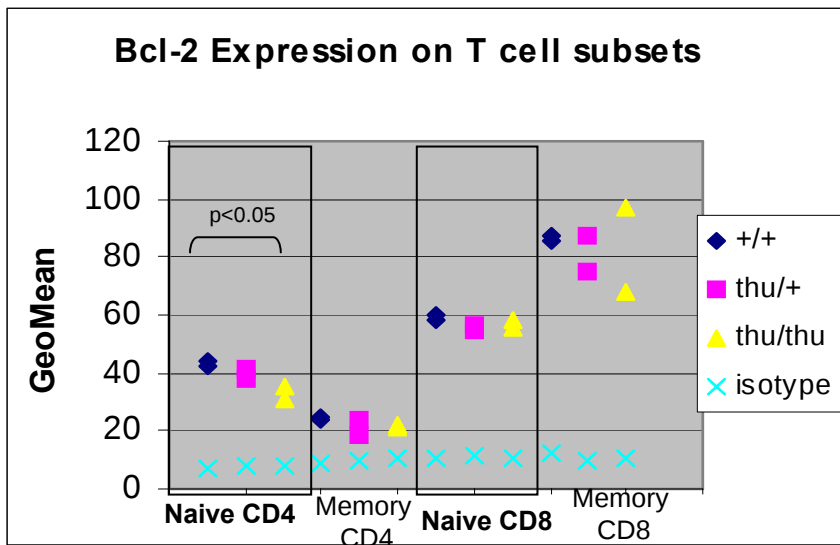
Figure 5.2.6 T cell apoptosis



- a. Dot plots showing Fas expression in the peripheral CD4⁺ T cells in correlation to the activation status represented by CD44 staining.
- b. Histogram overlay of CD4SP thymocytes, naïve CD4, and memory CD4 cells from wild-type (red), *thu/+* (green) and *thu/thu* (blue) showing Fas expression.
- c. Fas GeoMean of blood T cell subsets in wild-type (blue dots), *thu/+* (pink), and *thu/thu* (yellow). P value was from a Student's t-Tests.



f.



d. Dot plots showing Bcl-2 expression on naïve (CD44lo) and memory (CD44hi) CD4+ subsets in wild-type, $thu/+$, and thu/thu mice in the spleen.

e. Histogram overlays showing Bcl-2 expression in CD4SP thymocytes, naïve and memory CD4+ splenocytes. (isotype control=red and green, wild-type=blue, thu/thu =brown).

f. Bcl-2 GeoMean of spleen T cell subsets. P value from a Student's t-Test.

5.3 Investigating the role of CD45 in regulation of T cell homeostasis in *thunder* mice

CD45 is the most abundant membrane bound protein tyrosine phosphatase in lymphocytes playing important roles in initiating TCR signalling by dephosphorylating and activating tyrosine kinase p56Lck. Lck can then phosphorylate ITAMs (immune tyrosine activation motifs) of the TCR/CD3 complex, promote TCR signal transduction, and lead to cell activation and proliferation (Hermiston ML, Xu Z, Weiss A. 2003). It remains obscure how CD45 splice variants affect TCR signalling. The high molecular weight isoforms of CD45 (i.e. CD45RABC) which are expressed constitutively on the surface of *Hnrp11^{thu/thu}* T cells have been shown to have the highest level of phosphatase activity and therefore should be efficient in promoting TCR signalling (Chui, D. et al. 1994). We hypothesized that the phenotypic changes in TCR, CD3, CD5 and CD95 in naïve T cells of *thunder* mice might be contributed by increased CD45 enzymatic activity which could increase the strength of TCR signalling.

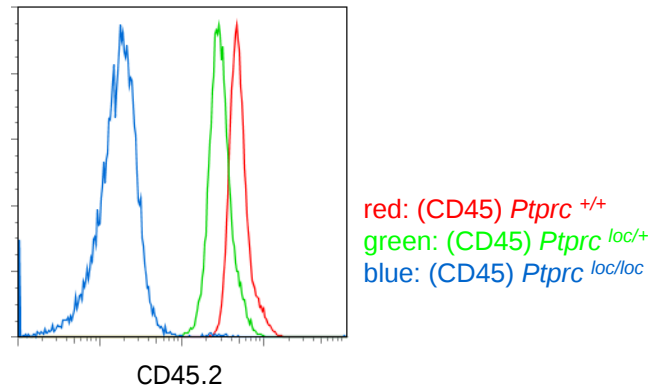
CD45 controls one of the earliest events in TCR signalling by dephosphorylating two critical tyrosine residues of the Lck protein (Hermiston ML, Xu Z, Weiss A. 2003). Antibody reagents have been generated to these phospho-peptides of the Lck protein and can be used to quantitate the level of phosphorylation in resting versus activated T cells by flow cytometry.

5.3.1. CD45 reduction in a previous identified ENU mutant lochy strain

To address the question of whether the defect in CD45 alternative splicing is correlated to loss of *Hnrpll*^{thu/thu} T cells, we crossed the *Hnrpll*^{thu/thu} mutation to a previously identified ENU mutant mouse strain, *lochy*, which has a point mutation (T to A conversion) of the intronic nucleotide in the 5' splice donor of exon 17 of the *Ptprc* gene (Yates, A.L. & Goodnow, C. unpublished). The mutation causes an alternative cryptic splice site adding 5 base pairs to the mRNA, causing a shift in the reading frame and introducing a premature stop codon. The premature termination of the CD45 mRNA leads to nonsense-mediated decay of the *Ptprc* mRNA and a 20-fold reduction in the total amount of CD45 expressed at the cell surface of lymphocytes (Yates, A.L. PhD thesis. 2003). The residual protein may arise from correctly spliced mRNA. It shows representative flow cytometric histograms of low CD45 expression on homozygous *Ptprc*^{loc/loc} CD4⁺ T cells compared to *Ptprc*^{+/+} and *Ptprc*^{loc/+} T cells (Figure 5.3.1 a), detected using a pan-CD45 antibody (anti-CD45.2). The relative expression of total CD45 is measured where the geometric mean expression of CD45 on *Ptprc*^{+/+} T cells is normalised to a value of 1.0 and *Ptprc*^{loc/+} mice have half the amount of CD45 at the surface of CD4⁺ cells, while the homozygous *Ptprc*^{loc/loc} mice have just 5% CD45 expression compared to wild-type T cells (Fig. 5.3.1 b).

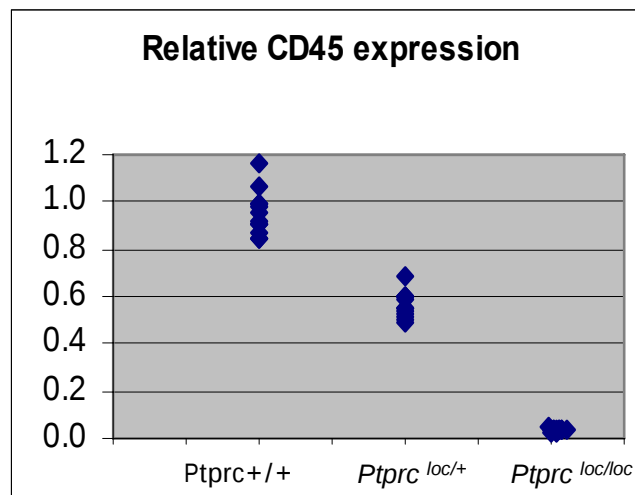
Figure 5.3.1 Reduced CD45 expression on T cells in an ENU mutant strain lochy

a



a. Total CD45 expression in the CD4⁺ T cells in a CD45/*Ptprc* mutant mouse strain, *lochy*, stained with a pan-CD45 antibody CD45.2. (*Ptprc*^{+/+}=red, *Ptprc*^{loc/+}=green, *ptprc*^{loc/loc}=blue). , *loc/loc* encode CD45 reduction (not null).

b



b. Relative expression of CD45 on CD4⁺ T cells in +/+, loc/+, and loc/loc littermates compared to normal B6 mice. Relative expression was calculated using CD45 GeoMean measured on the T cells divided by the average CD45 GeoMean of CD4⁺ T cells from a group of B6 mice.

5.3.2. Introducing *Hnrpll*^{thu/thu} mutation to lochy mouse shows similar CD45 splicing defects on T cells

To investigate if the changes in CD45 splicing might influence the loss of naïve T cells in the periphery of *thunder* mice, we bred the *thunder* mutation onto the *Ptpcr*^{loc/loc} background and examined T cell numbers of wild type, the *Ptpcr*^{loc/loc} *Hnrpll*^{+/+}, *Ptpcr*^{loc/loc} *Hnrpll*^{thu/+} and the *Ptpcr*^{loc/loc} *Hnrpll*^{thu/thu} mice in the thymus, spleen and peripheral blood of mice at 8-10 weeks of age by flow cytometry. As shown in Fig 5.3.2, it was still possible to observe different expression of the CD45RA, RB, and RC isoforms on the surface of T cells from the *Ptpcr*^{loc/loc} *Hnrpll*^{thu/thu} double mutant mice compared to the *Ptpcr*^{loc/loc} *Hnrpll*^{+/+} T cells. Although the expression of the CD45 isoforms on the double mutant T cells was relatively low it was still detectable and confirms that the residual CD45 mRNA in *Lochy* mice can still undergo alternative splicing which has not been previously documented.

5.3.3 CD45 reduction can not repress the action of *thu/thu* on peripheral T cell numbers

We have previously shown that the *thunder* mutation does not affect T cell development and *thu/thu* mutant mice have equivalent numbers of mature CD4 and CD8 single positive T cell numbers in the thymus compared to wild-type mice but reduced numbers of T cells in secondary lymphoid tissues. In correlation to the reports on CD45 deficient mice, the *Ptpcr*^{loc/loc} mice have defects in T cell maturation in the thymus resulting in decreased numbers of mature T cells in the thymus and

Figure 5.3.2 Alternative splicing with diminished CD45 in *Ptprc^{loc/loc} HnrpII^{thu/thu}* mice

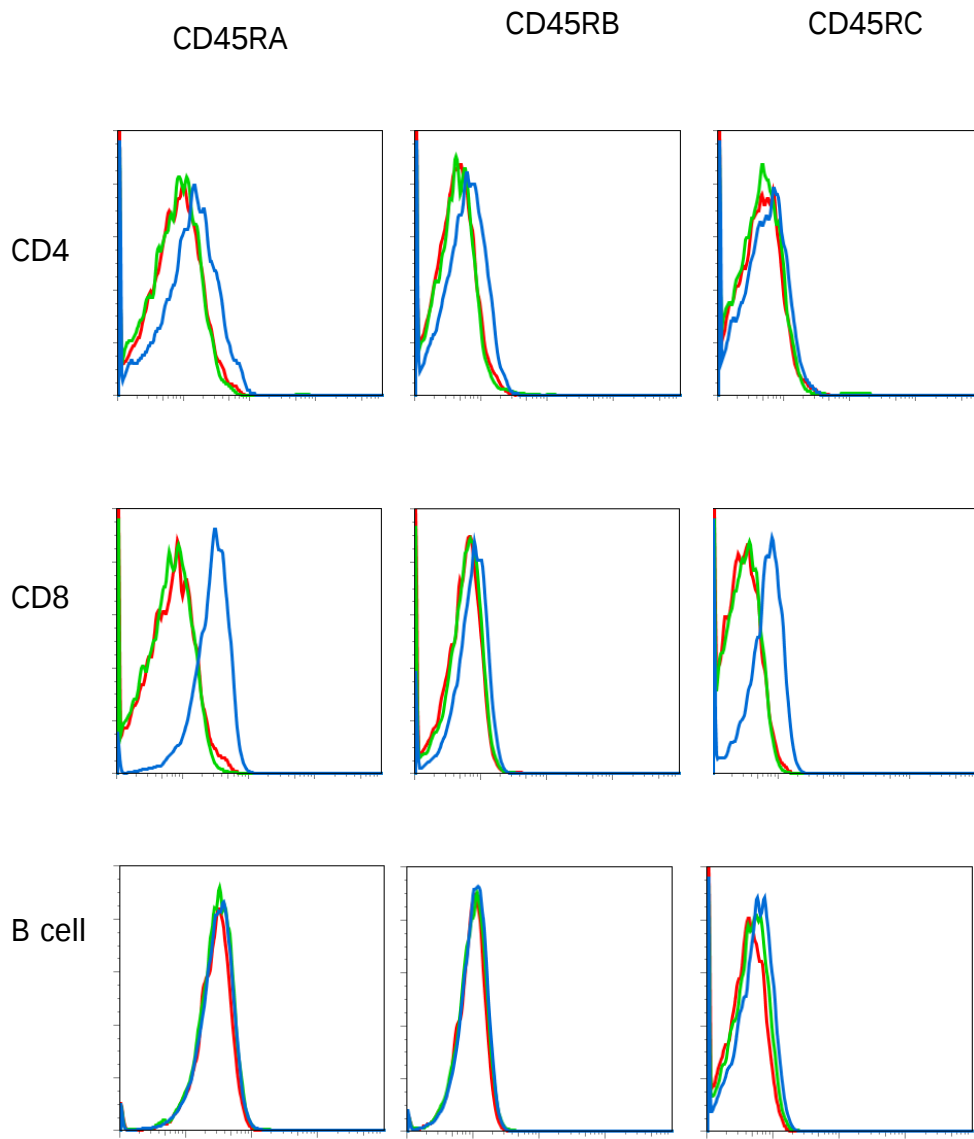


Figure 5.3.2 Histogram overlays of CD45RA, RB, and RC epitope specific staining in CD4, CD8, and B cells in *Ptprc^{loc/loc} HnrpII^{+/+}* (red), *Ptprc^{loc/loc} HnrpII^{thu/+}* (green), and *Ptprc^{loc/loc} HnrpII^{thu/thu}* (blue) mice.

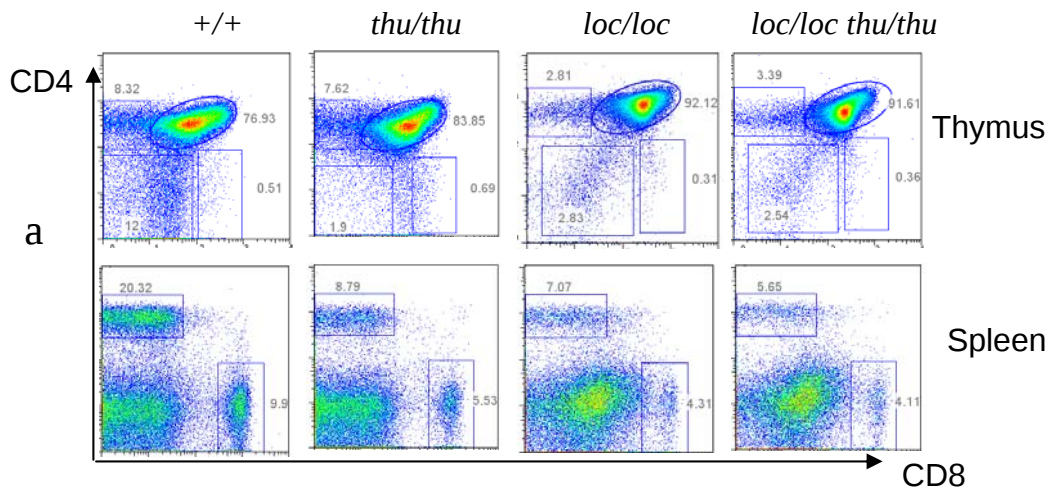
Red: *ptprc^{loc/loc} hnrpII^{+/+}*
 Green: *ptprc^{loc/loc} hnrpII^{thu/+}*
 Blue: *ptprc^{loc/loc} hnrpII^{thu/thu}*

reduced numbers of CD4⁺ and CD8⁺ cells in the spleen (Figure 5.3.3 a and b), consistent with the reduced activity of CD45 signaling in these mutant cells (Kishihara, K. et al. 1993; Stone, J.D. et al. 1997; Seavitt, J.R. et al. 1999; Baker, M. et al. 2000).

If the decrease in T cell numbers observed in the peripheral circulation of *thunder* mice is caused directly by the dysregulated splicing of CD45 then one prediction is that the *thunder* mutation would not affect T cell numbers in CD45-deficient mice. Thymus, spleen and peripheral blood samples were collected from *Ptprc*^{loc/loc} *Hnrpll*^{+/+}, *Ptprc*^{loc/loc} *Hnrpll*^{thu/+} and *Ptprc*^{loc/loc} *Hnrpll*^{thu/thu} double mutant animals and stained for flow cytometry analysis. The cell numbers were evaluated. As shown in figure 5.3.3 b *Hnrpll*^{thu/thu} mutant mice have normal thymic development and a decrease in peripheral CD4⁺ and CD8⁺ T cell numbers which we have shown in chapter 3. *Ptprc*^{loc/loc} mutation causes a 5-6 fold reduction in mature CD4⁺ and CD8⁺ SP thymocytes and reduced peripheral T cells as well (Figure 5.3.3 b). Importantly, CD4⁺ T cell numbers were significantly lower in the *Ptprc*^{loc/loc} *Hnrpll*^{thu/thu} double mutant mice compared to *Ptprc*^{loc/loc} mice without the *thunder* mutation, demonstrating that the *thunder* mutation still affect T cell numbers when CD45 was almost absent. As in the *thu/thu* single mutant mice, the *thunder* mutation had its greatest effect in loc/loc animals on the naive CD4 compartment (Figure 5.3.3 c and d).

Similar results were also observed in the blood of the *Ptprc*^{loc/loc} *Hnrpll*^{thu/thu} double mutant mice suggesting that the disruption to T cell homeostasis caused by the *thunder* mutation is independent of CD45 signals. Importantly the *thunder* mutation

Figure 5.3.3 CD45 reduction can not repress the action of *thunder* mutation on loss of naïve CD4+ but CD8+ T cells



b

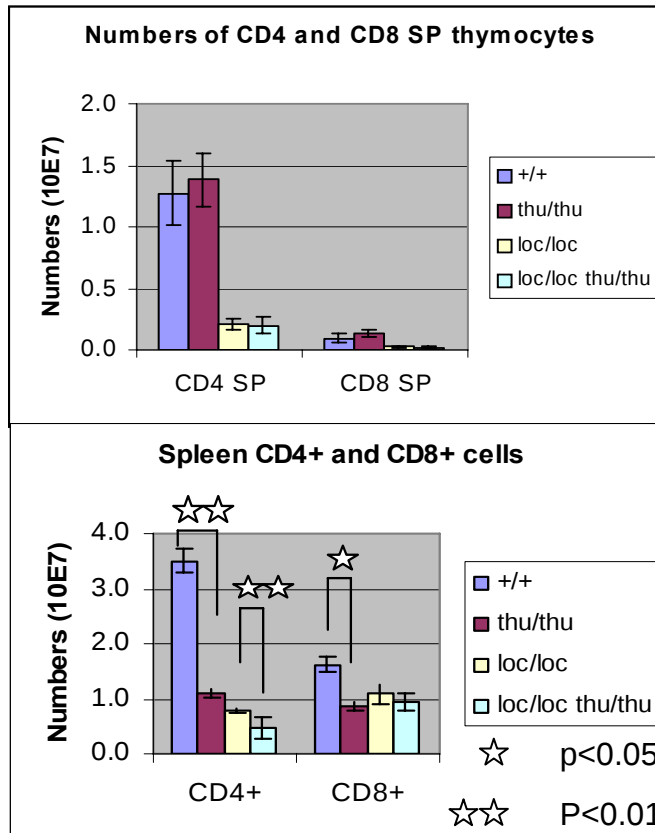


Figure 5.3.3 a. FACS plots showing CD4 and CD8 cells in the thymus and spleen of *Ptprc*^{+/+} *HnrpII*^{+/+}, *Ptprc*^{+/+} *HnrpII*^{*thu/thu*}, *Ptprc*^{*loc/loc*} *HnrpII*^{+/+}, and *Ptprc*^{*loc/loc*} *HnrpII*^{*thu/thu*} mice.

b. top: Total CD4 and CD8 SP thymocyte numbers in the wild-type (blue bar), *thu/thu* (red bar), *loc/loc* (yellow bar), and *loc/loc thu/thu* (green bar) mice. Bottom: Spleen CD4 and CD8 T cell numbers in the *thunder* and *lochy* mice. P values were calculated by a Student's t-Test.

