

UNRESTRICTED  
The role of the tyrosine phosphatase

CD45 in B and T cell development

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A thesis presented in partial requirement of the Open  
University for the degree of Doctor of Philosophy

November 1998

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*Jennifer and Daniel*

# Abstract

For many years the CD45 transmembrane tyrosine phosphatase has been known to be an important molecule in both B and T lymphocyte receptor signalling events. To understand the role of this molecule in lymphocyte ontogeny and function I have created mice which are homozygous for a targeted mutation of exon 12 of the *Cd45* gene. These mice have B cells that are unusual in that they appear immature on the basis of their expression of IgM and IgD receptors and yet have increased expression of other markers that are associated with increasing B cell maturation. In addition, CD45-deficient B cells have increased expression of B7.2, suggesting that these cells have been activated. Following BCR stimulation *in vitro* CD45-deficient B cells have a diminished proliferation response. B-1 cell populations have been found to be greatly reduced in these mice suggesting that CD45 is also a positive regulator of B-1 receptor signalling. The use of the 3-83 transgenic BCR demonstrates that 3-83/*Cd45*<sup>-/-</sup> B cells can be produced however these cells express altered levels of IgM and IgD. *Cd45*<sup>-/-</sup> B cells are deleted normally in the presence of the negatively selecting ligand for the transgenic receptor and undergo both Ig heavy and light chain rearrangements normally suggesting that CD45 is not required for these processes. Finally *Cd45*<sup>-/-</sup> B cells have a diminished but not absent Ca<sup>2+</sup> flux on receptor stimulation that could explain the abolition of some receptor mediated responses.

*Cd45*<sup>-/-</sup> mice have a severe deficiency in T cell development. Firstly there is a developmental block at the DN3 stage of thymocyte development, suggesting that CD45 plays a role in signalling from the pre-TCR. A second developmental block is at the DP stage in thymocyte developmental which is associated with the positive and negative selection events of T cell selection. Some *Cd45*<sup>-/-</sup> T cells are able to migrate to the periphery albeit in greatly reduced numbers. These cells are unusual in that they have an activated phenotype (CD44<sup>hi</sup>/L-selectin<sup>lo</sup>). To determine the role of CD45 during positive selection of thymocytes both class I and class II transgenic

TCR receptors were used. *Cd45*<sup>-/-</sup> mice were found to display a profound block in the development of mature T cells expressing the transgenic TCRs suggesting an important role for CD45 in the positive selection of these receptors. Moreover, this deficiency appears to be due to altered thresholds of TCR signalling as assessed using bi-specific antibodies to induce positive selection events in a dose dependent manner. The use of a transgenic TCR to investigate T cell negative selection events demonstrates that *Cd45*<sup>-/-</sup> thymocytes can undergo negative selection events induced by the cognate ligand. However, negative selection induced by endogenous (*Mtv*) and exogenous (SEB) superantigens are deficient in *Cd45*<sup>-/-</sup> thymocytes. In addition *Cd45*<sup>-/-</sup> T cells are found to have a profound block in their ability to mobilise Ca<sup>2+</sup> in response to antigen receptor stimulation.

# Acknowledgements

I would like to thank Dr. V. L. J Tybulewicz for advice, support and drive. Thanks to all members of the group past and present for friendship and laughs. I am particularly grateful to Dr. Martin Turner for helping to create the *Cd45<sup>-/-</sup>* mice and for much help and discussion during the project. I am also indebted to Chris Atkins for long hours over the FACS machine and Marian, Lesley, Alice and Francis for technical assistance. Thanks to Tom and Claire for providing excellent temporary accommodation during the writing of this thesis. Finally thanks to Dr. E. Schweighoffer, Dr. M. Walmsley and Dr. V. L. J Tybulewicz for proof reading.

I conducted all experiments within this thesis except those using neonatal thymic organ cultures which were conducted by Dr. A. Basson. I also acknowledge Dr. R. Zamoyska for breeding of *Cd45<sup>-/-</sup>* mice to the F5 transgenic animals, Dr. V. L. J. Tybulewicz for helping to make the CD45 targeting construct and for electroporation of ES cells, Dr. P. Costello for Western blotting and mast cell culture and finally Dr. M. Turner, L. Duddy and M. Quinn for help with Southern Blotting and ES cell work.

This work is funded in part by the Leukaemia Research Fund.

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# *Abbreviations*

APC	antigen presenting cell
$\beta$ 2-m	$\beta$ 2-microglobulin
BCR	B cell receptor
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
CD40L	CD40 ligand
CD45-AP	CD45 associated protein
cpm	counts per minute
DAG	diacylglycerol
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
EGF	epidermal growth factor
EGFR	EGF receptor
ES	embryonic stem cells
FCS	foetal calf serum
FDC	follicular dendritic cell
H	heavy chain
HEL	hen egg lysozyme
hi	high
Ig	immunoglobulin
IP <sub>3</sub>	inositol -1,4,5-trisphosphate
ITAMs	immunoreceptor tyrosine-based activation motifs
ITIMs	immunoreceptor tyrosine-based inhibitory motifs
kb	kilobase
kD	kilodalton

KIRs	killer cell inhibitory receptors
L	light chain
LCMV	lymphocytic choriomeningitis virus
lo	low
LTR	long terminal repeat
mAb	monoclonal antibodies
MAP	mitogen activated protein
MHC	major histocompatibility complex
Mls	minor lymphocyte stimulating
MMTV	mouse mammary tumour viruses
NP40	Nonidet P-40
NTOCs	neonatal thymic organ cultures
OD	optical density
PI-3K	phosphatidylinositol 3-kinase
PIP <sub>3</sub>	phosphatidylinositol-3, 4, 5-trisphosphate
PKC	protein kinase C
PLC $\gamma$	phospholipase C $\gamma$
PMA	phorbol-12-myristate-13-acetate
pre-TCR	pre-T cell receptor
pT $\alpha$	pre-T $\alpha$ chain
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
RAG	recombination activating gene
rpm	revolutions per minute
S/M	selecting peptide MHC complex
SEB	<i>Staphylococcus</i> enterotoxin B
sHEL	soluble HEL
SL	surrogate light chain
SP	single positive
SSC	standard saline citrate

TCR	T cell receptor
TD	thymus dependent
TI	thymus independent
TNF	Tumour necrosis factor
Tris	tris(hydroxymethyl)-amino-methane
<i>xid</i>	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

# Chapter One

## INTRODUCTION

### 1.1 The role of the immune system

#### **Adaptive immunity**

The major function of the mammalian immune system is to protect the host from a diverse array of potentially pathogenic organisms by recognising and reacting to non-self antigens (Janeway et al., 1996). To achieve this, the immune system has developed both innate and adaptive immunological responses to pathogens to aid their elimination.

The host's innate immune system is its first line of defence on exposure to foreign antigens. These innate responses pre-exist within the host and do not require prior exposure to antigen. There is no alteration in the ability of the innate immune system to respond specifically to that antigen on subsequent encounter (Fearon, 1997).

Adaptive immunity differs from innate immunity in that it is induced upon encounter with foreign antigen and gives rise to a long-lasting protection against subsequent encounter with the same pathogen. Both B and T lymphocytes play a major role in these adaptive immunological responses. The ability of B and T cells to respond to pathogenic infections relies on the presence of antigen receptors on the surface of these cells that are capable of recognising and responding to a wide variety of antigens. To achieve this diversity of antigen recognition every lymphocyte expresses an antigen receptor of unique specificity that when stimulated transmits a signal to the cell. Activated lymphocytes then expand clonally on

stimulation and differentiate into effector cells which mount responses to eliminate the pathogen (Janeway and Bottomly, 1994).

### **Major histocompatibility molecules**

The recognition of protein antigens by B cells involves a direct binding of the B cell receptor (BCR) to the native protein. T cell mediated immunity, however, relies on the binding of the T cell receptor (TCR) to intracellularly degraded peptides derived from pathogens, presented on the cell surface by membrane proteins of the major histocompatibility (MHC) class I and class II family (Engelhard, 1994). These molecules are highly polymorphic cell surface glycoproteins encoded by genes in a single gene cluster called the H-2 locus in the mouse. MHC class I and II molecules have different functions and are expressed on different cell types. MHC class I molecules are expressed on all nucleated cells and present peptides derived from intracellular antigens such as viruses. MHC class I molecules consist of two polypeptide chains, the  $\alpha$  subunit and  $\beta$ 2-microglobulin ( $\beta$ 2-m) which form a dimer. The  $\alpha$  subunit is encoded by three genes in the H-2 locus namely K, D and L whilst the  $\beta$ 2-m subunit is encoded in a separate locus. MHC class II molecules present peptides derived from extracellular antigens. MHC class II molecules consist of  $\alpha$  and  $\beta$  subunits as dimers and are exclusively expressed on specialised antigen presenting cells (APCs). The MHC class II  $\alpha$  and  $\beta$  subunits are encoded in the H-2 locus as two pairs of genes called I-E and I-A.