Figure 5.3.3 Alternative splicing with diminished CD45 in *Ptprc*^{loc/loc} *HnrpII*^{thu/thu} mice

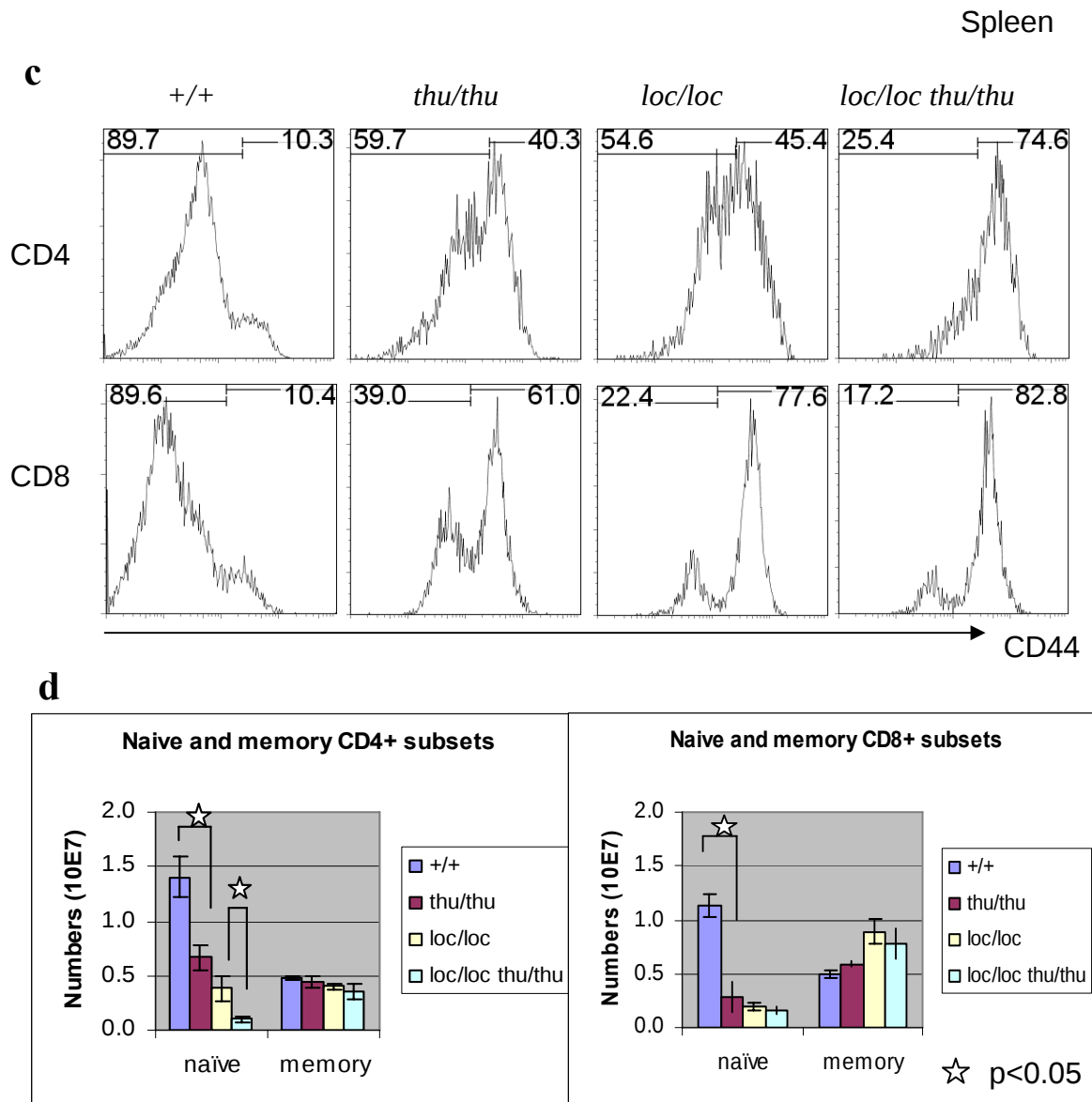


Figure 5.3.3 c. Histograms showing CD44 staining of CD4 and CD8 cells in the spleen of *Ptprc*^{+/+} *HnrpII*^{+/+}, *Ptprc*^{+/+} *HnrpII*^{thu/thu}, *Ptprc*^{loc/loc} *HnrpII*^{+/+}, and *Ptprc*^{loc/loc} *HnrpII*^{thu/thu} mice.

d. Naïve and memory T cell numbers in the wild-type (blue bar), *thu/thu* (red bar), *loc/loc* (yellow bar), and *loc/loc thu/thu* (green bar) mice. P values were calculated by a Student's t-Test.

does not change the total amount of CD45 expressed at the cell surface by CD4⁺ and CD8⁺ T cells (Fig. 3.3, chapter 3). These results do not rule out of the possibility that *thunder* mutation acts through CD45, since the *Lochy* mutant mice still have a low level of functional CD45 protein expression on the cell surface. A recent study has shown that as little as 3% of the total CD45 expression is required to allow normal T cell differentiation in mice and to dephosphorylate Lck505 (McNeill, L. et al. 2007). However, data from *lochy* mice showed that reduction of CD45 amount do not suppress the effect of *thunder* mutation in loss of peripheral T cells. We are currently breeding the *thunder* mutation with a CD45^{-/-} null mutation to address this remaining possibility.

5.3.4 CD45 splicing isoforms show different phosphatase activities when CD45 is reduced

As CD45 is the only known membrane bound protein phosphatase to activate Lck in T cells, we were interested to see how changes in CD45 splicing affect Lck activation in T cells. To confirm the specificity of the two antibodies it would be predicted that the staining intensity (i.e. geomean) of Lck pY505 and Lck pY394 antibodies should be higher in *loc/loc* T cells since they would have reduced levels of active CD45 protein compared to wild type T cells (discussed in chapter 1.3). This was indeed the observed result as *loc/loc* and *loc/loc:thu/thu* T cells (thymocytes or lymph node cells) showed higher levels of both Lck pY505 and Lck pY394 compared to wild type or *thu/thu* T cells (Figure 5.3.4).

Figure 5.3.4 Higher phosphatase activity of CD45RABC in T cells

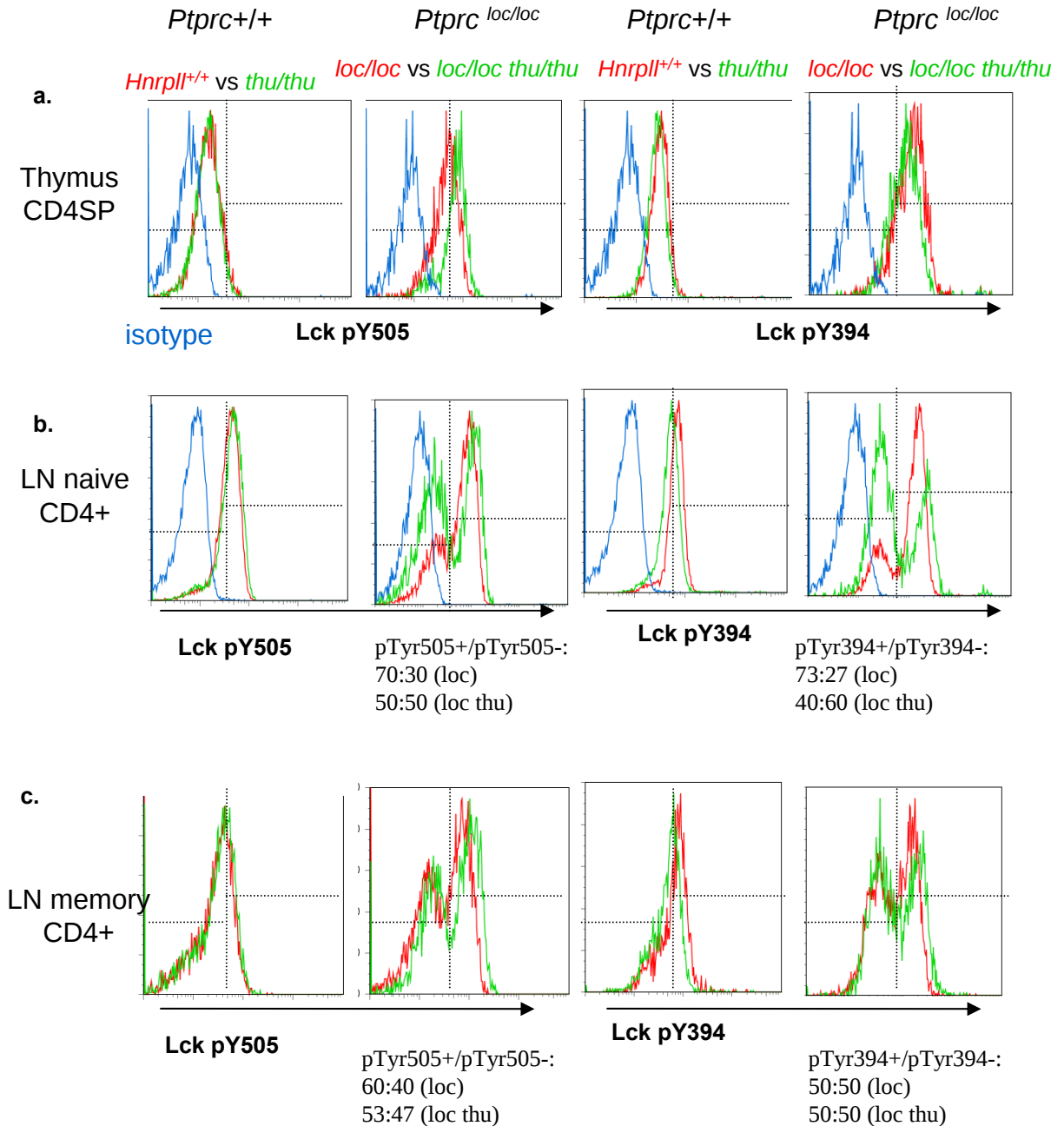


Figure 5.3.4 Intracellular staining of Lck pY505 (left) and pY394 (right) in the CD4SP thymocytes (a), LN naïve CD4 cells (b), and LN memory CD4 cells (c) in the *Hnrp1l*^{+/+} (red) and *Hnrp1l*^{thu/thu} (green) mice. Column 1 and 3 shows Lck pY505 and pY394 staining respectively in the *Ptprc*^{+/+} background. Column 2 and 4 compare phosphorylation of Lck Y505 and Y394 by the *thunder* mutation in the *Ptprc*^{loc/loc} background. Isotype control=blue.

When we compared thymocytes from *loc/loc* and *loc/loc:thu/thu* mice we observed no difference in the levels of Lck pY505 or Lck pY394 levels (Figure 5.3.4a). We could find no significant difference in the levels of pY505 and pY394 between wild-type and *Hnrp11^{thu/thu}* peripheral T cells irrespective of whether they were naïve or memory phenotype cells in the peripheral lymph nodes (Fig. 5.3.4a).

In contrast there was a dramatic shift to low Lck pY505 and Lck pY394 levels in the CD4⁺ CD44^{lo} (naïve) T cells in the lymph node of *loc/loc:thu/thu* double mutant mice (Figure 5.3.4 b), whereas there was no difference in the levels of Lck pY505 or Lck pY394 in CD4⁺ CD44^{hi} (memory) cells from the same mice (Figure 5.3.4 c). Similar changes were observed with the staining of pTyr394 in the double mutant naïve CD4⁺ cells. The same pattern of changes were also observed for CD8⁺ naïve T cells in the *loc/loc:thu/thu* mice compared to *loc/loc* mice (Figure 5.3.4 d and e). Importantly staining the same cells with a pan Lck antibody revealed no difference in abundance of the Lck protein which suggests the changes in Lck pY505 and Lck pY394 are real and reflect changes in CD45 activity (Figure 5.3.4 f).

Thus reducing the amount of total CD45 protein in *thu/thu* T cells it is possible to detect differences in the proximal TCR signaling events in CD4⁺ and CD8⁺ T cells. These changes with Lck activity correlate with the constitutive expression of the CD45 high MW isoforms in *thunder* T cells.

Consistent with the findings of lymph node T cells there was no difference in the levels of Lck pY505 in wild-type and *thu/thu* peripheral blood CD4⁺ and CD8⁺ T cells (Fig. 5.3.4 g and h). However, analysis of naïve T cells in *loc/loc:thu/thu* double

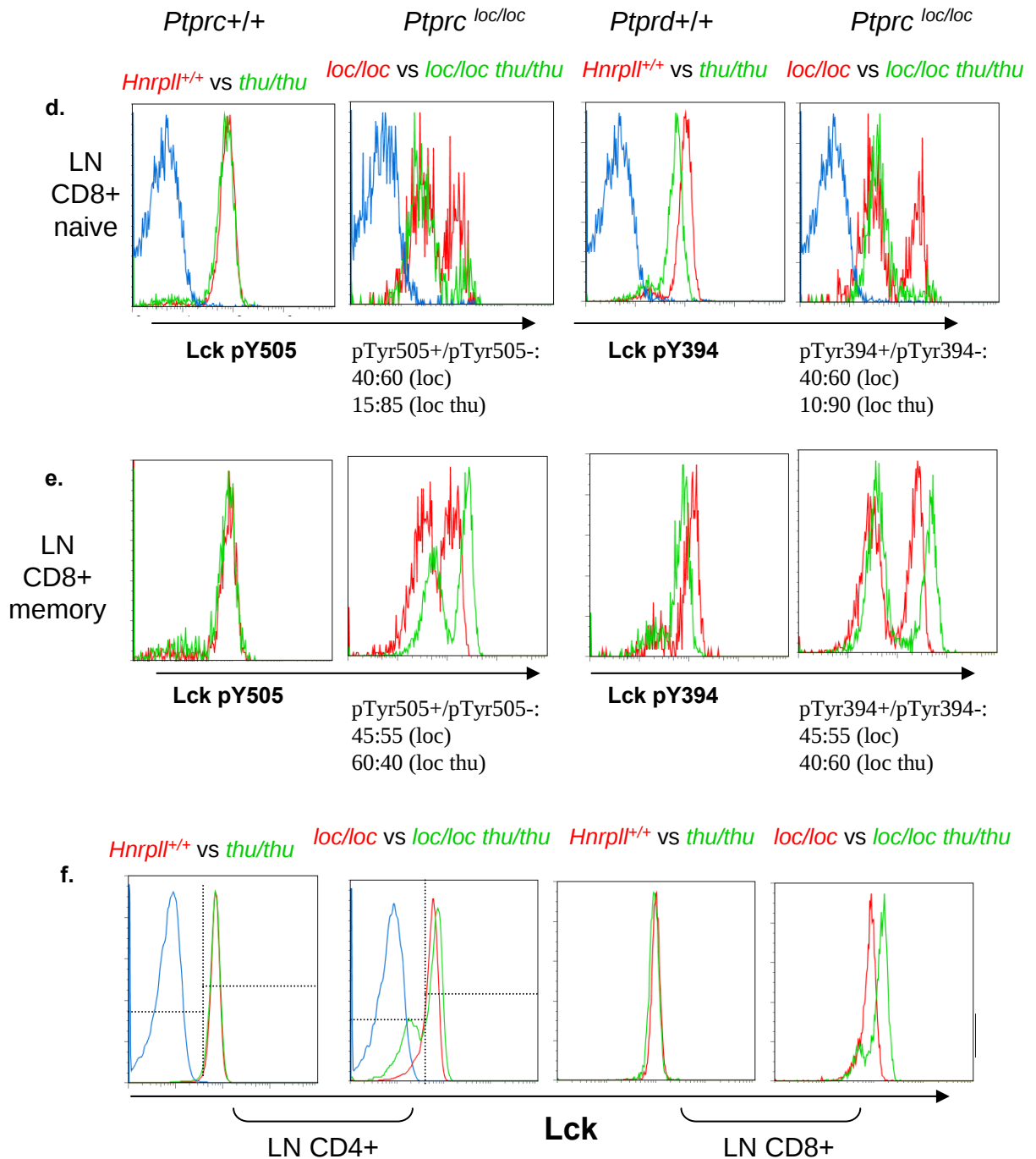


Figure 5.3.4 Intracellular staining of Lck pY505 (left) and pY394 (right) in the LN naïve CD8 cells (d) and LN memory CD8 cells (e) in the *Hnrp1*^{+/+} (red) and *Hnrp1*^{thu/thu} (green) mice. Column 1 and 3 shows Lck pY505 and pY394 staining respectively in the *Ptprc*^{+/+} background. Column 2 and 4 compare phosphorylation of Lck Y505 and Y394 by the *thunder* mutation in the *Ptprc*^{loc/loc} background.

f. Total Lck staining in LN CD4 and CD8 T cells. Isotype control=blue.

Figure 5.3.4 Higher phosphatase activity of CD45RABC in T cells

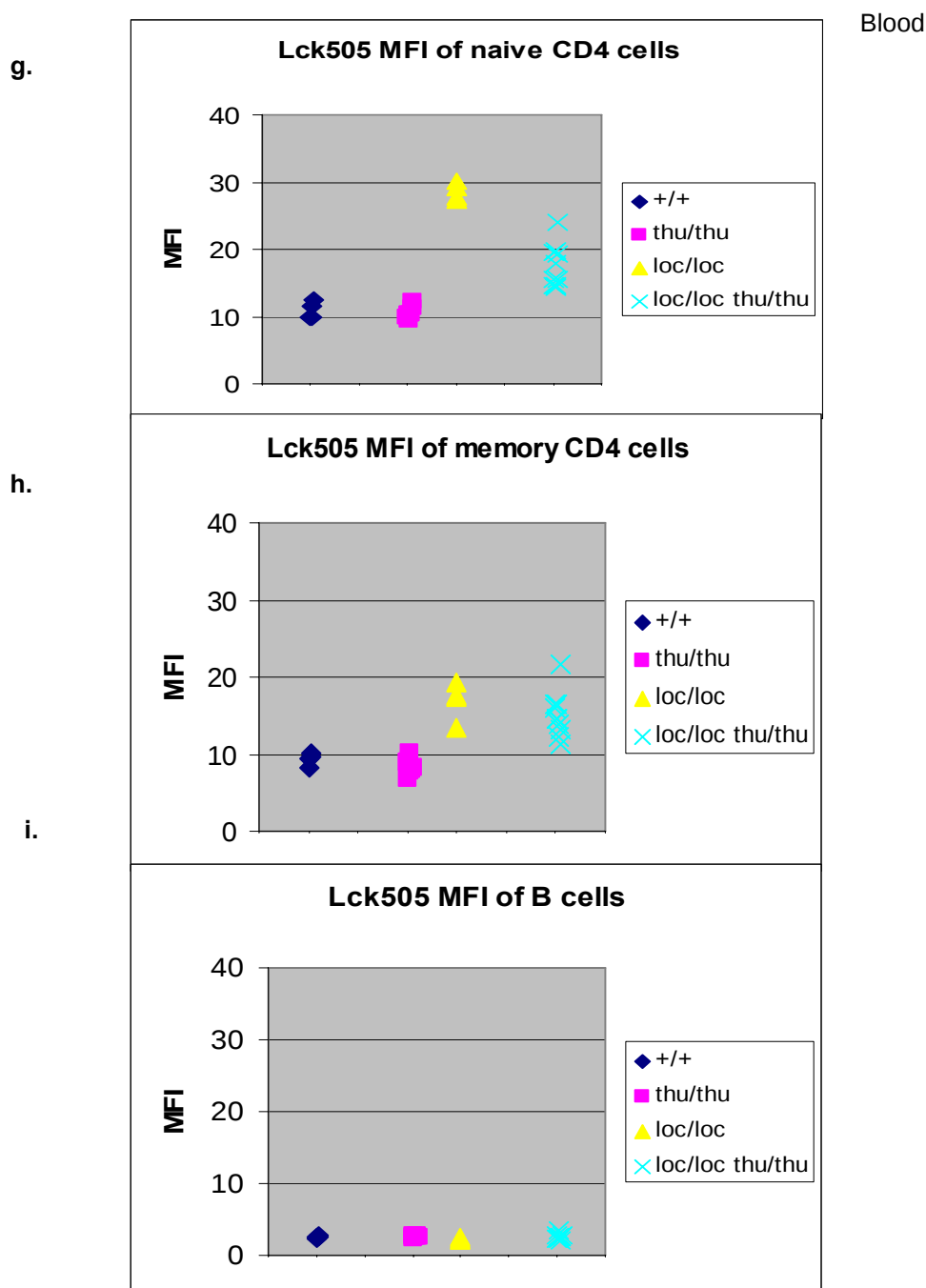


Figure 5.3.4 Intracellular staining of Lck pY505 in the blood naïve CD4 cells (g), memory CD4 cells (h), and B cells (i) in the *Hnrp1l*^{+/+} (blue dots), *Hnrp1l*^{*thu/thu*} (pink dots), *Ptprc*^{*loc/loc*} (yellow dots) and *Ptprc*^{*loc/loc*} *Hnrp1l*^{*thu/thu*} (green dots) mice. Each dot represents an individual mouse.

mutant mice revealed a significant decrease in Lck pY505 geomean levels compared to naïve *loc/loc* T cells (Fig. 5.3.4 g). There was no difference in Lck pY505 levels in memory T cells in *loc/loc* and *loc/loc:thu/thu* mice (Fig 5.3.4 h). Lck pY505 staining in B cells was unaffected by *thunder* and *lochy* mutations, since all mutant B cells showed equivalent low level of Lck pY505 to the wild-types (Fig 5.3.4 i).

5.3.5 Effects of *thunder* mutation on TCR coreceptor and IL-7 receptors in *Ptp^{loc/loc}* mice

Similar to the changes observed in T cells from *thu/thu* mice the *loc/loc:thu/thu* T cells also displayed lower levels of CD4 and CD8 co-receptor expression at the cell surface compared to T cells from the *loc/loc* mice (Fig. 5.3.5.a).

Furthermore the naïve CD4⁺ T cells in the *loc/loc:thu/thu* mice showed a decreased expression of CD127 on the cell surface compared to naïve T cells from *loc/loc* mice (Figure 5.3.5 b). The change is similar to the observations in naïve T cells of wild-type and *thu/thu* mice with normal amount CD45 expression (compare Fig. 5.2.1 c vs Fig. 5.3.5 b). There was no difference in the expression of CD127 on memory CD4 cells between *loc/loc* and *loc/loc:thu/thu* mice (data not shown).