Both MHC class I and II molecules are found to be very similar in their tertiary structure and both present peptides to T cells in a groove formed between subunits of their extracellular domains (Engelhard, 1994).

### **B and T cells: antigen recognition and interactions**

Bone marrow-derived T cell progenitors develop in the thymus into two distinct sub-types based on their expression of co-regulatory molecules. T cells expressing the CD8 co-regulatory molecule ( $CD8^+$ ) generally develop into cytotoxic

T cells which lyse infected cells. Peptides are presented to CD8<sup>+</sup> T cells in association with MHC class I molecules which activate them and cause the release of mediators, such as cytotoxins and cytokines, as well as the expression of surface molecules that lead to the death of the infected cell. T cells expressing the CD4 co-regulatory molecule (CD4<sup>+</sup>) develop into helper cells. Peptides are presented to CD4<sup>+</sup> T cells in association with MHC class II molecules and when activated these cells aid the destruction of pathogens by stimulating other cells of the immune system by producing growth factors and cytokines. Once activated CD4<sup>+</sup> T cells further develop into either T<sub>H</sub>1 or T<sub>H</sub>2 cells which express different cytokine profiles and have different functions (Murray, 1998).

Recognition of antigen by B cells leads to their activation and differentiation into plasma cells which produce antibody to fight infection (Rolink and Melchers, 1996). B cell activation can either be thymus-dependent (TD) or thymus-independent (TI), depending on the need for T cell help. TD antigens bound to BCRs are internalised and degraded into peptides that are presented in association with MHC class II molecules to CD4<sup>+</sup> helper T cells. This presentation triggers the CD4<sup>+</sup> T cells to produce cell bound and secreted effector molecules that synergise to elicit B cell activation. An early response of CD4<sup>+</sup> T cells to binding peptide/MHC complexes is the up-regulation of a T cell surface molecule, CD40 ligand (CD40L). CD40L binds to its receptor, CD40, a member of the Tumour Necrosis Factor (TNF) family that is expressed on the B cell surface (Durie et al., 1994). This interaction causes the B cell to increase expression of the immunoglobulin superfamily members CD80 (B7-1) and CD86 (B7-2) which bind to their ligand, CD28, present on the T cell surface (Thompson, 1995). Stimulation of CD28 strongly synergises with TCR stimulation and is necessary for full activation of the T cell. The activated CD4<sup>+</sup> T cell now releases cytokines such as IL-4, TGF- $\beta$  and IFN- $\gamma$  which mediate the development of these activated B cells to become antibody-secreting plasma cells.

TI B cell responses are stimulated directly by bacterial polysaccharides, polymeric proteins or lipopolysaccharides. Here T cell help is not required for plasma cell formation.

## 1.2 B cell development and function

### The B cell receptor structure and function

B and T cell antigen receptors (BCRs and TCRs) are essential for the signalling events required for lymphocyte development and activation (Janeway and Bottomly, 1994). Although distinct in structure, the B and T cell receptors share many common features (Borst et al., 1996). The BCR is a cell surface immunoglobulin (Ig) molecule consisting of a heavy (H) and light chain (L) each of which have a constant region ( $C_H$  and  $C_L$  respectively) and a variable region. The variable region is generated by somatic recombination events of the variable (V), diversity (D) and joining (J) gene segments of Ig H chains and V and J segments of Ig L chains which together give rise to extensive diversity of receptor specificity (Chen and Alt, 1993). These recombination events are initiated by the recombination activating gene-1 (RAG-1) and RAG-2 products (Alt et al., 1992; Weaver and Alt, 1997).

The BCR is part of a multi-subunit complex consisting of membrane Ig non-covalently associated with a disulphide-linked heterodimer of two transmembrane accessory proteins, CD79a ( $Ig\alpha$ ) and CD79b ( $Ig\beta$ ). These molecules have an Ig-like extracellular domain and a short intracellular domain which is responsible for early downstream signalling events upon receptor stimulation (see later and Roth and DeFranco, 1996).