The fact that these effects of *thu/thu* mutation on the coreceptors and IL-7R still occur in *Ptp^{loc/loc}* T cells argues against a role for CD45 in bringing them about. However, this will only be firmly excluded in crossing with *Ptp^{loc/loc}* null animals.

Figure 5.3.5 Changed CD4/CD8 coreceptor and IL-7R expression

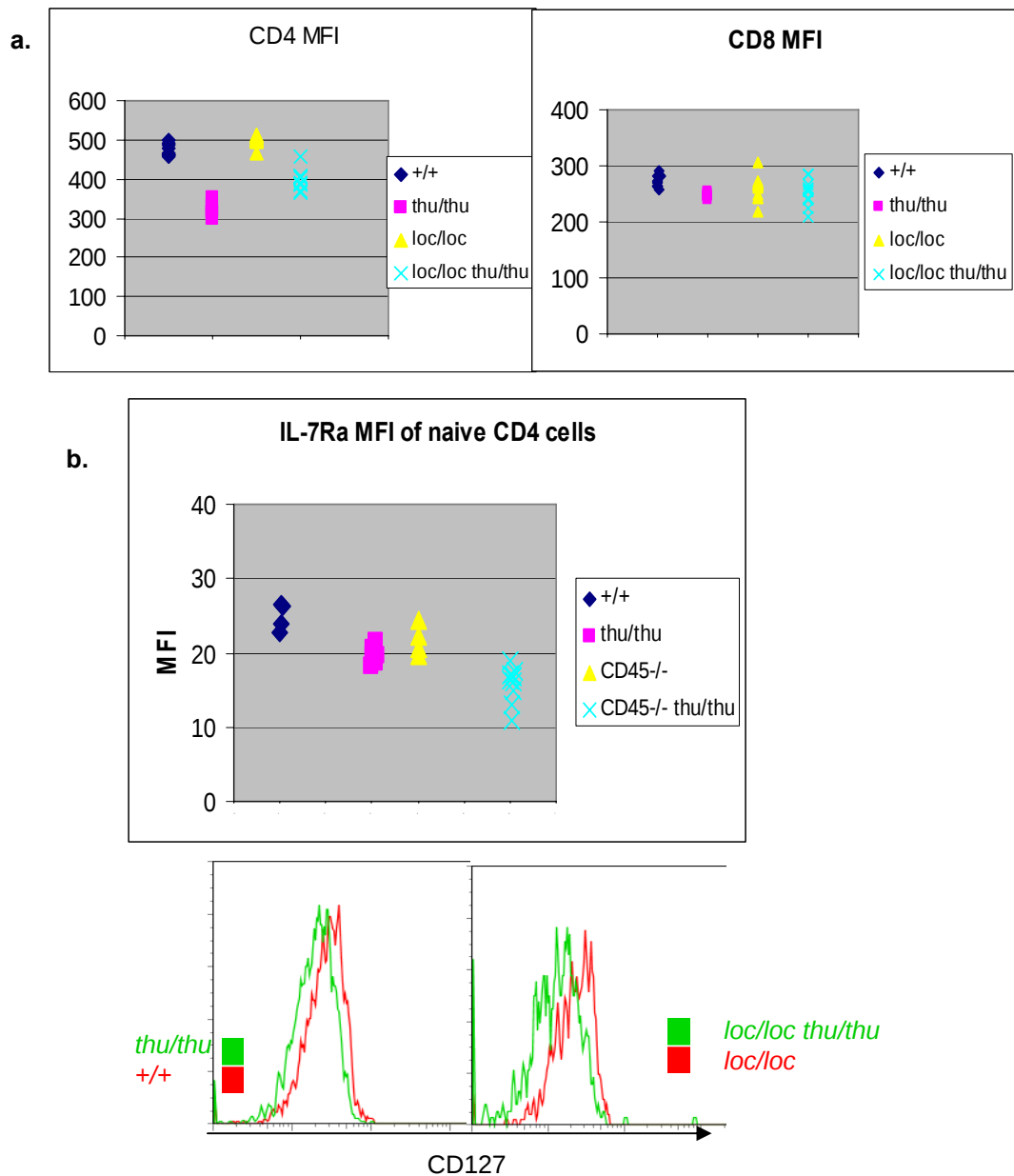


Figure 5.3.5 a. CD4 and CD8 GeoMean in the blood T cells in the *HnrpII*^{+/+} (blue dots), *HnrpII*^{thu/thu} (pink dots), *Ptprc*^{loc/loc} (yellow dots) and *Ptprc*^{loc/loc} *HnrpII*^{thu/thu} (green dots) mice. Each dot represents an individual mouse.

b. IL-7Ra (CD127) expression in the blood naïve CD4 cells in the *HnrpII*^{+/+} (blue dots), *HnrpII*^{thu/thu} (pink dots), *Ptprc*^{loc/loc} (yellow dots) and *Ptprc*^{loc/loc} *HnrpII*^{thu/thu} (green dots) mice. Each dot represents an individual mouse. Bottom left: histogram overlays comparing IL-7Ra expression in the wild-type (red) and *HnrpII*^{thu/thu} (green) naïve CD4 cells. Right: IL-7Ra expression in *loc/loc* (red) and *loc/loc thu/thu* naïve CD4 cells.

5.4 Discussion

5.4.1 *Hnrp11*^{th^u/th^u} T cells homeostasis: proliferation vs persistence

T cell homeostasis represents a dynamic balance between cellular proliferation and cell death. Heritable mutations that disable either of these key cellular checkpoints can have devastating outcomes on the long term health of an individual. A failure to control the basal proliferation of T cells could lead to T cell hyperplasia and predispose to malignancy, while unbridled expansion of self-reactive T cells due to lack of activation induced cell death could predispose to autoimmunity. During the first few weeks of postnatal life there is a controlled expansion of T and B cells to fill the lymphocyte compartments, but once these have reached their limits the cells are maintained at fixed numbers (Wilson, C.B. et al. 1992; Cahill, R.N. et al. 1997, 1999; Petrie, H.T. 2003).

Many studies on T cell homeostasis have focused on the homeostatic proliferation of naïve T cells after transfer to lymphopaenic hosts and established a clear role for both IL-7/IL-7R signaling and peptide/MHC –TCR signals (Surh, C.D. & Sprent, J. 2005). As discussed in the previous chapters the *thunder* strain was identified because of a loss of peripheral CD4⁺ and CD8⁺ T cells and we have identified the ENU-induced mutation in a previously uncharacterized member of the hnRNP protein family and showed it controls alternative splicing of CD45/*Ptp^{rc}* in T cells. In this chapter we have tried to analyse in more detail the mechanisms responsible for the loss of T cells in these mice.

Using an adoptive transfer assays we found that *thunder* T cells can proliferate normally when transferred together with wild type T cells into a lymphopaenic host (e.g. Rag^{-/-} or irradiated C57BL/6 mouse). The *Hnrpl*^{th^u/th^u} T cells could divide at the same rate as wild-type cells over the first week but they were unable to persist for long periods *in vivo*. In addition *Hnrpl*^{th^u/th^u} T cells were unable to persist in the circulation when transferred to a lymphoreplete recipient.

It is generally accepted that naive T cells require continuous contact with self-peptide/MHC complexes for long term survival *in vivo* enabling them to persist in the periphery (Surh, C.D. and Sprent, J. 2005; Dorfman, J.R. and Germain, R.N. 2002). Both wild-type and *Hnrpl*^{th^u/th^u} CD8⁺ T cells displayed lower proliferation rates in *Tap2* mutant mice compared to wild-type C57BL/6 recipients supporting the view that CD8⁺ T cells rely on peptide/MHCI-TCR signals to promote their expansion. But the data in figure 5.1.2d showed there is a subset of CD8⁺ T cells that can expand rapidly during the first week. Studies by Sprent and colleagues have established that this subset of CD8⁺ T cells are rapidly proliferating and rely on cytokine signaling rather than TCR signals *in vivo* (Judge, A.D. et al. 2002). So both the TCR dependent and cytokine dependent populations appear intact in the transferred *Hnrpl*^{th^u/th^u} CD8⁺ T cells, but once the homeostatic expansion phase is completed the CD8⁺ T cells are unable to persist.

Collectively, the results from these transfer assays identified that the *thunder* mutation acts in a cell autonomous manner to regulate the long term persistence of T cells *in vivo*. These data also provide an explanation for why *thunder* mice lose

peripheral T cells with age. In Chapter 3 we showed that there is no difference in T cell numbers in the periphery between wild type and *thunder* mice at 2-3 weeks of age, but by 4 weeks there is a significant decrease in CD4⁺ and CD8⁺ T cells in *thunder* mice (Figure 3.1.2). During the first 3 weeks of life there is a rapid filling of the peripheral pool with recent thymic emigrants until the T cell compartment is filled. By 6 weeks of age thymic production decreases and naïve T cell numbers in the periphery rely on IL-7 and peptide/MHC signals for maintenance. This coincides with the age that *thunder* T cell numbers fall below than wild-type counterparts.

5.4.2 *Hnrpll^{thu/thu}* naïve T cells display multiple cellular phenotypic changes

IL-7 signal is a key regulator for naïve T cell maintenance mediated through the IL-7R. Withdrawal of IL-7 signal leads to rapid cell death (Khaled, A.R. and Durum, S.K. 2002). IL-7 is produced by stromal cells at a constant rate whereas the consumption of IL-7 by T cells is mainly dependent on the number of T cells and the expression of IL-7R on the cell surface. We observed lower expression of IL-7R α chain on naïve *Hnrpll^{thu/thu}* T cells, and most importantly, down-regulation of IL-7R α expression in naïve T cells occurs in a cell autonomous manner as determined by mixed bone marrow chimeras and adoptive cell transfer assays. Decreased IL-7R α expression was detected at both the mRNA and protein levels but there is no evidence that the decrease in IL-7R α in *Hnrpll^{thu/thu}* T cells is caused by a change in alternative splicing.

Limiting the availability of trophic factors like IL-7 leads to a loss of expression of anti-apoptotic molecules such as Bcl-2 and arming of pro-apoptotic factors such as Bax and Bad. Consistent with the lower IL-7R α expression on naïve *Hnrp1l^{thu/thu}* T cells, there was also lower expression of Bcl-2 in *Hnrp1l^{thu/thu}* naïve T cells compared to memory T cells.

Activation induced cell death (AICD) through death receptors like Fas (CD95) is another pathway to eliminate activated T cells (Krammer, P.H. Arnold, R. and Lavrik, I.N. 2007). We observed that naïve *Hnrp1l^{thu/thu}* T cells display slightly enhanced Fas expression, suggesting that Fas may also play some roles in limiting the survival of *Hnrp1l^{thu/thu}* T cells in the periphery. However there is no increase in FasL expression by *thunder* T cells and so the change in Fas expression may be secondary to other changes that occur as a result of the mutation in the *Hnrp1l* gene. Moreover, the magnitude of the change is less than two-fold and there is no evidence that such small changes in Fas level are functional on their own.

Other characteristic changes that occur in *thunder* T cells include decreased expression of TCR & CD3 levels and an increase in CD5 expression. The level of CD5 expression at the cell surface mirrors the strength of TCR signalling and is dependent upon the activity of the Src-like kinases p56Lck and p59Fyn. Chronic TCR signaling in T cells can modulate TCR/CD3 levels at the cell surface. This is a natural physiological response by T cells that is thought to help decrease the strength of TCR signals in vivo in response to self-antigen-MHC complexes. The changes in TCR/CD3 were most significant in peripheral blood T cells, whereas T cells in lymph node or spleen had equivalent levels of TCR/CD3 compared to wild-type T cells. At

present we do not have a suitable explanation to explain the difference between TCR/CD3 expression on T cells in the blood and secondary lymphoid tissues but it will be interesting to pursue in the future.

5.4.3 CD45 alternative splicing and overall CD45 expression jointly modify its phosphatase activity

CD45 is the most abundant membrane tyrosine protein phosphatase in T cells and it is still not clear why there is so much CD45 on T cells considering a recent study suggests that a T cells only needs as much as 3% of the total CD45 to allow normal T cell development and function (McNeill, L. et al. 2007). The relevance of the different functional isoforms of CD45 and how this effects T cell differentiation in the peripheral circulation still remains a mystery.

TCR signalling is dependent upon the activation of Src like kinases in particular Lck. Several studies have highlighted the critical role for CD45 in this process (Ostergaard, H.L. et al. 1989; Koretzky, G.A. et al. 1990). There are two key tyrosine residues (Y505, Y394) located on the cytoplasmic tail of the p56Lck protein that need to be de-phosphorylated in order for Lck to become functional to interact with CD3-zeta chains (Ashwell and D'Oro, 1999; Palacios and Weiss, 2004).

We have used a unique ENU variant mouse strain (*lochy*) with a mutation in CD45 to investigate the link between CD45, TCR signalling and the T cell phenotype observed in *thunder* mice. The *lochy* mice have a mutation in a splice donor site in exon 17 of CD45 that results in a 20 fold reduction in CD45 protein at the surface of

lymphocytes because of non-sense mediated decay. This leads to an increase in pY505 and pY394 on the Lck protein in *lochy* T cells. We could find no significant difference in the levels of Lck pY505 when we compared wild-type or *Hnrpl^{thu/thu}* naïve CD4⁺ T cells. When we bred the *Hnrpl^{thu/thu}* mutation onto the *lochy* background to generate *Ptprc^{loc/loc} Hnrpl^{thu/thu}* double mutant mice we could detect significantly lower levels of pLckY505 in naïve CD4 T cells from the double mutant mice in comparison with T cells from the *Ptprc^{loc/loc}* mice.

The lower levels of Lck pY505 in double mutant T cells may be due to the alternative splicing of the small amount of CD45 on their surface. This correlates with previous data that enzymatic activity of the CD45 protein is enhanced by the RA, B, and C segments (Xu, Z. & Weiss, A. 2002; Dornan, S. et al. 2002). It is not clear at this stage why we cannot detect the intracellular differences in the level of Lck pY505 in *thunder* T cells until the level of CD45 is titrated below a critical threshold. It is also intriguing that no differences are observed in thymocytes or memory T cells, implying that the effect of CD45 isoforms is developmentally restricted.

Consistent with a reduced function of CD45 in *lochy* mice there is a significant reduction in the numbers of thymocytes and peripheral T cells. Combining the *lochy* mutation with the *thunder* mutation on the same background still leads to a further reduction in peripheral CD4⁺ T cells, especially naïve CD4⁺ T cells. The *lochy* mutant mice have a significant increase in the numbers of peripheral CD8⁺ T cells and it is not understood why this occurs. However, *Ptprc^{loc/loc}, Hnrpl^{thu/thu}* double mutant mice have a decreased number of peripheral CD8⁺ compared to the *Ptprc^{loc/loc}* mice. Taken together these results suggest that the loss of peripheral naïve T cells

caused by the *thunder* mutation is not simply caused by the increase in CD45 activity. The loss of peripheral T cells in *thunder* mice is likely to be more complex and may involve the effects on multiple genes.

5.4.4 Interaction of TCR signalling and IL-7 signalling?

The survival of naïve T cells is dependent upon IL-7 and peptide/MHC-TCR signals (Boyman, O. et al. 2007). Circulating T cells are likely to have low affinity for self antigens since they were developmentally selected to be tolerant of self antigens. The intensity of TCR signals stimulated by self antigens affects the ability of naïve T cells to survive in the periphery that is determined by both TCR affinity and co-receptor expression (Marrack, P. & Kappler, J. 2004). As the level of co-receptor expression helps to set the level of the TCR signalling threshold, high co-receptor expression increases signalling by low affinity TCRs, whereas low coreceptor expression limits signalling by high avidity TCRs (Viola, A. et al. 1996; 1997).

During T cell homeostasis the level of IL-7R is modulated to allow limiting amounts of IL-7 to be shared amongst the maximal possible number of T cells (Mazzucchelli, R. & Durum, S.K. 2007). Signalling through IL-7R can decrease IL-7R α transcription, while T cells that do not signal through IL-7 display increased levels of IL-7R α . Thus the numbers of T cells that survive in the periphery reflect the amount of available IL-7. A recent study by Singer and colleagues identified that the level of CD8 co-receptor expression is not set developmentally during T cell development in the thymus but is determined by the level of IL-7 and other common γ chain cytokines in the periphery. The capacity of CD8 + T cells to transduce common

γ chain cytokines is impaired by TCR signals induced by self antigens. These authors observed an inverse relationship between CD8 co-receptor expression and IL-7R signalling (Park, J.H. et al. 2007).

The modulation of CD8 co-receptor expression is termed co-receptor tuning and is thought to promote CD8⁺ T cell survival but only within the limits which are permitted by self tolerance. (Park, J.H. et al. 2007). Coreceptor tuning is proposed to work in CD4⁺ cells but perhaps via a different mechanism. In CD4⁺ cells, coreceptor modulation is mediated by ligand induced TCR signals via activation of Lck that does not affect CD8 expression levels.

It was showed that TCR and IL-7 signals can act independently or synergize to promote naïve T cell proliferation (Seddon, B. and Zamoyska, R. 2002). It was also well demonstrated in mouse and human that TCR stimulation cause down-regulation of IL-7R expression in T cell surface (Kaech, S.M. et al. 2003; van Leeuwen, E.M. et al. 2005). As shown here in this chapter we have observed down-regulated IL-7R expression in *thunder* naïve T cells, as well as lower TCR:CD3 complex, higher CD5, higher Lck activity (on the Lochy background) and lower coreceptor expression on both CD4⁺ and CD8⁺ T cells. Collectively these phenotypic changes may reflect stronger TCR signalling in *thunder* mice under resting conditions.

As shown here in this chapter we have observed down-regulated IL-7R expression in *thunder* naïve T cells, as well as lower TCR:CD3 complex, higher CD5, higher Lck activity (on the Lochy background) and lower coreceptor expression on both CD4⁺

and CD8⁺ T cells. Collectively these phenotypic changes may reflect stronger TCR signalling in *thunder* mice under resting conditions.

Although the dysregulated CD45 signalling may not on its own account for the loss of peripheral T cells in *thunder* mice, it nevertheless may contribute in a significant way to altering the strength of TCR signalling and thus the long term survival of T cell in the periphery.

~Chapter 6~

*Effects of $Hnrpl^{thu/thu}$ mutation on
thymic T cell selection and
the fate of transgenic T cells in the periphery*

Introduction

TCR signal strength is critical for both T cell development in the thymus and activation in the periphery. Pre-TCR plays important roles during early thymocyte development whereas the mature $\alpha\beta$ TCR is the primary trigger for signal transduction through contact with peptide/MHC and is critical for both clonal selection in the thymus and clonal expansion in the periphery (Moisge, L. and Zamoyska, R. 2007; Davis, et al. 1998). If the antigen receptor on double positive (CD4⁺ CD8⁺) and early single positive (CD4⁺CD8^{lo} or CD4^{lo}CD8⁺) thymocytes recognizing self-peptide/MHC strongly are induced into cell apoptosis, whereas those do not recognize self-peptide/MHC generate no or weak TCR signals die by neglect. Only weak peptide-MHC recognizing and intermediating TCR signals lead to positive selection and survival (Love, P.E. and Chan, A.C. 2003).

In this chapter, we wanted to examine the effect of the *thunder* mutation on positive and negative selection using a TCR-transgenic model so that we can track the fate of antigen-specific T cells throughout their development in the thymus and during the circulation in the peripheral immune system.

6.1 Normal positive and negative selection in the thymus

To examine if *Hnrp11*^{thu/thu} mutation affects either positive or negative selection of antigen-specific CD4⁺ T cells in the thymus, we bred *Hnrp11*^{thu/thu} mice to a well characterised 3A9 TCR transgenic mouse strain expressing a rearranged TCR $\alpha\beta$ receptor specific for the immunodominant epitope of Hen Egg Lysozyme (HEL

Figure 6.1 Normal positive and negative selection

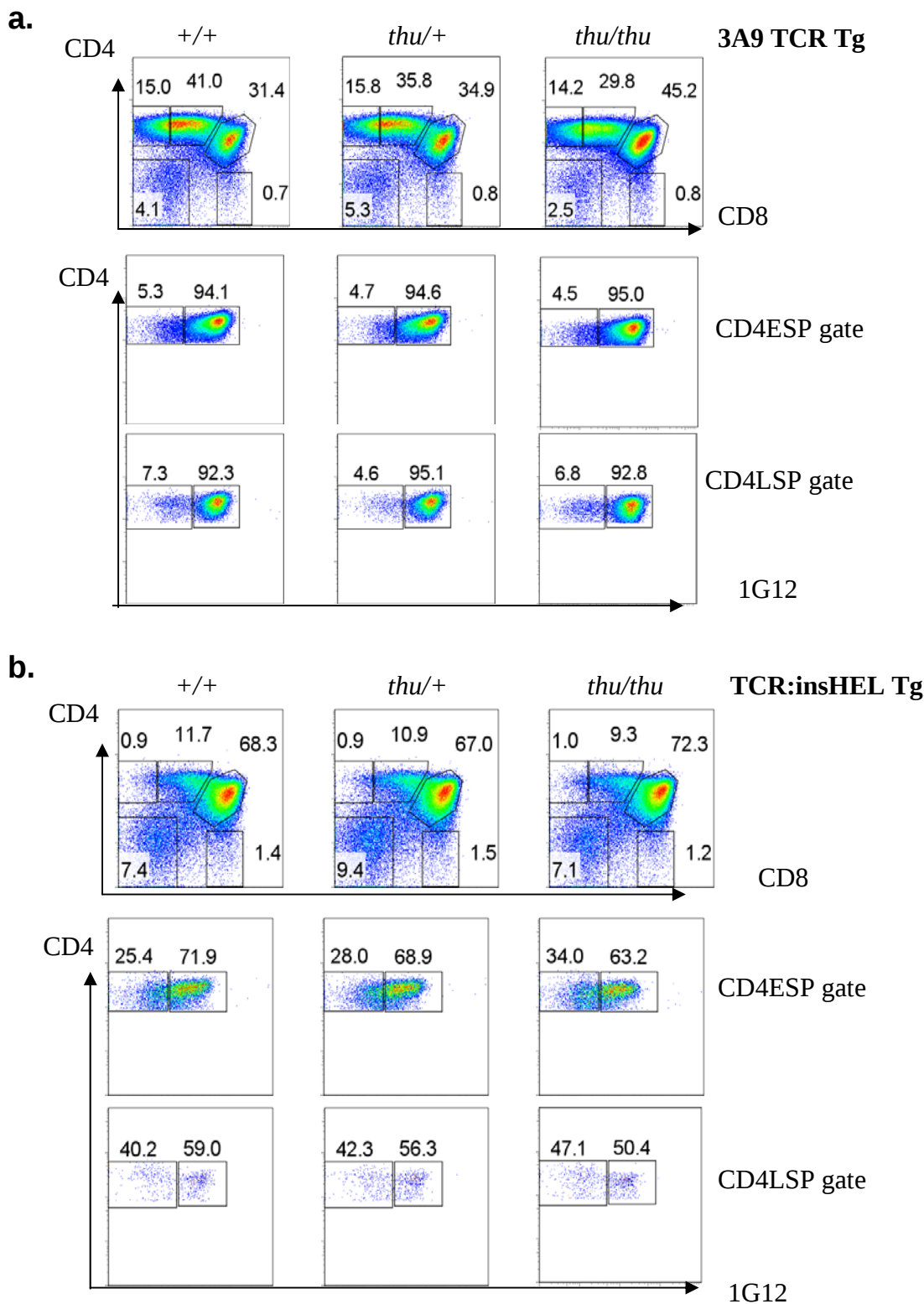


Fig. 6.1 Representative FACS profiles showing T cell development and clonotype positive CD4⁺ T cell thymic selection in TCR Tg (a) and TCR:insHEL Tg (b) mice.

peptide 46-63) in association with I-A^k. An advantage of this model is that the HEL-specific CD4⁺ T cells can be detected by a clonotype monoclonal antibody (1G12) which can be used to track the developmental fate in the thymus (Liston, A. et al. 2003). Normally these T cells are positively selected into CD4 fate. A second transgene was added to evaluate negative selection. In TCR:insHEL double transgenic mice HEL protein is expressed under the control of the Rat Insulin Promoter in pancreatic beta cells and thymic medullary epithelial cells. Under these conditions the HEL protein is regarded as a neoself antigen. 3A9 HEL-specific T cells are mostly deleted in the thymus during negative selection (Liston, A. et al. 2003; Akkaraju, S. et al. 1997).

In wild-type 3A9 TCR transgenic mice there is strong positive selection of CD4⁺ T cells that express the clonotype TCR. More than 90% CD4 late single positive (LSP) thymocytes expressing the transgenic TCR. When the *Hnrp11*^{th^u/th^u} mutation was bred onto the 3A9 TCR transgenic background, positive selection of the 3A9 TCR transgenic T cells was normal and there was no difference in the percentage of DP or CD4SP cells between wild-type and *Hnrp11*^{th^u/th^u} mice (Figure 6.1 a). The majority of CD4 single positive cells (about 90-95% ESP and LSP) expressed the transgenic $\alpha\beta$ TCR (Figure 6.1 a). The numbers of thymocytes showed no change in *Hnrp11*^{th^u/th^u} 3A9 transgenic mice from DN stage to SP stage (Figure 6.1 c and d). This contrasts with subtle mutations in Zap70 which severely cripple positive selection in this 3A9 TCR strain (Siggs, O. et al. 2007).

In TCR:insHEL double transgenic mice the expression of neoself antigen HEL which is under the control of the rat insulin promoter directing the majority of the

Figure 6.1 Normal positive and negative selection

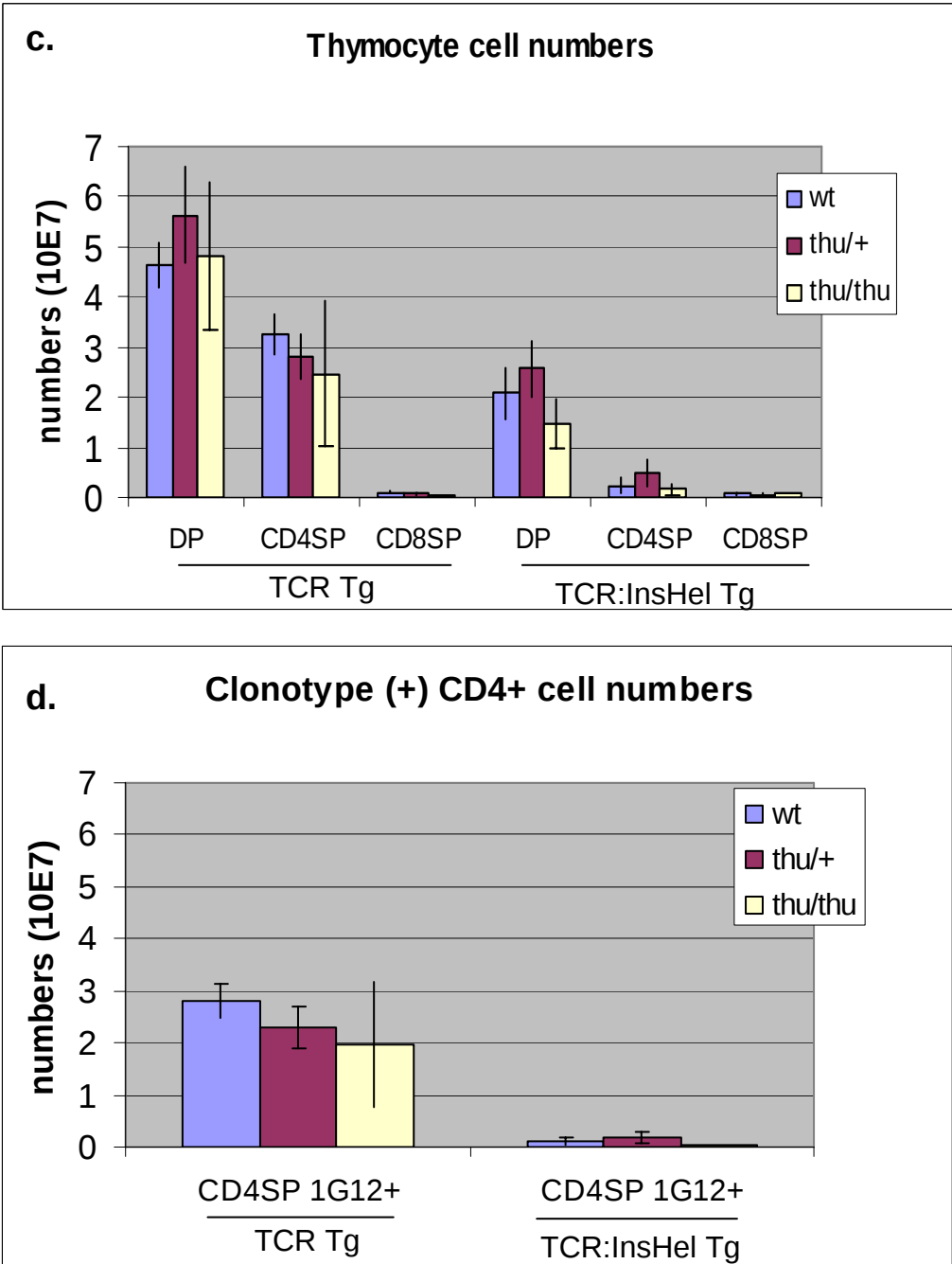


Fig. 6.1 c. Thymocyte numbers from DP to SP stage in wild-type (blue bar), *thu/+* (red bar), and *thu/thu* (yellow bar) TCR and TCR:insHEL transgenic mice. 3 mice in each group. Student's t-Tests show no significant difference.

d. Clonotype positive CD4+CD8- cell numbers in the thymus of TCR Tg mice and TCR:insHEL mice. 3 mice in each group. No significant difference between wildtype and *thu/thu* mice from Student's t-Tests.

protein expressed in the pancreatic beta cells. HEL antigen is also ectopically expressed in medullary thymic epithelium cells (mTECs) under the control of the autoimmune regulatory gene AIRE that allows deletion of the organ-specific colonies in the thymus and the development of immune tolerance. In both wild-type and *Hnrpl^{thu/thu}* TCR:InsHEL mice the vast majority of CD4⁺ T cells are deleted (compare Fig 6.1 a vs 6.1 b), leading to a decrease in the frequency and absolute numbers of CD4⁺ SP T cells in the thymus (Fig. 6.1 a, b, c, and d). During the early stage of CD4 single positive cell differentiation approximately 60-70% of CD4⁺ cells express the HEL-specific receptor in TCR:insHEL double transgenic mice. However, the vast majority of these cells are eliminated during maturation in the late single positive stage. There was no difference in the frequency or absolute cell number of HEL reactive CD4⁺ cells in the thymus of wild-type and *Hnrpl^{thu/thu}* TCR:InsHEL mice (Figure 6.1 b and d). These results suggest *thunder* mutation does not affect the process of negative selection of autoreactive T cells in the thymus, and contrasts with the lesions in negative selection when AIRE or NOD mutations are introduced (Liston, A. et al. 2003) (Liston, A. et al. 2004).

CD69 is a membrane receptor transiently expressed on lymphocytes during activation (Testi, R. et al. 1994; Sancho, D. et al. 2005). It is also induced in thymocytes by TCR engagement to self-peptide/MHC complex during positive and negative selection and declines as the cells complete their maturation and exported from the thymus into the periphery. The only significant phenotypic change that we have been able to reliably detect in the thymus of *Hnrpl^{thu/thu}* TCR transgenic mice is the low expression of CD69 on the CD4⁺ 1G12⁺ cells at the late single positive stage of CD4⁺ T cell development (i.e. CD69⁻) where there is a significant expansion in the

Figure 6.1 Normal positive and negative selection

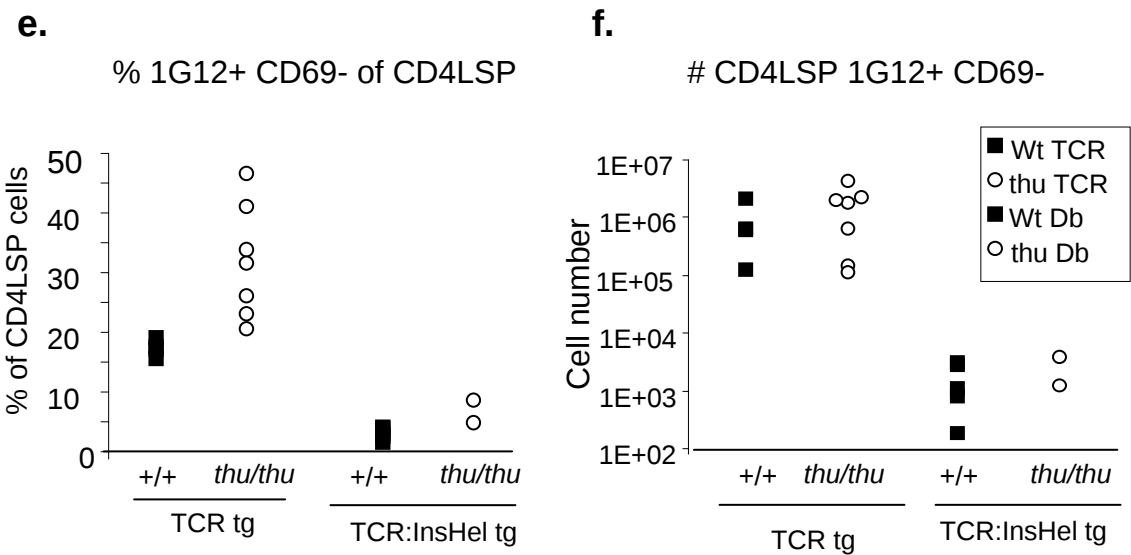


Fig. 6.1 e. Percentages of 1G12+ CD69- cells of CD4 LSP thymocytes in wild-type (filled square) and *thu/thu* (cycles) in TCR transgenic and TCR:insHEL double transgenic mice.

f. Numbers of CD4LSP CD69- 1G12+ cells in wild-type (filled square) and *thu/thu* (cycles) in TCR transgenic and TCR:insHEL double transgenic mice. Student's t-Tests showed no significant difference.

percentage of CD4⁺ 1G12⁺ CD69⁻ thymocytes which represent the most mature CD4 cells that are ready for export to the periphery (Fig. 6.1 e and f). However there is no difference in the absolute number of mature CD4⁺ 1G12⁺ CD69⁻ T cells in the thymus compared to wild-type 3A9 TCR tg mice (Fig.6.1 f). No difference of this subset of mature CD4⁺ T cells was observed in the TCR:insHEL double transgenic mice either, in both of percentages and absolute cell numbers (Fig. 6.1 e and f). Collectively, these results indicate that *Hnrp11*^{thu/thu} mutation does not affect positive and negative selection of antigen specific T cells during thymic development. The subtle shift between CD69^{hi} and CD69⁻ in CD4 LSP cells may indicate enhanced maturation after positive selection which may be caused by increased strength of TCR signalling due to the constitutive expression of high molecular weight CD45 isoforms by the *thunder* cells but this requires further analysis. This could be investigated by breeding the 3A9 TCR tg model onto the CD45^{-/-} background.

6.2 Different fate of *thu/thu* clonotype positive and clonotype negative T cells in the periphery

6.2.1 *Hnrp11*^{thu/thu} transgenic T cells are maintained normal in the peripheral lymphoid tissues

As discussed in the previous chapters, the *Hnrp11*^{thu/thu} mutation leads to a loss of peripheral T cells. The loss of T cell numbers is not due to their ability to undergo homeostatic proliferation, but instead is caused by an intrinsic failure of *Hnrp11*^{thu/thu} T cells to persist *in vivo*, particularly the naïve compartment. The data presented above show that *Hnrp11*^{thu/thu} mutation does not interfere with either positive or negative

thymic selection of antigen-specific CD4⁺ T cells. Therefore we were interested in determining if *Hnrp11*^{th^u/th^u} mutation would interfere with the persistence of TCR transgenic cells in the peripheral circulation. Because the TCR and TCR:InsHEL strains are on a *Rag1* sufficient background, peripheral T cells will express either the clonotype-specific TCR or the TCRV β transgene in association with an endogenous TCRV α gene. Both 1G12⁺ and 1G12⁻ cells coexist in each mouse so we can study the homeostasis of both the clonotype HEL-specific CD4⁺ cells and the non clonotype CD4⁺ T cells in the same animals. The latter cell population in the *th^u/th^u* TCR or TCR:InsHEL mice are expected to behave like polyclonal T cells in a C57BL/6 *th^u/th^u* mouse where naive T cells are unable to survive in the periphery and shift to the CD44^{hi} phenotype.

We examined the number of CD4⁺ 1G12⁺ T cells in the spleen of TCR and TCR:InsHEL transgenic mice on a wild-type and *Hnrp11*^{th^u/th^u} mutant background. When compared to the CD4SP population in the thymus where more than 90% of mature CD4⁺ T cells express the 1G12 TCR, the percentage of cells bearing this receptor drops to approximately 70% in peripheral CD4 cells in the TCR transgenic mice; whereas in the TCR:insHEL double transgenic mice 1G12⁺ cells are effectively eliminated from 50% of CD4LSP thymocytes to only 5% of CD4⁺ T cells in the spleen expressing transgenic TCR (Fig. 6.2.1 e and f).

The peripheral CD4⁺ T cells pool in the TCR and TCR:InsHEL strains are composed of both CD4⁺ 1G12⁺ cells and CD4⁺ 1G12⁻ cells. These two cell populations showed different fates in the peripheral lymphoid tissues in the transgenic mice. In *Hnrp11*^{th^u/th^u} TCR transgenic mice, clonotype positive CD4⁺ cells have

Figure 6.2.1. Transgenic *HnrpII^{thu/thu}* CD4+ T cells are maintained in the periphery

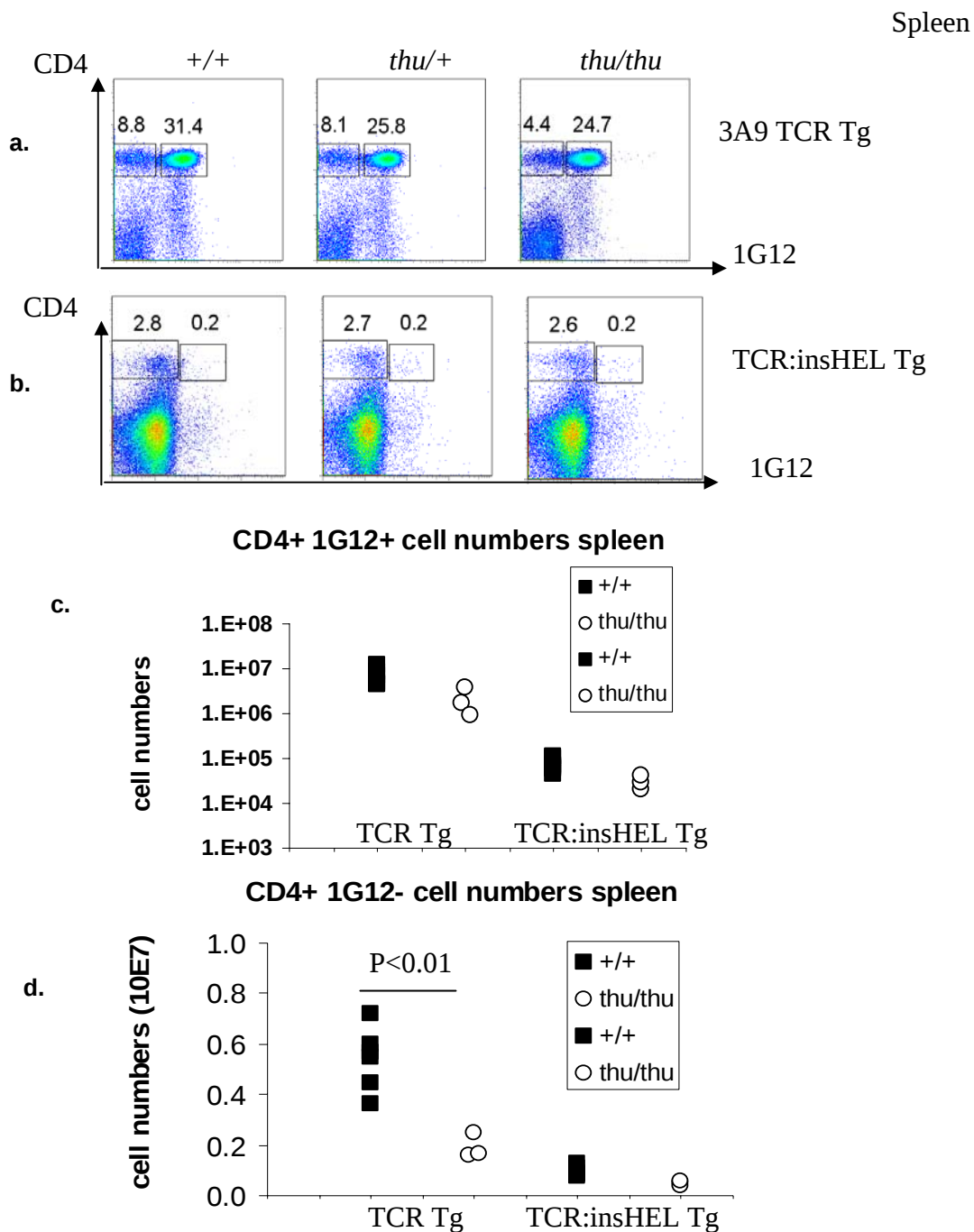


Fig. 6.2.1 a. Representative FACS profile of CD4 vs 1G12 staining of lymphocyte gate in the spleen of wild-type (left), *thu/+* (middle), and *thu/thu* (right) TCR transgenic mice.

b. Representative FACS profile of CD4 vs 1G12 staining of splenocytes of *+/+* (left), *thu/+* (middle), and *thu/thu* (right) TCR:insHEL transgenic mice.

1G12⁺CD4⁺ (c) and 1G12⁻CD4⁺ (d) cell numbers in TCR transgenic (left) and TCR:insHEL mice (right). Wild-type=filled squares, *thu/thu*=open cycles. Each symbol represents an individual mouse.