When activated, mature B cells expressing membrane bound IgM/D differentiate into plasma cells that secrete antibodies. This is initially achieved by the alternative splicing of exons encoding a carboxy-terminus portion of IgM/IgD from that of a membrane bound form to that of a secreted form. Therefore activated

B cells initially secrete IgM and IgD antibodies. The cells can then undergo further recombination events between sequences known as “switch regions” that are located adjacent to other C<sub>H</sub> regions of the Ig locus which allows for the production of other secreted immunoglobulin isoforms. This process is called “isotype switching” and the type of secreted immunoglobulin (either IgG, IgM, IgA, or IgE) that a plasma cell secretes depends on the type of cytokines produced by the helper T cell during B cell activation (Lorenz et al., 1995; Lorenz and Radbruch, 1996).

B cell memory is an important element in the development of a strong humoral response to repeated exposure to antigen. B cells that encounter foreign antigen for the first time are likely to have a low affinity to the antigen, however on secondary and subsequent exposure to the same antigen, the responding antibodies have increased affinities, a process known as affinity maturation (Wagner and Neuberger, 1996). During a primary antibody response activated B cells proliferate to form a primary focus in the T cell area of lymphoid tissues where they differentiate and secrete antibody that helps to localise the antigen on the surface of follicular dendritic cells (FDCs). This stimulates the formation of germinal centres where B cells that have not undergone terminal differentiation enter a second phase of proliferation. At this stage the B cells undergo somatic hypermutation of the Ig V regions whereby there is an accumulation of mutations within these regions altering the specificity's of their receptors. Somatic hypermutation results in B cells that may have BCRs with higher or lower affinities for the antigen. B cells must have their BCR bound to antigen if they are to survive in the germinal centres. Those B cells that have the highest affinity after somatic hypermutation compete more efficiently for antigen displayed on the FDCs and this ensures that only those cells with increased affinity to the antigen after somatic hypermutation survive. During secondary responses, antibody produced during the primary response also competes with B cells binding to antigen on FDCs amplifying this effect such that secondary responses produce higher antibody affinities than primary responses.

## **B cell coreceptor**

The B cell coreceptor consists of a complex of four transmembrane signalling molecules CD19, CD21, CD81 and Leu-13 molecules that are closely associated with the BCR. The function of these molecules is to modulate the responsiveness of B cell signals from the receptor (Doody et al., 1996; Fearon, 1993). B cells from transgenic mice that over-express human CD19 are found to be hyper-responsive to antigen crosslinking (Engel et al., 1995) whilst CD19-deficient mice have decreased proliferative responses to mitogens, reduced serum Ig and fail to form germinal centres (Engel et al., 1995; Sato et al., 1997). These studies indicate a positive role for CD19 in the signal transduction mechanisms during BCR stimulation. The CD21 component of the coreceptor is a receptor for iC3b, C3dg and C3d fragments of C3 the third component of complement (Sato et al., 1997; Tedder et al., 1997). These complement components are involved in the innate immunological responses to microbial infection. CD21 is also thought to have a positive role in BCR signal transduction and therefore plays an important role during B cell encounter with microbial antigens. CD21-deficient mice have severely diminished T-dependent antibody responses consistent with its positive regulatory role. CD81 is a ubiquitously expressed cell surface glycoprotein which is a member of a family of proteins containing four transmembrane spanning regions (Maecker and Levy, 1997). CD81 also appears to play a positive role in regulating the thresholds of receptor signalling. Mice deficient in CD81 have a reduced level of CD19 expression and are defective in signal transduction on CD19 crosslinking (Tsitsikov et al., 1997). In conclusion, the role of the coreceptor molecules is to regulate the strength of receptor signalling on BCR engagement. The exact roles and the contribution that each of these coreceptor molecules make to BCR signal transduction during encounter with antigen is not clear (DeFranco, 1996).