Figure 6.2.1. Transgenic *HnrpII*^{thu/thu} CD4+ T cells are maintained in the periphery

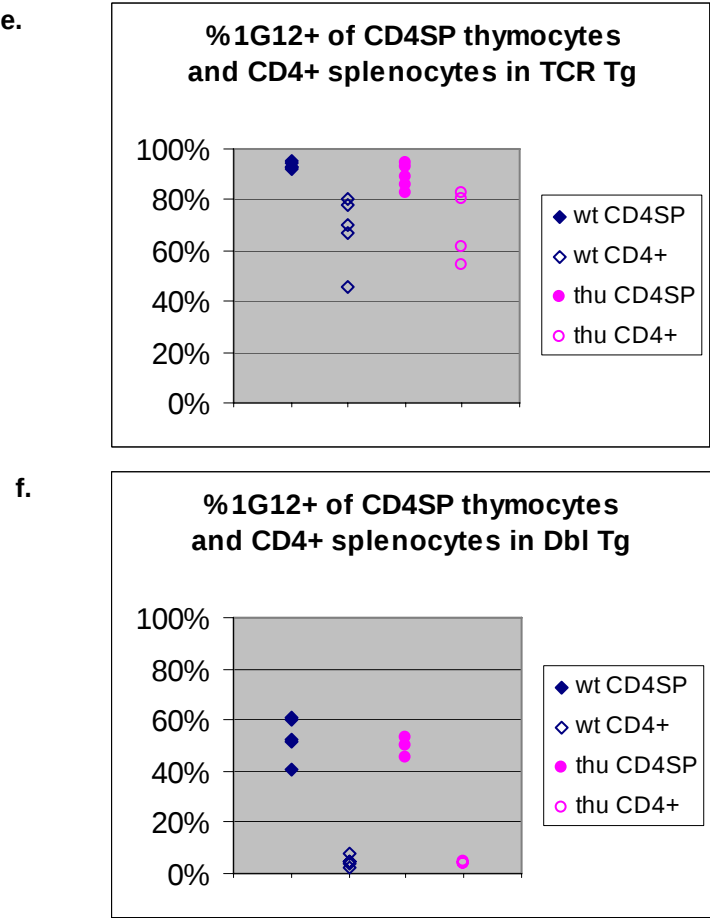


Fig. 6.2.1 e. Percentages of CD4+1G12+ cells within the CD4SP thymocyte and CD4+ splenocyte compartment of TCR transgenic mice.

f. Percentages of CD4+ 1G12- cells of CD4SP thymocyte and CD4+ splenocyte compartment of TCR:insHEL double transgenic mice. Each symbol represents an individual mouse.

normal percentages and absolute numbers in the spleen (Fig. 6.2.1 a and c), in contrast, the number of 1G12- mutant CD4⁺ cells decrease (Fig. 3.2.1 a and d), similar to that observed in the *thunder* strain on a non-transgenic background. In the TCR:insHEL double transgenic mice, *Hnrp1l*^{thu/thu} CD4⁺ 1G12⁺ cells are equally eliminated to remain in a very low level in the periphery. In the wild-type TCR:InsHEL mice (Figure. 3.2.1 b and c), the number of CD4⁺ 1G12⁻ cells are also very low but there appears no further reduction of this population in the mutant mice (Figure 6.2.1 b and d).

Similar phenotypes were observed in the peripheral blood. *Hnrp1l*^{thu/thu} mutant mice have relative larger proportion of CD4⁺ 1G12⁺ cells and fewer CD4⁺ 1G12⁻ cells compared to their wild-type counterparts in the TCR transgenic mice (Figure 6.2.1 g and i). In the TCR:insHEL double transgenic mice there was an increase in the percentage of *thu/thu* CD4⁺ 1G12⁺ cells in the blood while no difference was observed in the frequency of CD4⁺ 1G12⁻ cells between wild type and *Hnrp1l*^{thu/thu} mutant mice (Figure 6.2.1 h and j).

6.2.2 Transgenic TCR restores *Hnrp1l*^{thu/thu} naïve T cells in the periphery

Next we wanted to know if the HEL-specific T cells in the periphery of the TCR and TCR:InsHEL mice remain in a naïve state (i.e CD44^{lo}). In the TCR and TCR:insHEL transgenic mice, CD4⁺ T cells bearing the clonotype TCR are maintained in a naïve status as the majority of cells were CD44^{lo}. We presume this happens because the transgenic TCR has low avidity to the endogenous peptide/MHC complex. In both TCR (Figure 6.2.2 a, b) and TCR:insHEL (Figure 6.2.2 c, d)

Figure 6.2.1. Transgenic *HnrpII*^{thu/thu} CD4+ T cells are maintained in the periphery

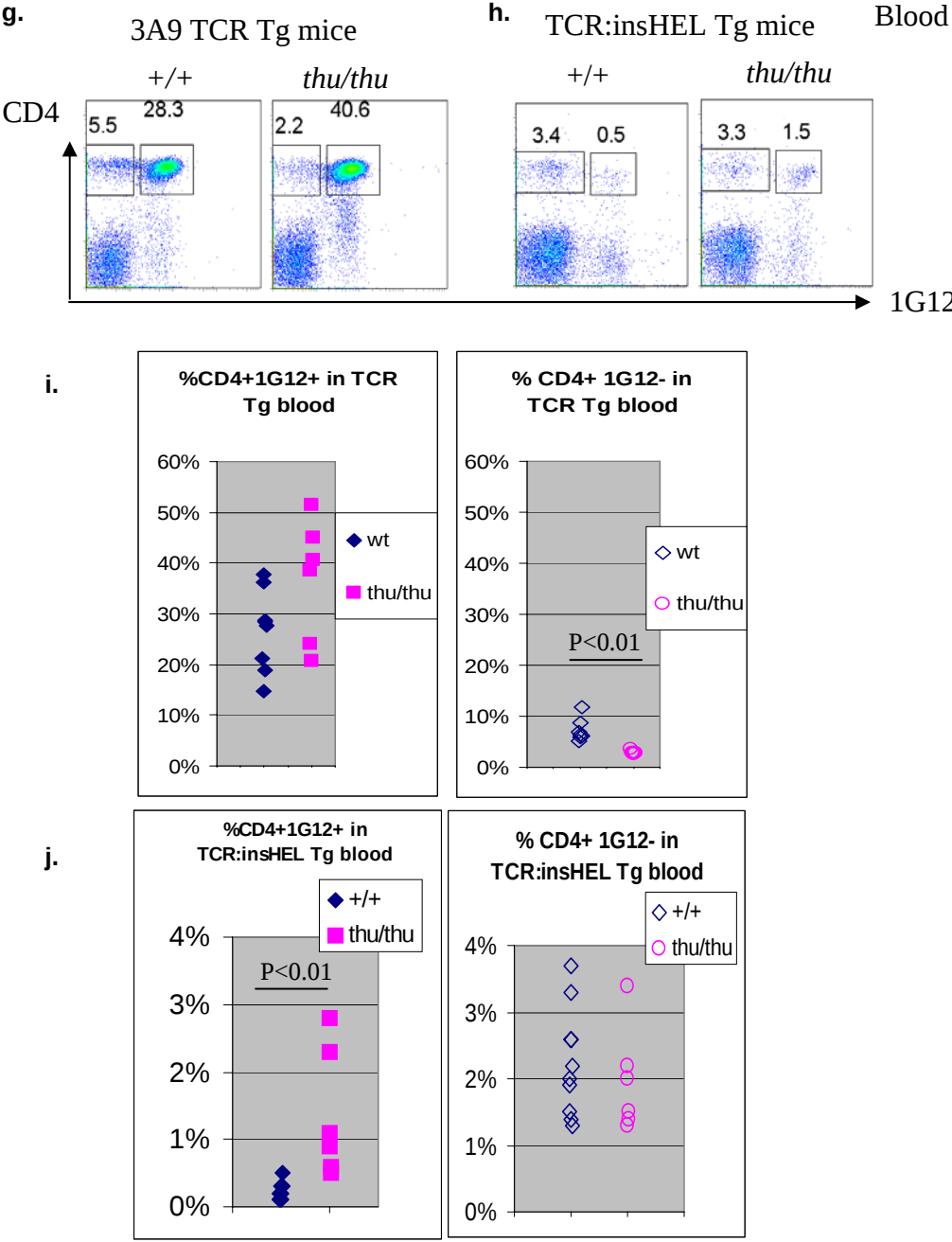


Fig. 6.2.1 g. Representative FACS profile of CD4 vs 1G12 staining of blood lymphocytes in wild-type and *thu/thu* TCR transgenic mice.

h. Representative FACS profile of CD4 vs 1G12 staining of blood lymphocytes of +/+ and *thu/thu* TCR:insHEL transgenic mice.

i. Percentages of CD4+ 1G12+ and CD4+ 1G12- cells in the blood of TCR transgenic mice. Each symbol represents an individual mouse. Student's t-Tests were used for statistic analysis.

j. Percentages of CD4+ 1G12+ and CD4+ 1G12- cells in the blood of TCR:insHEL double transgenic mice. Each symbol represents an individual mouse. P values were calculated from Student's t-Tests.

transgenic mice, *Hnrp11*^{thu/thu} 1G12+ CD4 T cells display a CD44^{lo} naïve phenotype like the wild-type CD4+ 1G12+ cells. The CD44^{lo} status of CD4+ 1G12+ cells in the TCR:InsHEL background would indicate that the HEL-specific T cells are tolerant to HEL antigen in the periphery. In both transgenic strains the *thu/thu* CD4+ 1G12- cells showed a higher proportions of CD44^{hi} cells that was statistically significant (Fig. 6.2.2 b,d). It is notable that there is a strong shift to a CD44^{hi} phenotype in the CD4+ 1G12- cell populations of both wild-type and *thu/thu* TCR:InsHEL mice but the *thu/thu* CD4+ 1G12- cells have an even larger proportion of CD44^{hi} than the wild-type.

We have shown in chapter 5 that *thunder* naïve T cells express lower levels of IL-7R α in a cell autonomous manner potentially affecting T cell longevity. So we examined the expression of IL-7R on CD4+ 1G12+ and CD4+ 1G12- T cells in the spleen and blood of TCR and TCR:InsHEL mice on a wild-type and *thunder* background. Figure 6.2.2 e shows that 1G12+ and 1G12- *Hnrp11*^{thu/thu} CD4+ T cells expressed lower IL-7R α compared to the wild-type CD4+ 1G12+ and 1G12- cells in the TCR transgenic mice. In the double transgenic mice, IL-7R α expression was only significantly lower on the *thu/thu* clonotype negative CD4 cells (Figure 6.2.2 f) there was no difference in the levels of IL-7R α on CD4+ 1G12+ cells.

Figure 6.2.2. Transgenic TCR restores *HnrpII*^{thu/thu} CD4⁺ naive T cells

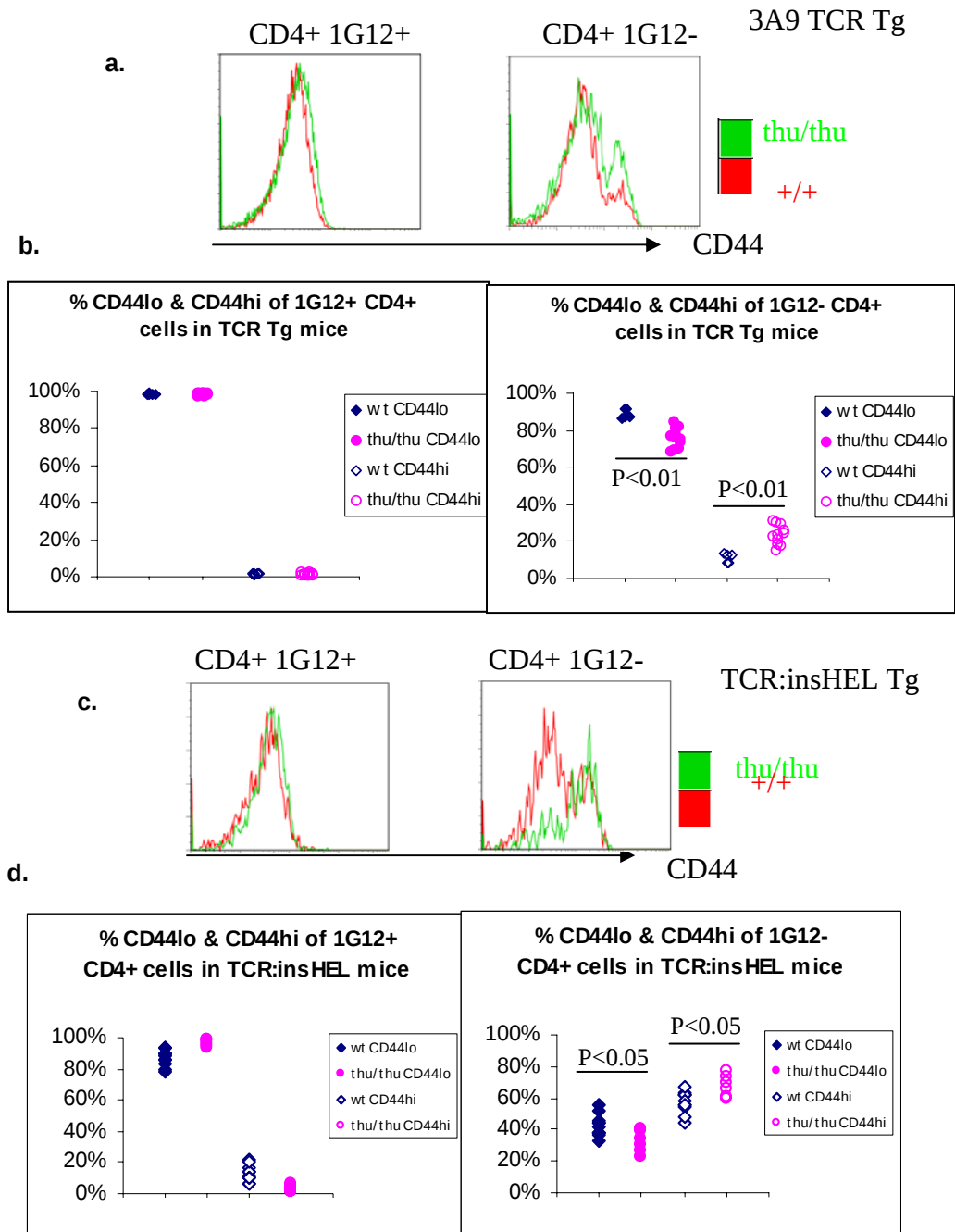


Fig. 6.2.2 a. Histogram overlay of 1G12⁺ (left) and 1G12⁻ (right) CD4⁺ splenocytes showing CD44 staining in +/+ (red) and *thu/thu* (green) TCR transgenic mice.
b. Dot plots showing CD44^{lo} (filled) and CD44^{hi} (open) percentages of 1G12⁺ CD4⁺ (left) and 1G12⁻ CD4⁺ (right) splenocytes in +/+ (blue) and *thu/thu* (red) TCR transgenic mice.
c. Histogram overlay of 1G12⁺ and 1G12⁻ CD4⁺ T cells showing CD44 expression. Wildtype (red), *thu/thu* (green).
d. Percentages of CD44^{lo} (filled) and CD44^{hi} (open) subsets in 1G12⁺ (left) and 1G12⁻ (right) CD4⁺ T cells in TCR:insHEL transgenic mice (right). +/+ (blue), *thu/thu* (red). Each symbol represents an individual mouse. P values from Student's t-Tests.

Figure 6.2.2. Transgenic TCR restores *HnrpII^{thu/thu}* CD4⁺ naive T cells

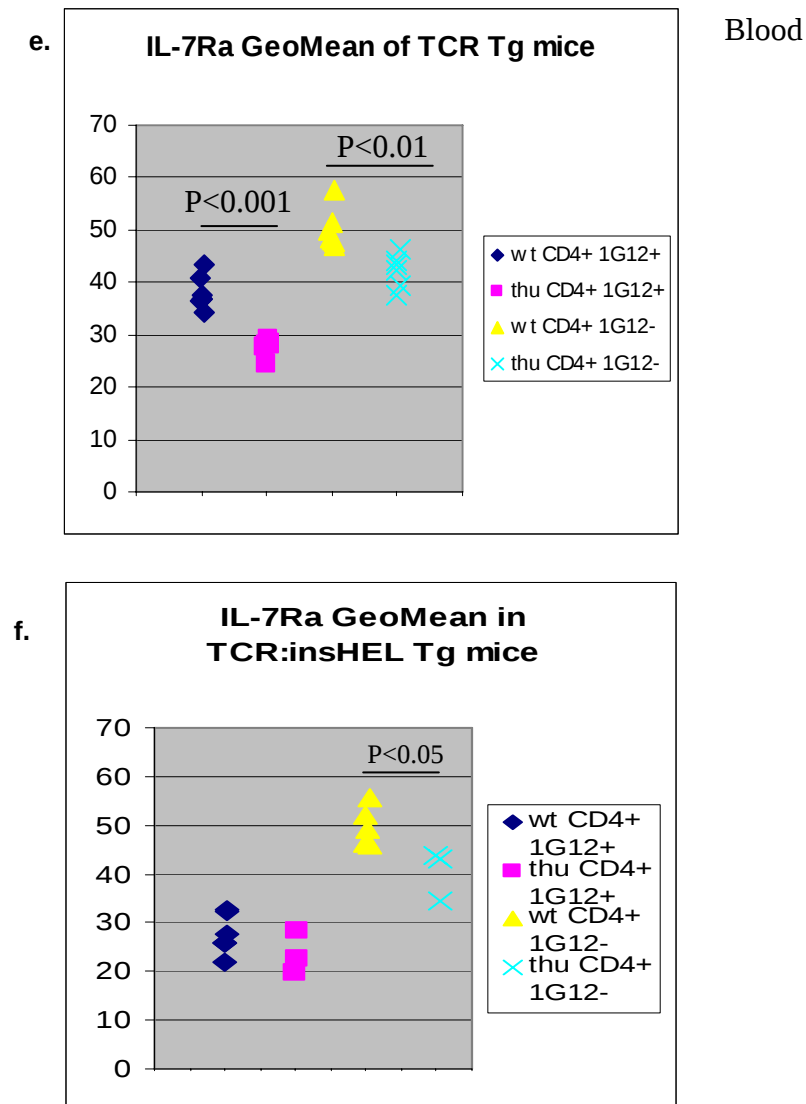


Figure 6.2.2 e. IL-7Ra (CD127) GeoMean of blood CD4⁺ T cells in the TCR transgenic mice.

f. IL-7Ra GeoMean of blood CD4⁺ T cells in the TCR:insHEL double transgenic mice.

Wild-type 1G12⁺ (blue), *thu/thu* 1G12⁺ (red), wt 1G12⁻ (yellow), *thu/thu* 1G12⁻ (light blue). Each symbol represents an individual mouse. P values were calculated by Student's t-Tests.

6.3 Discussion

Transgenic TCR, T cell selection and T cell persistence in the periphery

The results from the analysis of the TCR and TCR:insHEL transgenic mice confirmed that *Hnrp11*^{thu/thu} mutation does not affect positive and negative selection in the thymus. This is in accordance with the phenotypic analysis performed on the non-transgenic *thu/thu* mice we described in chapter 3 and is consistent with the observation that *Hnrp11*^{thu/thu} mice remain healthy throughout life despite the T cell lymphopaenia.

As discussed in the previous chapter, *Hnrp11*^{thu/thu} naïve T cells fail to persist in the peripheral lymphoid tissues after leaving thymus. What makes the findings in this section interesting is that *Hnrp11*^{thu/thu} mice have equal numbers of CD4⁺ 1G12⁺ T cells in the spleen and even higher percentages of these cells in the blood of TCR transgenic mice while those mutant CD4⁺ T cells not bearing clonotypic TCR decrease in both the spleen and blood.

It is thought that the peptide required to signal naïve CD4⁺ 1G12⁺ cells is limiting in the periphery, therefore the CD4⁺ 1G12⁺ cells do not homeostatically expand very efficiently in the periphery, compared to TCR tg cells of other specificities (e.g. OT-1 or DO-10-11). The ability of CD4⁺ 1G12⁺ cells to persist in the periphery of TCR or TCR:InsHel mice is somewhat of a paradox since in all other studies (e.g. analysis of *thunder* B6 mice or adoptive cell transfer studies or mixed bone marrow chimeras) *thunder* T cells consistently show an inability to persist in the circulation. One

possibility is that the *thunder* TCR Tg cells still undergo attrition in the periphery but this is masked by the high thymic output of TCR tg cells from the thymus. In both TCR and TCR:InsHEL mice the major CD4⁺ cell that leaves the thymus is the CD4⁺ 1G12⁺ cell.

Since commencing the write up of this thesis further studies in the laboratory have gone onto show that indeed the *thunder* 3A9 CD4⁺ TCR Tg cells, as well as *thunder* OT-I CD8⁺ TCR Tg cells, fail to persist in the periphery when adoptively transferred to either a RAG1^{-/-} host or even a lymphoreplete C57BL/6 host (Chan, Yabas and Hoyne unpublished). Similar to the studies presented in Chapter 5 the *thunder* CD4⁺ 3A9 TCR Tg cells and CD8⁺ OT-1 T cells can compete effectively with wild-type 3A9 TCR Tg or OT-I TCR Tg cells during the homeostatic proliferation phase in RAG1^{-/-} recipients, but once the T cell compartment is filled the *thunder* TCR tg cells are rapidly lost from the peripheral circulation.

Therefore the attrition of the 3A9 TCR tg cells in the peripheral circulation of the *thunder* TCR and TCR:InsHEL mice could be masked by the constant export of mature CD4⁺ 1G12⁺ cells into the peripheral circulation. An alternative possibility that is not mutually exclusive is that the *thunder* TCR Tg cells may homeostatically expand in the periphery due to the loss of the clonotype negative T cell population and this helps to fill the peripheral T cell compartment.

Hnrp11^{thu/thu} TCR transgenic CD4⁺ T cells maintain a naïve phenotype of CD44^{lo} as the wild-type cells do. Lack of specific peptide helps to keep the cells in a naïve state. We could show that in the same animal the *Hnrp11*^{thu/thu} TCR transgenic T cells

remained in a naïve state whereas the mutant non-transgenic (i.e. CD4⁺ 1G12⁻) T cells showed a tendency to transit to the memory phenotype. The only difference between these two populations in the mice is the expression of the transgenic TCR which have different avidity to environmental peptides. This implies that some changes in TCR signalling may contribute to the loss of naïve T cells in the intact mouse.

IL-7R expression on T cells is regulated by the binding of IL-7 to its receptor and TCR signalling where both types of signals lead to a decrease in IL-7R expression at both the mRNA and protein level (Mazzucchelli, R. & Durum, S.K. 2007). Lymphocyte survival and death is controlled by multiple factors and through different pathways (Khaled, A.R. & Durum, S.K. 2002; Marrack, P. & Kappler, J. 2004). The expression of IL-7R α is decrease on both CD4⁺ 1G12⁺ and CD4⁺ 1G12⁻ cells in *thunder* TCR tg mice. In the *thunder* TCR:InsHEL mice only the CD4⁺ 1G12⁻ cells showed lower IL-7R α expression. The lower IL-7R α expression on *thunder* T cells observed on a non transgenic and TCR Tg mice background is intriguing. One possibility for this is that *thunder* T cells are more actively engaged in consuming IL-7 to help them to survive in the periphery. Alternatively stronger TCR signalling caused by the change in CD45 isoform expression may drive down IL-7R expression. Further work is required to understand why *thunder* T cells have the lower IL-7R expression. The phenotypic changes that occur in the *thunder* strain could be caused by the change in function of a number of *Hrnpl1* target genes and it is through the combined action of these genes that will affect the survival of *thunder* T cells in the periphery.

6.4 Chapter summary

In this chapter, I have confirmed that the mutation in the *Hnrp11* gene does not affect positive or negative selection of *thunder* thymocytes in the 3A9 TCR and TCR:insHEL transgenic mouse. The TCR tg cells display lower levels of IL-7R expression but the CD4⁺ 1G12⁺ T cells appear to persist in the periphery of both TCR and TCR:InsHEL mice.

~Chapter 7~

General discussion

7.1 ENU mutagenesis: a non-biased approach to identify genes important in immune cell development, function and homeostasis.

ENU mutagenesis screens are phenotype-driven approaches designed to identify genes in a nonbiased manner that control key biological processes. The Goodnow group has pioneered the use of ENU mutagenesis screens to identify genes which influence lymphocyte development and function (Jun, J.E. et al. 2003; Miosge, L.A. et al. 2002; Papathanasiou, P. et al. 2003; Vinuesa, C.G. et al. 2005; Siggs O.M., et al. 2007). My studies of the *thunder* mouse strain have led to the discovery of a mutation in a novel gene, *Hnrpll*, which is involved in nascent mRNA alternative splicing. The biology of the *thunder* mouse strain highlights the important and nonredundant role that the *Hnrpll* gene plays in regulating T cell homeostasis. It also provides the starting point for an unprecedented insight into the role for alternative splicing in controlling the differentiation of naïve and memory T cells.

Since SR proteins and hnRNP proteins are ubiquitously expressed throughout development and in multiple tissues, this has made *in vivo* analysis of the roles of these proteins difficult to address. Mutations in SR or hnRNP proteins normally give rise to embryonic lethal phenotypes (Jumaa, H. et al. 1999; Michaud, E.J. et al. 1993; Roshon, M.J. & Ruley, H.E. 2005), therefore scientists have had to rely on conditional gene targeting to study the role of these genes *in vivo* in a cell type or tissue specific manner. However, for most SR and hnRNP proteins studies have been restricted to *in vitro* analysis on transfected cell lines expressing mutant forms of the protein of interest. Fortunately, the ENU screen gave rise to the ENU 126 pedigree carried a

point mutation in the *Hnrpll* gene. Although it disrupts the function of the hnRNP LL protein, mice carrying the mutation are viable and can breed to propagate the mutation. From the data presented in this thesis, it is not possible to determine whether the *thunder* mutation is a hypomorphic mutation that retains some essential function in other cells, or if it is a null allele.

Structural studies on the mutant hnRNP LL protein carried out in collaboration with Prof Gottfried Otting and colleagues revealed that the mutated Val136 residue lies beneath the RNA binding domain formed by the RNP1 and RNP2 motifs. The V→D mutation in *thunder* mice destabilizes the L1 loop of the RRM1 domain on which the V136 amino acid resides and the non-conservative change makes the RRM1 domain highly unstable at physiological temperatures. We went onto show that the mutant RRM1 domain can still bind to an RNA oligonucleotide consisting of the ARS sequence of exon 4 of *Ptprc* with micromolar affinity that is not dissimilar to the binding affinity of the wild type RRM1 domain of hnRNP LL. Although the NMR structure of several hnRNP proteins has been solved the studies here provide the first evidence for the importance of the conserved Val136 residue in the RRM1 domain of hnRNP LL as the amino acid is conserved across multiple species and also in other hnRNP proteins such as PTB and hnRNP L. This result shows that the *thunder* RRM1 domain unfolds at 37°C in vitro, and this could cause the protein to be degraded creating an almost null allele. To resolve the question, it will be necessary to develop antibodies for western blotting, and to produce a targeted null allele.

7.2 *Thunder* mutation affects naïve T cell longevity

The number of T cells in the peripheral circulation of mice and humans remains remarkably stable throughout life and the peripheral repertoire is controlled through a process of T cell homeostasis that represents a dynamic balance between cellular proliferation and cell death (Sprent, J. & Surh, C. 2005). After birth T cell numbers increase through homeostatic proliferation due to the initial lymphopaenic environment of newborn mice. The proliferation of the recent thymic emigrants is dependent upon TCR signals by recognition of a self peptide/MHC complexes on the surface of APCs as well as survival signals mediated through IL-7/IL-7R signalling. Once the compartment is filled T cells will become quiescent and they continue to circulate through secondary lymphoid tissues and the blood until they contact their specific ligand. T cell homeostasis can be defined by two distinct stages (i) the homeostatic proliferation phase and (ii) the persistence phase. There is a large body of evidences showing that naïve T cells require TCR/pMHC and IL-7/IL-7R signals for long term viability. Studies on the *thunder* strain highlights an additional layer of regulation in T cell homeostasis involving alternative mRNA splicing that is critical to allow naïve T cells to persist in the peripheral circulation.

Thunder mice have normal thymic development and T cell maturation but specifically lose naïve T cell in the periphery. Spleen T cells from *Hnrpl^{thu/thu}* mice proliferate normally in response to anti-CD3/CD28 stimulation *in vitro* indicating that TCR signalling in mature T cells is normal. Furthermore, *thunder* thymocytes were able to compete successfully during the homeostatic proliferation phase with wild-type T cells when mixtures of wild-type and *thunder* cells were transferred to

lymphopaenic hosts. However it was apparent that as the T cell compartment was filled the *thunder* T cells were selectively lost from the periphery. These experiments imply that the *Hnrpll^{thu/thu}* naïve T cells have a specific survival defect and cannot persist for long periods in the peripheral circulation *in vivo*.

Studies with IL-7 or IL-7R deficient mice revealed the critical role of this cytokine in thymic T-cell development (Peschon, J.J. et al. 1994; von-Freeden-Jeffry, U. et al. 1995; Maraskovsky, E. et al. 1996), and in naïve T cell proliferation and survival in the periphery (Schluns, K.S. et al. 2000; Tan, J.T. et al. 2001; Surh, C.D. & Sprent, J. 2005). In light of the importance of IL-7 signalling in naïve T cell biology, we decided to examine if there is any changes in IL-7/IL-7R signalling in *Hnrpll^{thu/thu}* T cells. Firstly we found that *Hnrpll^{thu/thu}* naïve T cells had consistently lower IL-7R expression both of the cell surface protein and mRNA levels. Using mixed irradiation bone marrow chimeras as well as T cell transfer experiments we were able to confirm that the lower IL-7R expression on *Hnrpll^{thu/thu}* T cells is a cell intrinsic phenotype, rather than a secondary change to the altruistic inhibition by circulating IL-7. More recent studies in the laboratory have shown that that *Hnrpll^{thu/thu}* T cells can bind and modulate the IL-7R from the cell surface as efficiently as wild type cells. This indicates that the *thunder* mutation does not affect the membrane proximal events of IL-7/IL-7R interactions. However *thunder* T cells are less responsive to the pro-survival effects of IL-7, suggesting that the *thunder* mutation affects naïve T cell survival somewhere downstream of the IL-7R (Yabas and Hoyne Unpublished observations).

It is intriguing that lower expression of IL-7R in *thunder* naïve T cells influences their longevity rather than homeostatic proliferation. One hypothesis is that TCR signalling is the primary driving force for T cell proliferation. This was evident from the observation in TAP2 mutant *jasmine* mice that failure in presenting MHC class I peptides leads to weaker CD8 proliferation. Another possibility is that *thunder* mature thymocytes have normal IL-7R expression as the fact so that the recent thymic emigrants have normal response to IL-7.

Besides cytokine signals, naïve T cells require a second signal from continuous contact with self-peptide/MHC complexes for the long term survival *in vivo* (Surh, C.D. and Sprent, J. 2005; Dorfman, J.R. and Germain, R.N. 2002). We confirmed the requirement of peptide/MHC complex for the naïve T cell homeostatic proliferation as we showed that disruption of class I peptide presentation by the mutation in TAP2 lead to weaker CD8⁺ T cell expansion, although it was not completely abolished. This is probably because that the TAP2 *jasmine* mutation is not a null allele and there may be residual expression of a low amount of Class I MHC proteins in the periphery of the *jasmine* mice. In addition the CD8⁺ T cells could expand in the *jasmine* hosts in response to IL-7 signalling and the expansion would be independent of TCR signalling (Surh, C. & Sprent, J. 2005).

IL-7 signalling leads to activation of the serine/threonine kinase AKT and this in turn phosphorylates the Foxo3a transcription factor targeting it for degradation. Withdrawal of IL-7 in wild-type cells leads to decreased activity of AKT and the failure to phosphorylate foxo3a which translocates to the nucleus where it promotes expression of a range of target genes including Bim, FasL, Trail and Bcl-6 (Bosque, A.

et al. 2005; Sabbagh, L. et al. 2006; Wojciechowski, S. et al. 2007; Riou, C. et al. 2007; Weant, A.E. et al. 2008; Williams, M.A. et al. 2008; Ichii, H. et al. 2007). Down regulated Bcl-2 expression in IL-7 deprived *thunder* naïve T cells would from one arm lead to a shift in the balance of anti-apoptotic Bcl-2 vs pro-apoptotic Bim to a situation that would favour apoptosis.

From another aspect we have preliminary results from all exon arrays showing that the *Hnrp11*^{thu/thu} mutation leads to a change in the alternative splicing of Bim favouring expression of the pro-apoptotic BimS isoform. BimS is the smallest isoform generated by alternative splicing and, skipping of exons 3 and 4 which encode the Dynein-binding domain. Absence of the Dynein-binding domain results in failure to bind Dynein-light-chain (DLC) proteins and stronger apoptotic activity of BimS (O'Connor, L. et al. 1998; Bouillet, P. et al. 2002). Taken together, the reduced Bcl-2 expression and constitutive expression of the alternatively spliced pro-apoptotic BimS isoform may be important factors that contribute to decreased survival of *Hnrp11*^{thu/thu} naïve T cells.

However, Bcl-2 and Bim are not the only genes that modulate apoptosis in *Hnrp11*^{thu/thu} T cells. Preliminary data from exon arrays showed that the alternative splicing of a number of genes involved in regulation of apoptosis are affected in *Hnrp11*^{thu/thu} naïve T cells including, *Itsn1*, *Lrdd*, *Apaf1*, *Nalp1* and *Madd*. The expression of the Fas receptor is increased on the surface of *Hnrp11*^{thu/thu} naïve T cells too. Therefore the loss of naïve T cells in the *Hnrp11*^{thu/thu} mice may not be the result of a single gene defect. Instead the loss of naïve T cells could be due to the combined

effects of multiple genes and it will take a lot more time to decipher the exact mechanism(s) responsible for the loss of naïve T cells in *thunder* mice.

7.3 CD45 expression, alternative splicing, and TCR signalling

CD45 is the prototypic membrane bound protein tyrosine phosphatase (PTP). It is the most abundant PTP in T cells covering 10% of total cell surface area (Hermiston, M.L. Xu, Z. Weiss, A. 2003). A recent study suggests that as little as 3% of the total CD45 is required for T cell development and function *in vivo* (McNeill, L. et al. 2007). CD45 undergoes alternative splicing to silence the expression of three variable exons in T cells upon activation (Lynch, K.W. 2004). It has not been fully appreciated why there is so much CD45 on T cells, and what roles the various CD45 spliced isoforms have on T cell function.

Activation of Src family kinases in particular p56Lck is the primary event initiated by CD45 during TCR signal transduction. CD45 can dephosphorylate two key tyrosine residues pY394 and pY505 of p56Lck tyrosine kinase, suggesting a dual role of CD45 and complex regulation of Lck activity (Ostergaard, H.L. et al. 1989; Koretzky, G.A. et al. 1990; Ashwell and D'Oro, 1999; Palacios and Weiss, 2004). In CD45 deficient animals, the Y505 and Y394 residues of Lck remains highly phosphorylated and Lck is functionally inactive, resulting in a severe defective T cell development in the thymus (Kishihara, K. et al. 1993; Wallace, V.A. et al. 1997; Mee, P.J. et al. 1999; Baker, M. et al. 2000).

We have used a unique mouse strain that was identified from an ENU mutagenesis screen called *lochy* characterized by markedly reduced expression of CD45 on lymphocytes. The *lochy* strain was used to investigate the link between CD45 splicing isoforms and TCR signalling with different amounts of CD45 being expressed on the T cell surface. We found no difference in the phosphorylation of pY505 and pY394 of Lck in the C57BL/6 wild-type and *Hnrpll*^{thu/thu} T cells when the normal level of CD45 is expressed. However, it was markedly increased when total CD45 levels are reduced to just 5% in the *lochy* mice. Homozygosity for the *thunder* mutation on this background of limiting CD45 in *Ptprc*^{loc/loc}, *Hnrpll*^{thu/thu} mice decreased the phosphorylation of Y505 and Y394 residues. This result is considered with published data (Chui, D. et al. 1994; Dornan, S. et al. 2002; Xu, Z. & Weiss, A. 2002) that the CD45RABC isoforms are more catalytically active in T cells than the lower molecular weight CD45 forms. It was recently reported that expression of CD45RO, RB, and RABC at intermediate levels (10-40% of wild-type) had no measurable differences as assessed by T cell development or TCR signalling (Salmond, RJ. et al. 2008). This is an agreement with our own observations that no functional difference between CD45RO and CD45RABC when total CD45 levels are normal. Since there does appear to be different in CD45 isoform activity under limiting conditions, perhaps these differences only become relevant in parts of the TCR synapse when CD45 is mostly excluded.

Despite the difference in Lck phosphorylation between *loc/loc* and *loc/loc thu/thu* T cells, the number of mature single positive thymocytes was not detectably altered between *lochy* and *lochy thunder* mice. The fact that positive selection continues, although inefficiently, despite the drastically reduced level of CD45 expression is

consistent with the previous studies by McNiel et al who showed that as little as 3% of the normal CD45 levels on T cells is sufficient for some positive selection to occur (McNiel et al 2007). In the context of TCR synapse that may form during positive selection, changing the CD45 isoforms does not appear to have functional consequences.

Ptp^{loc/loc} mice had normal numbers of memory CD4⁺ T cells and even slightly more CD8⁺ memory T cell in the spleen compared with *Ptp^{+/+}* mice, but the naïve CD4⁺ and CD8⁺ T cells are reduced. The numbers of naïve CD4⁺ T cells in *Ptp^{loc/loc} Hnrpl^{thu/thu}* mice are further decreased, suggesting that factors other than CD45 alternative splicing must account for the loss of naïve T cells in *Hnrpl^{thu/thu}* and *Ptp^{loc/loc} Hnrpl^{thu/thu}* mice. However, since some CD45 remains on the double mutant T cells, and is altered in isoforms and Lck phosphorylation, it remains possible that the *thunder* mutation acts on T cell survival through CD45. Future experiments in *thu/thu* CD45^{null} mice will be needed to resolve this issue.

Trans-acting factors involved in CD45 alternative splicing

Although it has been known for more than 30 years that *Ptp^{rc}* undergoes alternative splicing, knowledge of the trans-acting regulatory factors that control the regulated splicing of the 3 variable exons of *Ptp^{rc}* remains obscure. I have shown that loss of hnRNP LL function in T cells results in failure to silence CD45 exons 4, 5, and 6, which can be detected at the exon level on microarrays, amplified transcripts (RT-PCR), and protein level (monoclonal antibody staining). The closely related hnRNP L protein was recently identified as one of three hnRNP proteins that bound to the ESS1

sequence within the variable exons of the human PTPRC gene (Rothrock, C. et al. 2003; Rothrock, C. et al. 2005; Tong, A. et al. 2005; House, A.E. & Lynch, K.W. 2006; Melton, A.A. et al. 2007). Due to the high degree of conservation between the *Hnrpl* and *Hnrpll* proteins, especially within the RRM1 domain, it is conceivable in the previous studies that implicated roles of hnRNP L in CD45 alternative splicing are actually performed by hnRNP LL rather than hnRNP L binding to the ESS to induce exon skipping of CD45. In *Hnrpll^{thu/thu}* mice, hnRNP L exhibits normal mRNA expression yet it does not compensate the effects of *Hnrpll^{thu/thu}* mutation in CD45 splicing in T cells. This suggests hnRNP LL has an essential and nonredundant role in regulation of CD45 alternative splicing in T cells in mice. It is equally plausible that hnRNP L and hnRNP LL act in concert to regulate *Ptprc* splicing. However, hnRNP LL undergoes developmental- and activation-induced changes in mRNA expression that parallel changes in *Ptprc* splicing. Thus it is certain that hnRNP LL is the regulated component of the process. Future work will need to examine mice or cells where hnRNP L has been mutated or knock out.

7.4 Interplay of TCR signal and IL-7 signal pathways?

TCR signals and IL-7 signals are essential for normal T cell development and differentiation. Different strengths and duration of these signals direct T cells to different fates. This is exemplified in thymocytes high avidly binding to self-peptide/MHC causing strong TCR signals and apoptosis; no binding to self-peptide/MHC causes death by neglect; while intermediate binding and meek TCR signals leads to positive selection down the appropriate CD4/CD8 lineage, survival and differentiation of mature T cells (Love, P.E. & Chan, A.C. 2003).

Circulating naive T cells emerging from thymic positive and negative selection have low affinity for self-peptides. The potency of TCR signal transduction plays a role in the naive T cell differentiation in the periphery with a resting threshold determined for activation. The intensity of TCR signals is determined by both TCR affinity and co-receptor expression. As the level of co-receptor expression helps to set the level of the TCR signalling threshold, high co-receptor expression increases signalling by low affinity TCRs, whereas low co-receptor expression limits signalling by high avidity TCRs.

IL-7 is a limiting factor for T cell development and persistence secreted by stromal cells in a constant rate. Serum IL-7 concentration is determined by the rate of its consumption from T cells. IL-7R expressed on T cells not only determines the efficiency of IL-7 consumption but also the strength of the responses of T cells to IL-7 signal. IL-7R expression is correlated to the development of T cells. It is also

regulated on mature T cells by extrinsic stimuli such as antigens and common γ chain cytokines including IL-7. IL-7 down-regulates its receptor expression on exposed T cells enabling other T cells to acquire IL-7 (Mazzucchelli, R. & Durum, S.K. 2007).

It is interesting TCR stimulation in peripheral T cells down-regulates IL-7R α expression. Lefrancois and colleagues found down-regulated IL-7R expression in OT-1 transgenic CD8⁺ T cells after activation when investigating roles of IL-7 in T cell homeostatic proliferation and survival (Shluns, K.S. et al. 2000). O'Shea's group also reported that TCR-mediated activation up-regulates IL-2R expression whereas down-regulates IL-7R expression (Franchimont, D. et al. 2002). These results indicated that there is potentially interplay between TCR signalling and IL-7 signalling pathways. A recent study from Singer and colleagues identified that the level of CD8 co-receptor expression reflecting TCR signalling was determined by the level of IL-7 and other common γ chain cytokines in the periphery (Park, J.H. et al. 2007). They showed that the capacity of CD8⁺ T cells to transduce common γ chain cytokine signals is impaired by TCR signals induced by self antigens. They also observed an inverse relationship between CD8 co-receptor expression and IL-7R signalling.