## **B-1 cells**

B cells can be subdivided into B-1 and B-2 cells based on their location and the expression of surface markers (Tarakhovsky, 1997). The developmental relationship between these B-1 and B-2 cells is not clear though they appear to be descended from distinct progenitor populations (Borrello and Phipps, 1996; Hamilton et al., 1994). B-1 cells are present in lower numbers than B-2 cells, being only about 1% of the mature B cell population. They reside predominantly in the peritoneal and pleural cavities where they are thought to be the primary defence against microbial antigens. They can be distinguished from B-2 cells by their expression of CD11b, a marker normally expressed on macrophages. B-1 cells also have a higher IgM:IgD ratio than B-2 cells and are thought to be the major source of serum IgM that is present in the absence of antigenic challenge (Tarakhovsky, 1997). B-1 cells can be further subdivided into B-1a and B-1b subtypes on the basis of their expression of CD5, a marker more commonly associated with T development (Forster et al., 1991). B-1a cells express CD5 whilst B-1b cells do not. Whether there are any functional differences between these B-1 cell subtypes is not clear (Tarakhovsky, 1997).

## **B-2 cell development**

B cell precursors in the marrow undergo a series of developmental steps prior to maturing to IgM<sup>+</sup>IgD<sup>+</sup> peripheral recirculating B cells (Hentges, 1994). All B cells can be recognised by antibodies to the B cell specific isoform of CD45 (B220). When used in conjunction with antibodies that recognise IgM and either the IL-2 receptor  $\alpha$  chain (CD25) (Rolink et al., 1994) or CD43 (Hardy et al., 1991), B cell development can be divided into pro-B, pre-B and immature B cell populations. B220<sup>+</sup>IgM<sup>+</sup>CD25<sup>-</sup>CD43<sup>+</sup> pro-B cells begin the process of immunoglobulin rearrangement of the D and J segments of their heavy chain (D<sub>H</sub> and J<sub>H</sub>) (Weaver and Alt, 1997). This is followed by joining V<sub>H</sub> to D<sub>H</sub>J<sub>H</sub> segments to complete V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> recombination. Successful rearrangement results in expression of the  $\mu$  heavy chain which associates with the surrogate light chain components,

VpreB and  $\lambda 5$ , to form the pre-BCR (Kitamura et al., 1992). Functional expression of the pre-BCR drives the development of these cells into B220<sup>+</sup>IgM<sup>-</sup>CD25<sup>+</sup>CD43<sup>-</sup> pre-B cells. This also stops the rearrangement of heavy chain genes and induces the V<sub>L</sub> and J<sub>L</sub> rearrangement of receptor light chains,  $\kappa$  or  $\lambda$ . Successful rearrangement of these genes results in the production of a light chain which associates with the heavy chain to form a functional BCR, leading to in the formation of B220<sup>+</sup>IgM<sup>+</sup>CD25<sup>+</sup>CD43<sup>-</sup> immature B cells. Immature B cells responsive to autoantigens are deleted by negative selection at this stage in the bone marrow to avoid autoimmunity. Non-autoreactive immature B cells migrate to the red pulp and T zones of the spleen where they either die or are induced to mature to IgM<sup>+</sup>IgD<sup>+</sup> recirculating B cells and to enter B cell follicles. This final step of B cell maturation may involve signals from the BCR which select only a subset of immature B cells into the recirculating pool (Torres et al., 1996; Turner et al., 1997). This may represent the positive selection of B cells, analogous to the more extensively studied T cell process.

### **B cell developmental checkpoints and allelic exclusion**

The ultimate goal of B cell development is to produce B cells expressing a BCR of a single unique specificity that does not recognise autoantigens. To achieve this, BCR construction occurs in a stepwise fashion with developmental checkpoints to monitor the success of each step. As discussed earlier, pro-B cells firstly undergo V<sub>H</sub> D<sub>H</sub> J<sub>H</sub> rearrangement at one of the Ig heavy chain alleles. Only if this rearrangement leads to production of a functional  $\mu$  H chain does it pair with the surrogate light chain (SL) components VpreB and  $\lambda 5$ , to form the pre-BCR which signals to the cell to suppress further rearrangement at the second Ig HC allele - a process known as allelic exclusion (Loffert et al., 1996). Successful H chain rearrangement and assembly of the pre-BCR also promotes the differentiation of pro-B cells into pre-B cell (and/or their clonal expansion). Only if rearrangement of the first allele fails to produce a functional pre-BCR is the second allele rearranged, a mechanism that allows the B cell a second chance to make a functional receptor