We observed down-regulated IL-7R expression in *Hnrp11*^{th^u/th^u} naive T cells, as well as lower TCR:CD3 complex, lower co-receptor expression, and higher CD5 expression on both CD4⁺ and CD8⁺ T cells. Collectively these phenotypic changes may reflect increased TCR signalling and cause decreased IL-7 signalling in *Hnrp11*^{th^u/th^u} naive T cells under resting conditions. Although there is no obvious difference in Lck phosphorylation in normal CD45 background, it is mostly possible

that *HnrpII*^{th^u/th^u} mutation may affects some other molecules in TCR signal pathway that strengthen TCR signalling and lead to hyper-activation status of resting T cells.

This hypothesis also applies to the 3A9 TCR transgenic mice. The transgenic TCR has strong affinity to HEL-46-61 peptide. However it has apparently very weak avidity to endogenous peptide/MHC present in the peripheral lymph nodes and spleen, because the 3A9 cell die about 10 days after thymic emigration and do not undergo homeostatic proliferation in lymphopaenic hosts despite the presence of positive selecting I-A^k. *HnrpII*^{th^u/th^u} T cells bearing the same TCR still showed lower IL-7R expression. This result suggests that stronger signalling by the TCR does not account for decreased IL-7R on *thunder* T cells.

7.5 Preliminary evidence that *Hnrpll* is a global regulator of pre-mRNA alternative splicing in T cells

I have shown that *Ptprc* exon silencing is disrupted by the *thunder* mutation, but a key question that follows is that whether or not this is the only target of hnRNP LL. Recently, a new generation of Affymetrix Arrays have been developed with sets of probes for each annotated exon in the mouse genome. Hybridizing labelled cRNA from *thunder* vs wild-type T cells to these arrays would provide a powerful way to address this question. While writing up this thesis, I have initiated the all exon array experiment in collaboration with Professor Alan Aderem's group in the Institute for System Biology in Seattle of the United States.

From our preliminary analysis of this exon array data, generated from naïve and memory T cells and CD4SP and CD8SP thymocytes isolated from *thu/thu* vs *+/+* mice, hnRNP LL regulates splicing of many T cell mRNAs (Fig. 7.1a and b). While this work is organising, some of the preliminary results are mentioned here.

In addition to *Ptprc*/CD45 (Figure 7.2.1a, b, and c), other important molecules of the TCR signalling pathway also appear to be alternatively spliced by hnRNP LL and are differentially spliced in memory T cells, hence are likely to modulate their responsiveness to antigen. These include other transmembrane protein tyrosine phosphatases, *Ptprrt*, and *Ptprrk*, the CD3-zeta subunit (*Cd247*) (Tsuzaka, K. et al. 2006), phospholipase c gamma 1 (*Plcg1*) (Patterson, R.L. et al. 2005), downstream of kinase-2 (*Dok2*) (Dong, S. et al. 2006), and Abelson tyrosine kinase (*Abl1*) (Wong, W.

Figure 7.1a All exon array experiment design:

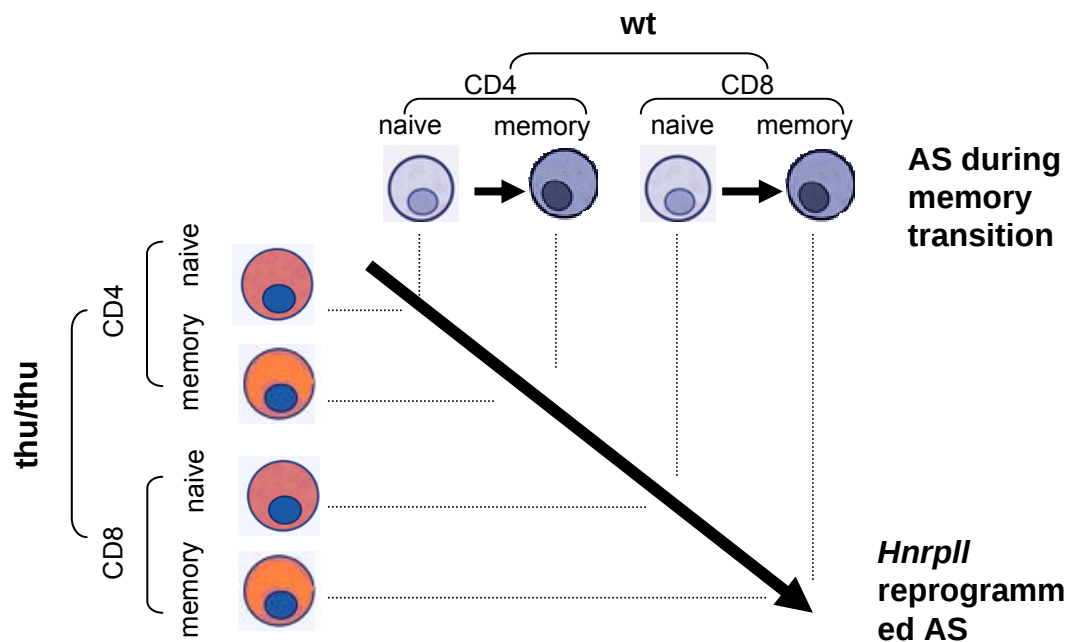
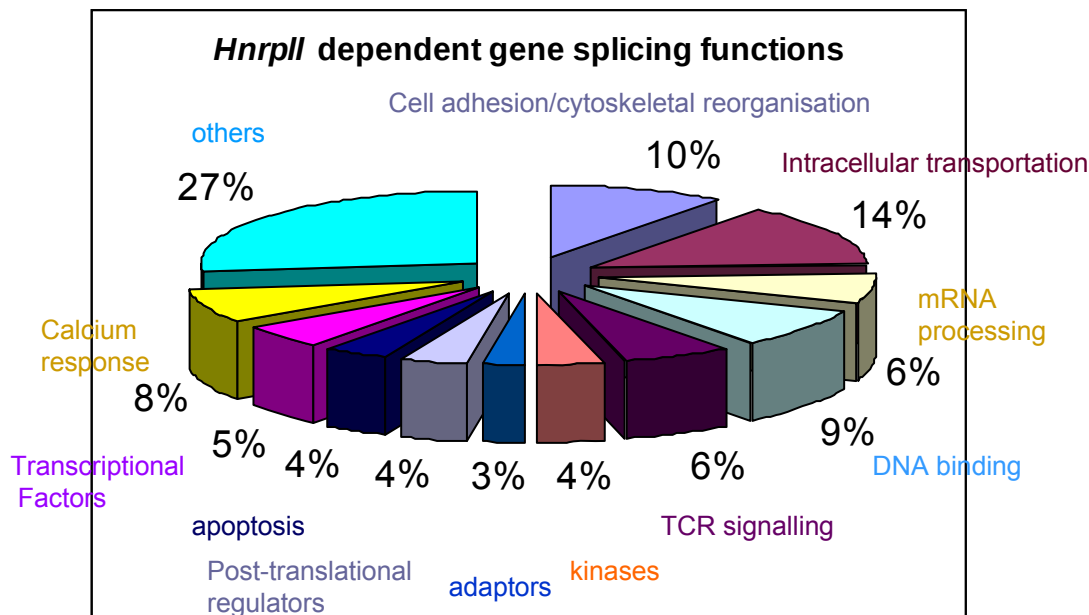


Figure 7.1b Gene functional families alternatively spliced by *Hnrp11*



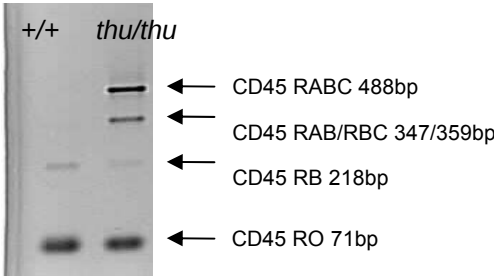
& Witte, O.N. 2004), and the transcription factor *Nfatc1* (Figure 7.2.3a and b). *Hnrpll* promotes differential promoter usage in the *Nfatc1* gene. This could have important functional consequences in T cells because there is evidence that the long isoforms of NFATc1/ β B or β C can promote apoptosis whereas the shorter isoform NFATc1/ α A produced from the inducible P1 promoter plays an important role in gene transcription and T cell differentiation (Serfling, E. et al. 2007).

With respect to T cell responsiveness upon re-infection, one of the top scoring *Hnrpll*-regulated genes in the array analysis is *Nalp1* (Figure 7.2.1d, e, and f). This encodes a cytoplasmic receptor for bacterial cell wall products that signals interleukin-1 production, inflammation, and apoptosis in innate immune cells (Church, L.D. et al. 2008; Tschopp, J. et al. 2003), but has not previously been linked to T cell function or adaptive immunological memory.

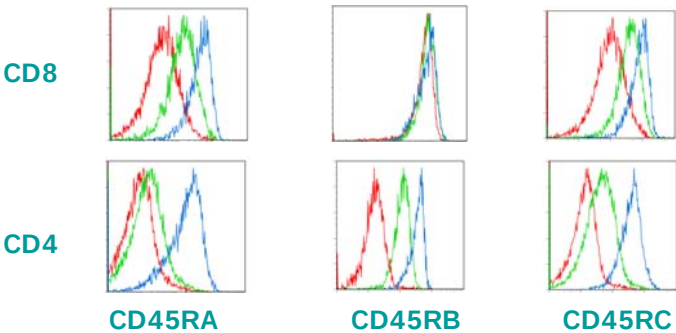
Many essential genes controlling T cell responsiveness and longevity are among the hnRNP LL regulated gene set. These include some core regulators of apoptosis, Apoptotic Protease Activating Factor-1 (*Apaf1*) (Fig. 7.2.2f) (Cain, K. Bratton, S.B. & Cohen, G.M. 2002), Cytochrome-c (*Cyc1*) (Orrenius, S. Gogvadze, V. & Zhivotovsky, B. 2007), survival motor neuron (*Smn1*) (Sumner, C.J. 2007), and Bim (*Bcl2l11*) (Figure 7.2.2a, b, and c) (Youle, R.J. & Strasser, A. 2007). The pro-apoptotic function of Bim is known to be modulated by altered splicing (O'Conner, L. et al. 1998). Splicing of these apoptotic molecules may potentially enhance cell apoptosis and contribute to the loss of naive T cells in *thunder* mice.

Figure 7.2.1 *HnrpII* induces exon silencing

a. CD45 transcripts



b. CD45 isoform expression



c. Exon level detection

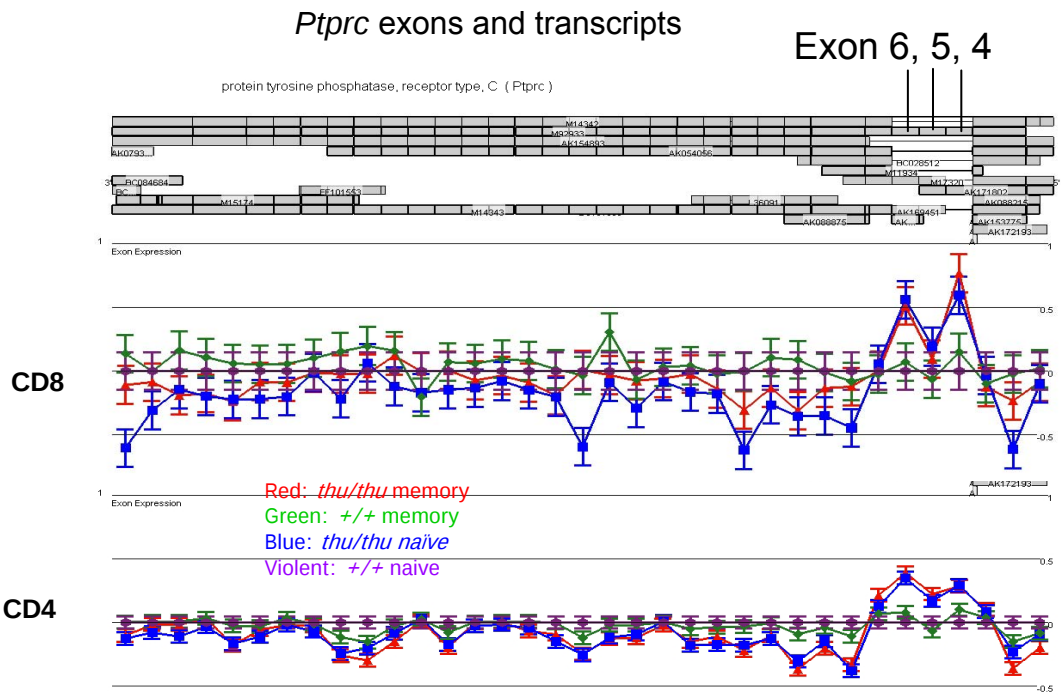
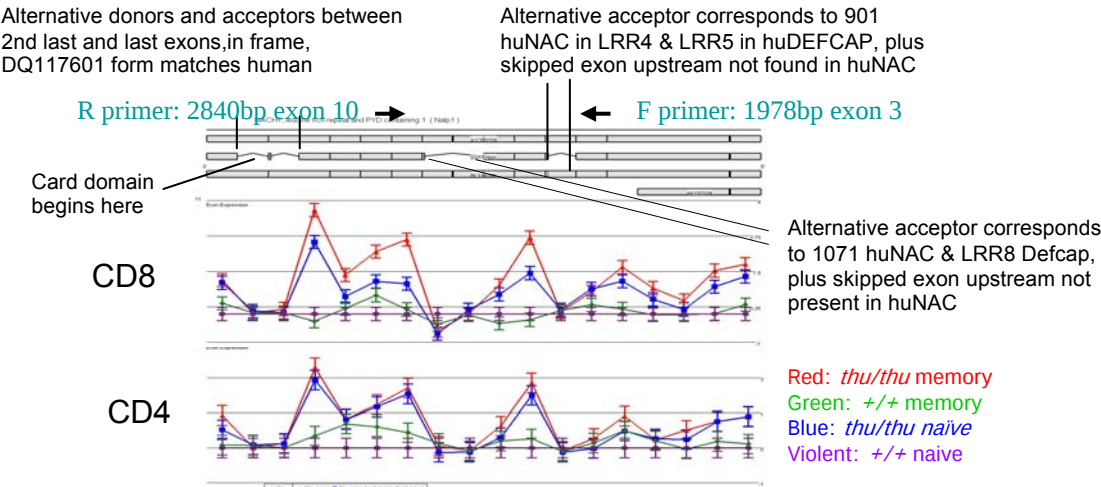


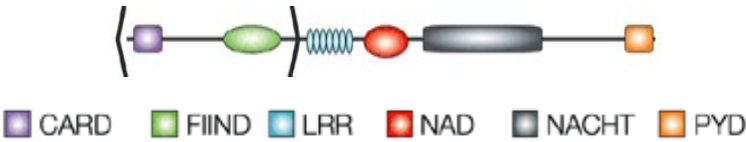
Fig. 7.2.1a. RT-PCR showing amplified CD45 isoforms from thymic cDNA.
b. CD45 isoforms detected by epitope specific antibodies.
c. Exon level detection of *Ptpsrc* transcripts. Dots represent average exon specific probeset intensity from 4 samples.

Figure 7.2.1 Hnrpl induces exon silencing

d. Nalp1 transcripts and exons



e. Schematic Nalp1 protein (modified from Tschopp, J. et al. 2003):



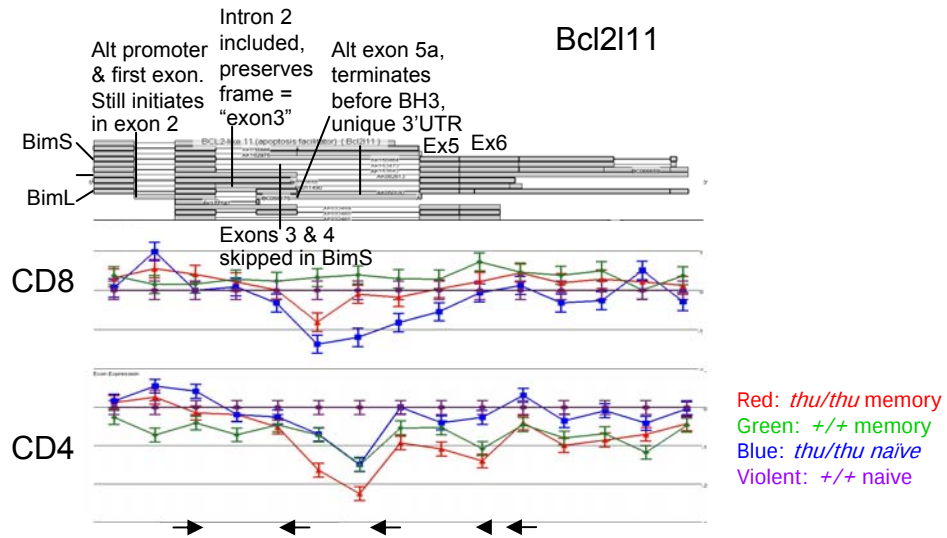
f. RT-PCR: Nalp1 isoforms



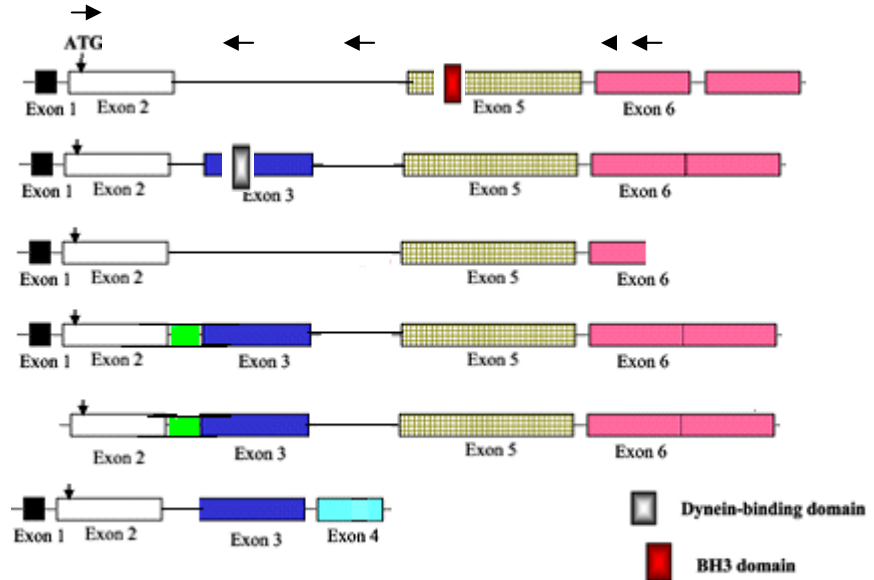
Fig. 7.2.1d. . Exon level detection of Nalp1 transcripts. Dots represent average exon specific probeset intensity from 4 samples.
e. Schematic structure of Nalp1 protein.
f. RT-PCR showing *Nalp1* transcripts in the thymus of wildtype and *thu/thu* mice.