(Loffert et al., 1996). Failure to make a functional H chain from this allele results in the death of the pro-B cell (Kitamura and Rajewsky, 1992; Kitamura et al., 1991). The presence of a functional pre-BCR is not only essential for the process of allelic exclusion but is required for the further transition of these B cells into the pre-B cell compartment. Mice deficient in  $\lambda 5$  ( $\lambda 5^{-/-}$ ) cannot efficiently form a functional pre-BCR and therefore cannot efficiently transit the pro-B/pre-B cell developmental checkpoint (Kitamura et al., 1992; Rolink et al., 1993). However, some B cells do accumulate in these mice due to either some light chain rearrangement occurring before  $V_H$  to  $D_HJ_H$  rearrangements or possibly due to  $V_{preB}$  and  $\mu$  forming a pre-BCR in absence of  $\lambda 5$ .

The  $Ig\alpha$  and  $Ig\beta$  components of the pre-BCR are known to play an important role in signal transduction from the BCR. These molecules are expressed early in B cell development at the pro-B cell compartment prior to  $V_H D_H J_H$  recombination and are therefore available to complex with the pre-BCR and contribute to signal transduction pathways (DeFranco et al., 1995). Interestingly, mice deficient in  $Ig\beta$  are able to undergo  $D_H$  to  $J_H$  recombination but fail to rearrange their  $V_H$  to  $D_H J_H$  gene segments (Gong and Nussenzweig, 1996). This may suggest there is a checkpoint at this point during B cell development that requires signal transduction events mediated by  $Ig\beta$ . Mice expressing only a truncated form of the  $Ig\alpha$  chain that cannot contribute to signal transduction have a partial block in the transition from the immature B cell to the mature recirculating B cell (Torres et al., 1996). In conclusion,  $Ig\alpha$  and  $\beta$  subunits may contribute to signal transduction during different B cell developmental stages (Roth and DeFranco, 1996).

Allelic exclusion is not restricted to the H chain genes but also occurs during L chain gene rearrangement. Pre-B cells first rearrange  $\kappa$  L chains which substitute for SL to form the BCR (Melchers et al., 1993). Formation of a functional BCR acts to suppress further  $\kappa$  chain gene rearrangement as well as rearrangement of  $\lambda$  L chain genes and allows the transition of the B cells into immature  $IgM^+$  cells that exit the bone marrow (Loffert et al., 1996). Only in the absence of the production of a

functional rearrangement of  $\kappa$  chain genes at this stage of development does  $\lambda$  L chain rearrangement then occur.

## **B cells and autoantigens**

I have discussed how B cells undergo a series of defined differentiation steps on their way to becoming mature antigen-reactive B cells. During this development it is necessary to check that not only has a functional BCR been constructed but also that the BCR is non-autoreactive (Goodnow, 1996; Hentges, 1994).

B cells expressing autoreactive Ig receptors are thought to be negated either by deletion or inactivation (Goodnow, 1992). Clonal deletion of self-reactive B cells has been investigated using transgenic mice expressing BCRs in the presence or absence of their cognate ligands (Nemazee et al., 1991). The two best studied BCR transgenic mouse models are the 3-83 $\mu\delta$  mice which have transgenes encoding IgM and IgD that bind MHC class I H-2K<sup>k</sup> or H-2K<sup>b</sup>, and the anti-HEL transgenic mice which express IgM and IgD that bind hen egg lysozyme (HEL) (Goodnow, 1992). In both these models self-reactive B cells that recognise a membrane-associated form of the cognate ligand with high affinity in the bone marrow are deleted such that no B cells expressing the autoreactive BCR enter the periphery of these animals (Nemazee et al., 1991). This deletion is driven by the induction of apoptosis leading to the death of these cells in the bone marrow. It is unclear whether this deletion is caused by apoptosis-inducing intracellular signals from the receptor itself, or whether the presence of autoreactive receptors leads to the failure of secondary signals that induce B cell maturation, apoptosis being a default pathway (Goodnow, 1996).

It is possible that B cells may not encounter all autoantigens during their development in the marrow. To counteract this B cells must have a mechanism that allows for the deletion or inactivation of self-reactive cells in the periphery. BCR transgenic mice expressing the cognate antigen for the receptor exclusively in

peripheral tissue have been used to investigate this issue. For example 3-83 $\mu\delta$  mice crossed to mice that express the deleting ligand H-2K<sup>b</sup> solely in the liver, delete B cells efficiently from the periphery demonstrating that autoreactive B cells are still susceptible to negative selection after leaving the marrow (Russell et al., 1991).

Alternatively, B cells can avoid deletion on exposure to autoantigens by either altering the specificity of the antigen receptor. Receptor editing is the term given to autoantigen-driven alterations in the specificity of a BCR by the substitution of the light chains of the receptor (Hertz and Nemazee, 1998; Pelanda et al., 1997). This phenomenon has been particularly well studied in transgenic mouse models. 3-83 $\mu\delta$  transgenic mice in the presence of the deleting H-2K<sup>k</sup> or H-2K<sup>b</sup> antigen mainly undergo clonal deletion of their B cells. However, a proportion of their B cells survive and enter the periphery. These B cells have predominantly undergone rearrangement of their endogenous k or l genes resulting in a light chain which pairs with the heavy chain of the transgene resulting in altered, non-autoreactive BCR specificity (Gay et al., 1993; Pelanda et al., 1997; Tiegs et al., 1993).

The use of transgenic mice expressing rearranged receptors to autoantigens has also revealed mechanisms other than receptor editing to avoid deletion without inducing autoimmunity. For example, anti-HEL transgenic animals do not delete their B cells when crossed to mice that secrete small amounts of a soluble form of HEL (sHEL) (Goodnow et al., 1989). No autoimmune disease develops in these mice although their B cells are able to produce anti-HEL antibodies in response to the antigen *in vitro* (Goodnow et al., 1989). This phenomenon is described as clonal ignorance, and is probably due to the low level of receptor stimulation provided by the small amounts of cognate antigen present in these animals.

Clonal anergy is another mechanism by which autoimmunity is avoided (Goodnow, 1992). This process is characterised by the presence of B cells that express BCRs that recognise self-antigen but have become functionally inactivated. This process has also been studied using the anti-HEL transgenic mouse model. When these mice are crossed to transgenic mice expressing sHEL at higher levels

than those that induce clonal ignorance then clonal anergy occurs (Goodnow et al., 1988). B cells reach the periphery of these mice in near normal numbers but they exhibit a loss of many normal cellular responses such as a reduction in mitogen-induced B cell proliferation, Ig secretion and they have a characteristic twenty-fold reduction in the level of BCR expression (Cyster et al., 1994; Goodnow, 1992; Mason et al., 1992). When the fate of these anti-HEL expressing anergic B cells was explored in the context of a polyclonal repertoire where they were mixed with non-autoreactive B cells and used to reconstitute lethally irradiated recipients, the anergic cells were found to be excluded from migrating into primary follicles and died in the outer T cell zone (Cyster and Goodnow, 1995; Cyster et al., 1994). This suggests that transition through the outer T cell zone where they compete with naive B cells for survival may be an important selection event that prevents the accumulation of anergic B cells.

The induction of B cell deletion, ignorance or anergy is probably dependent on the signals elicited from the receptor on engagement. In the model proposed by Goodnow (Goodnow, 1996), B cells are essentially "tuned" in their responsiveness to antigen and the outcome of encounter with antigen depends on the amount of stimulating antigen, the avidity of the receptor recognition, the timing of the exposure to the antigen as well as the level of co-stimulation the receptor receives. These factors contribute to the strength of signal from the BCR which determines the fate of the developing B cell.

### **Signal transduction from the BCR**

Signal transduction from the BCR is essential for B cell responses to foreign antigens and for passage through development checkpoints during B cell development as discussed above. The immunoglobulin domain of the BCR has only a short cytoplasmic tail and relies on the Ig $\alpha$  and Ig $\beta$  components of the receptor to elicit signalling responses (Cronin et al., 1998; Teh and Neuberger, 1997). One of the earliest events following receptor engagement is a rapid increase