Figure 7.2.2 *Hnrp1l* induces exon inclusion

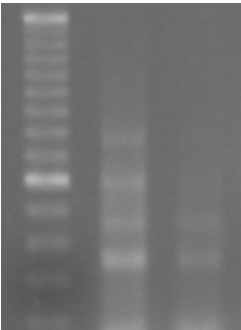
a. Bim transcripts and exons



b. Mouse transcripts of Bim



c. RT-PCR validation

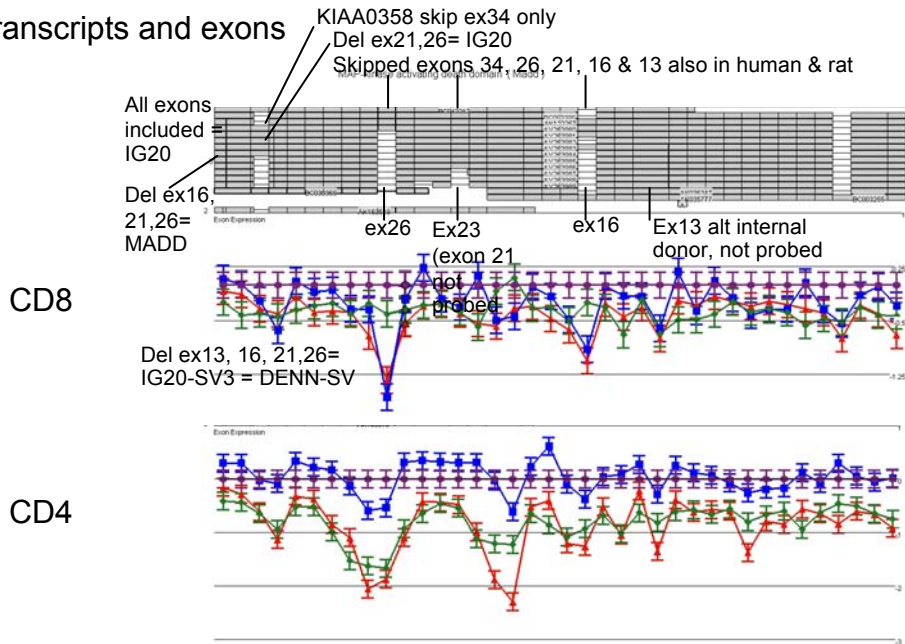


F primer: 232bp exon 2;
R1 primer: 706bp exon 6 after transcript terminus
R2 primer: 386bp start of exon 3;
R3 primer: 486bp start of exon 6;
R4 primer: 530bp middle exon 4;

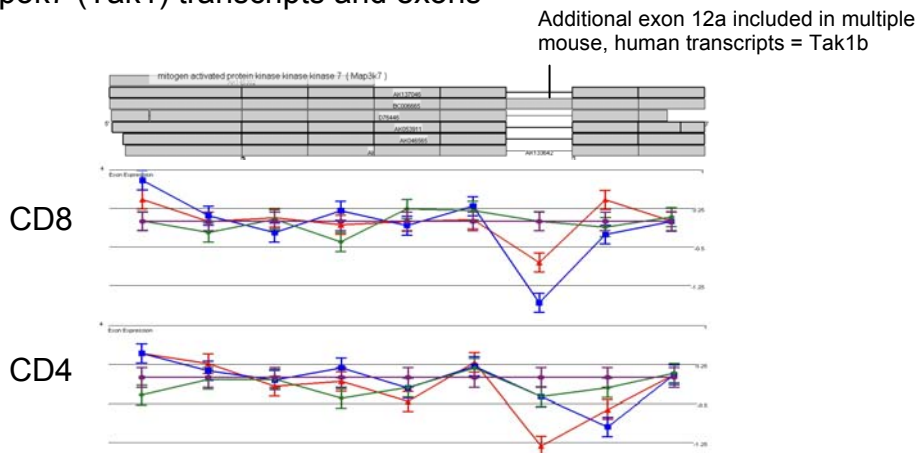
- a. Transcript views of Bim from Exon array.
- b. Schematic transcript variants of Bim.
- c. RT-PCR validation of Bim splicing. (Arrows indicate location of the primers for amplification.)

Figure 7.2.2 *Hnrp1l* promotes exon inclusion

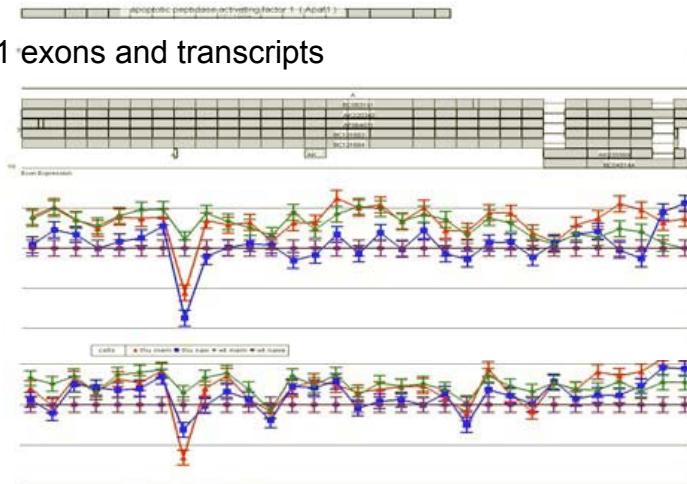
d. Madd transcripts and exons



e. Map3k7 (Tak1) transcripts and exons



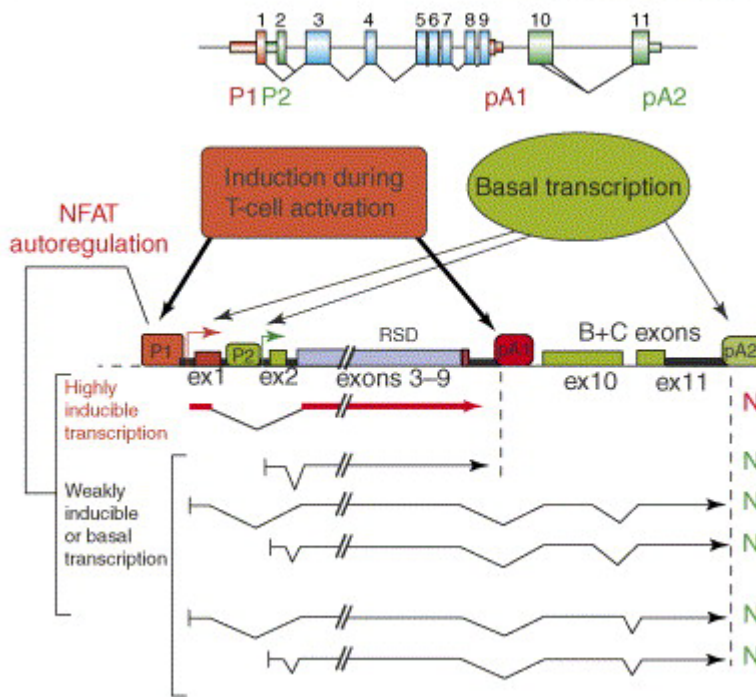
f. Apaf1 exons and transcripts



d. Transcript views of Madd.
e. Transcript views of Map3k7 (Tak1).
f. Transcript views of Apaf1.

Figure 7.2.3 *HnrpII* modifies differential promoter usage

a. Schematic *Nfatc1* transcripts:



Schematic *nfatc1* gene and transcript variants.

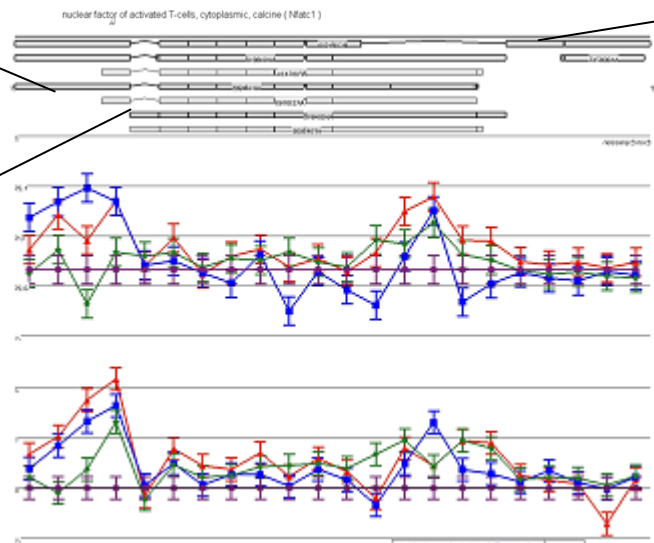
Red indicates NFATc1/αA, green represents βB or βC isoforms. In resting (or naive) T cells, P2 activity results in the transcription of exon 2 and generation of three β isoforms. T-cell activation causes a promoter switch from P2 to P1, the transcription of exon 1 and generation of αA isoform.

Serfling, E. et al. 2006

b. NFATc transcripts and exons

P1 autoinducible promoter - NFATc1-A and C

P2 Alternative start, constitutive promoter - NFATc1-B



CD8

CD4

Transcript views from exon array showing autoinducible P1 and *Nfatc1*/αA isoform in *thu/thu* naïve T cells

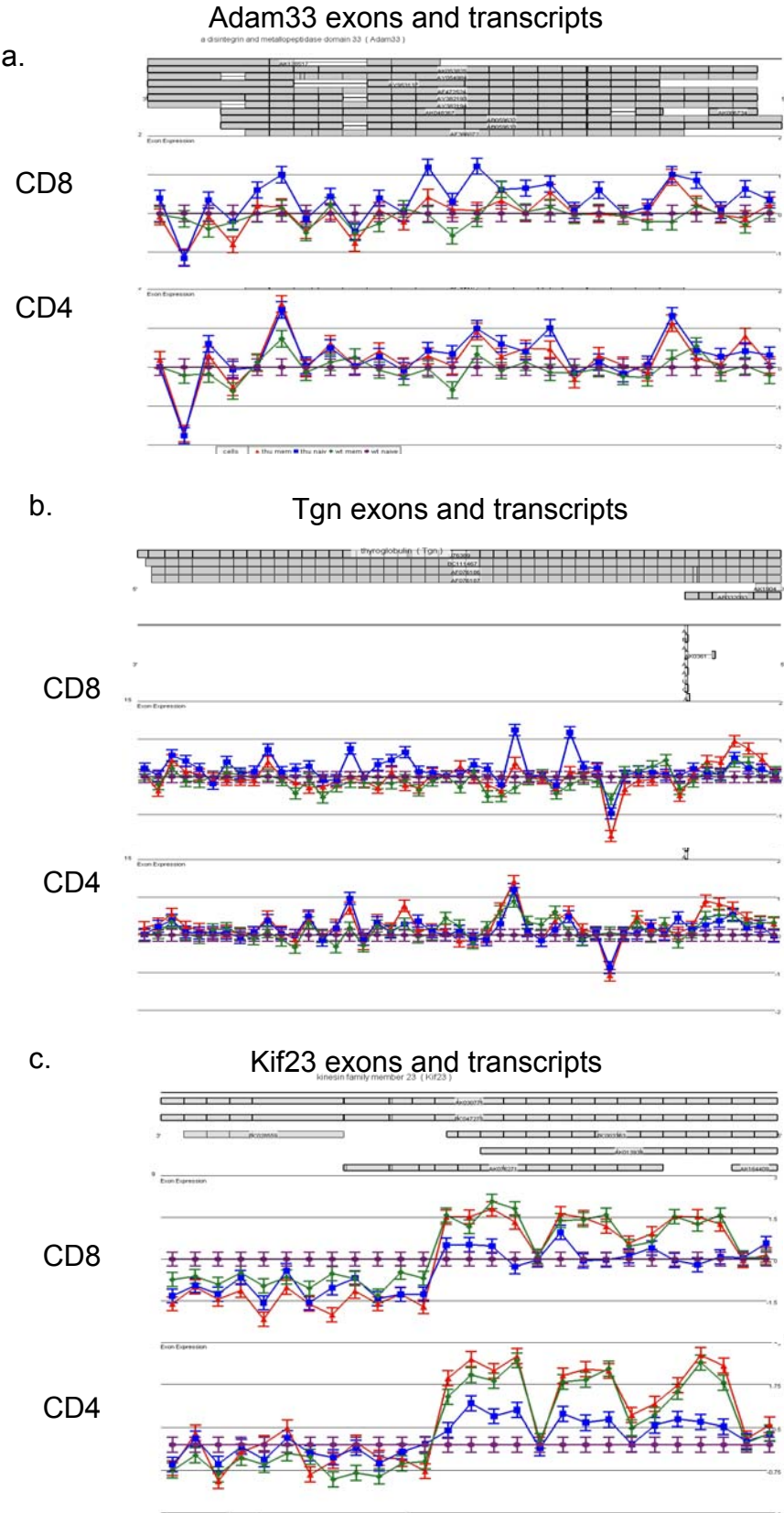
The change in hnRNP LL function caused by the *thunder* mutation is likely to have additional downstream effects on the program of alternative splicing since the *thunder* mutation caused alteration in splicing of a number of splicing regulators including *Smn1*, *Sfrs11*, *Cpsf4*, *Prpf4b*, *Ccnl2* and *Thrap3*,

Differential expression of adhesion molecules is one of the cell surface features of memory T cells representing homing capabilities. Several cell adhesion molecules were alternatively spliced in memory T cells as well as in *Hnrpll^{thu/thu}* naïve T cells. *Itga9* (Integrin alpha 9) was recently reported to enhance granulocyte colony-stimulate factor receptor (CSF-R) signalling (Chen, C. et al. 2006), surprisingly both *Itga9* and *Csf1r* (Colony stimulating factor 1 receptor) are alternatively spliced by hnRNP LL. This spliced Integrin-CSF signalling pathway may play some roles in memory T cell formation. *Stab2* (Stabilin-2) has been shown involving in lymphocyte adhesion to hepatic sinusoidal endothelium (Jung, M.Y. et al. 2007). *Stab2* has a striking spliced isoform expression in memory T cells comparing to their naïve counterparts (Fig. 7.2.5). The roles of *Stab2* splicing in memory T cells remain to be elucidated.

CD46 was initially characterised as a complement regulatory molecule on all nucleated haematopoietic cells. Recent data suggested that CD46 is also involved in T cell proliferation and differentiation (Marie, J.C. et al. 2002; Kemper, C. et al. 2003). CD46 expression of alternative spliced isoforms is in tissue-specific manner (Russell, S.M. et al. 1992). So, differential spliced CD46 in memory T cells and *Hnrpll^{thu/thu}* naïve T cells may contribute to the autonomous transition from the mutant naïve T cell to memory phenotype.

Another interesting target is *Itsn1* (Intersectin 1, SH3 domain protein 1A) (Fig. 7.2.5). Intersectin 1 (*Itsn1*) is a multidomain adaptor protein involved in endocytosis, regulation of actin polymerization, and Ras/MAPK signaling. It was recently reported that ITSN-1 in human cells function as a negative regulator of the mitochondrial pathway-dependent apoptosis secondary to activation of the Erk1/2 survival signaling pathway. (Predescu, S.A. et al. 2007). *Itsn1* ubiquitously expresses a short transcript *Itsn1-s* in mammals comprising two Esp15 homology (EH) domains and five Src-homology 3 (SH3) domains, whereas the long form *Itsn1-l* has neuron-specific expression with a Dbl-homology (DH) or RhoGEF, pleckstrin homology (PH), and putative calcium-interaction domains in the C terminus (Tsyba, L. et al. 2004, 2008). There is currently nothing known about the role of *Itsn1* in T cells and it remains unclear how changes in *Itsn1* alternative splicing affects naive and memory T cells.

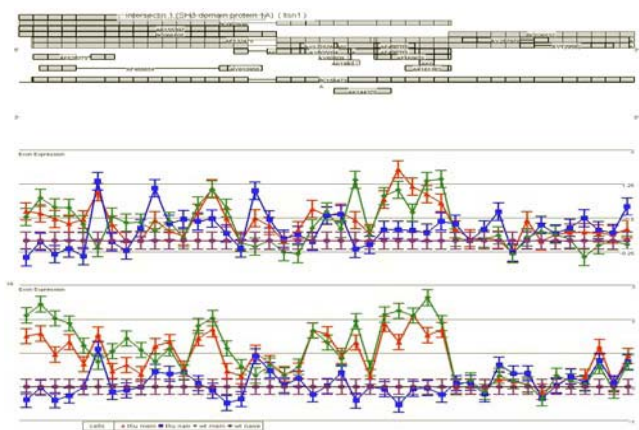
Figure 7.2.4 *Hnrp1l* causes some complex splice patterns



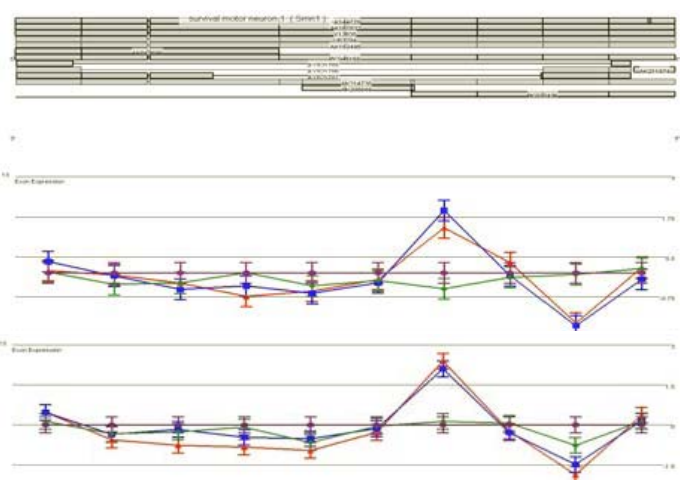
a. b. and c. Complex patterns of alternative splicing in Adam33, Tgn, and Kif23 transcripts.

Figure 7.2.5 Some other *HnrpII* spliced genes of interest

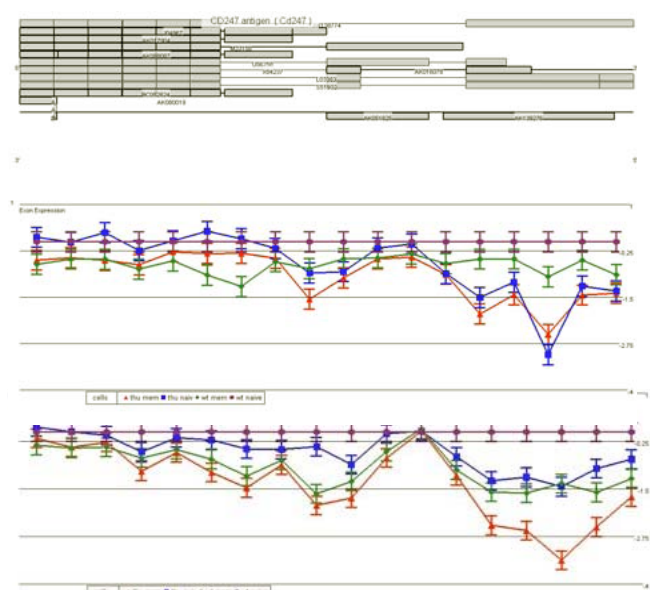
Itsn1 exons and transcripts



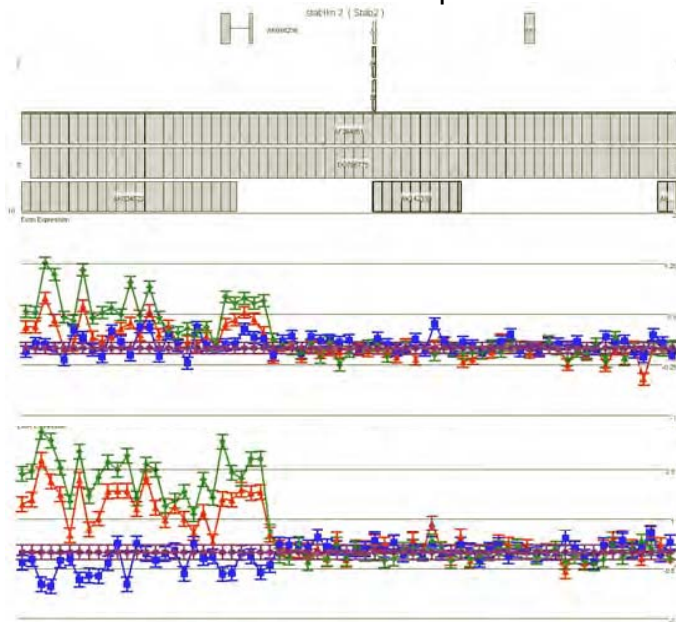
Smn exons and transcripts



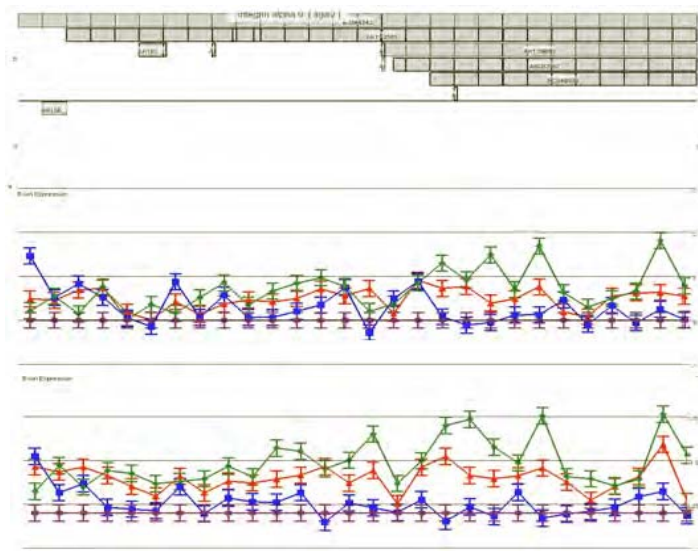
CD247 exons and transcripts



Stab2 exons and transcripts



Itga9 exons and transcripts



Concluding Remarks

Transcriptional regulation of gene expression during the course of immune reaction and T cell transition from naïve to effector/memory subsets has been well studied, but post-transcriptional modifications like alternative splicing have not been examined except for a few genes like CD45, CD44, and IL-7R α in man.

I found that there is higher expression of hnRNP LL in T cells than in B cells, and higher expression of *Hnrpll* in memory T cells compared to naïve T cells. Since the heterozygous *Hnrpll* mutant had a striking effect on CD45 isoforms, this implies that induction of *Hnrpll* coordinates a program of alternative splicing as naïve T cells differentiate to the memory cell fate. The use of the Affymatrix exon array technology has begun to reveal an unexpected diversity of alternative splicing that occurs between naïve and memory T cells. These data revealed that the change in widely used CD45 splicing represents just the tip of an iceberg and that hundreds of other genes undergo alternative splicing between the naïve and memory T cells and many are controlled by hnRNP LL. The exploration for how this mRNA diversity contributes to adaptive immunity and T cell memory will be a challenging but fascinating problem for future research. Although many differentially spliced genes in *Hnrpll*^{th^u/th^u} naïve / memory T cells were detected by exon arrays I have yet to confirm them by other methods such as RT-PCR or quantitative real time PCR.

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