in Src-family protein tyrosine kinase (PTK) activity (Campbell and Sefton, 1990; Gold et al., 1990). The BCR itself does not have any intrinsic kinase activity and instead depends on the recruitment of kinases to the receptor. Specifically Lyn, Blk and Fyn Src-family kinases have been shown to be associated with the BCR, and to become phosphorylated and activated following BCR stimulation (Brown et al., 1994; Burkhardt et al., 1991; Sefton and Taddie, 1994). Engagement of the BCR by antigen results in the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) with the consensus sequence YXXL<sub>6,8</sub>YXXL (single letter amino acids code, X represents any amino acid) within the cytoplasmic portion of the Ig $\alpha$  and Ig $\beta$  molecules by Src-family members (Thomas, 1995). The phosphorylated ITAMs act as docking sites for the SH2 domains of the Syk-family kinases. This binding leads to the activation of these kinases and to further downstream signalling events such as the activation of the Erk, JNK and p38 mitogen-activated protein (MAP) kinase pathways (Cambier et al., 1994). BCR antigen engagement is also associated with a rapid increase in the concentration of intracellular Ca<sup>2+</sup>. This response is driven by the activation of phospholipase C, which results in the hydrolysis of phosphoinositide lipids, and the production of the second messengers inositol -1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> in turn binds to IP<sub>3</sub> receptors in the endoplasmic reticulum leading to the release of Ca<sup>2+</sup> from intracellular stores into the cytoplasm. In contrast DAG is thought to activate the serine/threonine protein kinase C (PKC) (Bazzi and Nelsestuen, 1989). The consequence of these signalling events is to ultimately alter the activity of many different transcription factors such as NF-AT, NF- $\kappa$ B and AP-1, leading to altered gene transcription activity which elicits a B cell response, whether that be activation, proliferation or apoptosis (May and Ghosh, 1998).

The signals emerging from the activated BCR are subject to negative regulation by cell-surface proteins which include the IgG constant region receptor Fc $\gamma$ RIIB and the  $\alpha$ 2,6-sialoglycoprotein binding, immunoglobulin superfamily member CD22. These molecules contain tyrosine-based inhibitory motifs (ITIMs) which contain the tyrosine phosphatase SHP-1/2 binding domain consensus sequence V/IXYXXL/V (Isakov, 1997; Unkeless and Jin, 1997). Co-aggregation

of the BCR and Fc $\gamma$ RIIB inhibits BCR-mediated proliferation and antibody responses (D'Ambrosio et al., 1995). Moreover Fc $\gamma$ RIIB-deficient mice have elevated responses to both T-dependent and T-independent antigens demonstrating the negative role of this molecule has on BCR signalling (Takai, 1996; Takai et al., 1996). CD22 appears to function as a constitutive regulator of B cell function without requiring co-ligation with the receptor. CD22-deficient mice are found to have elevated serum Ig, enhanced BCR-mediated proliferative responses and increased Ca<sup>2+</sup> mobilisation on receptor stimulation (Nitschke et al., 1997; O'Keefe et al., 1996; Otipoby et al., 1996; Sato et al., 1996). The importance of SHP-1 in the negative regulation of receptor signalling is evident from the naturally occurring *motheaten* mice which have a defect in SHP-1 and show severe autoimmune disease (Tsui et al., 1993). SHP-1 has a negative effect on downstream signalling events such as inositol phospholipid hydrolysis and Ca<sup>2+</sup> flux that occur on receptor stimulation, however its *in vivo* substrates are not known and therefore it is not clear how SHP-1 mediates its inhibitory effect (Unkeless and Jin, 1997). Coligating Fc $\gamma$ RIIB with the BCR has been used to study how the inhibitory signalling pathways interact with signals from the BCR. These studies demonstrate that these pathways integrate through phosphorylation events of CD19. Receptor ligation normally causes the phosphorylation of CD19 and this is reversed by the recruitment of Fc $\gamma$ RIIB to the receptor (Buhl and Cambier, 1997; Hippen et al., 1997; Kiener et al., 1993). CD19 phosphorylation causes the association and activation of phosphatidylinositol 3-kinase (PI-3K). PI-3K activity results in the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP<sub>3</sub>) which ultimately gives rise to the enhanced production of IP<sub>3</sub> by Phospho Lipase C $\gamma$  (PLC $\gamma$ ) (Falasca et al., 1998; Scharenberg and Kinet, 1998). Thus CD19 dephosphorylation results in the termination of IP<sub>3</sub> production and Ca<sup>2+</sup> flux. In addition to the ability of Fc $\gamma$ RIIB-1 to recruit SHP-1 and SHP-2 which function to prevent the production of PIP<sub>3</sub> it also recruits a PIP<sub>3</sub> 5'-phosphatase called SHIP whose function is to degrade PIP<sub>3</sub> and therefore contribute to the inhibition of these signals.

The role of the Src-family members in membrane-proximal signalling events has been further analysed in mice deficient in these kinases. Lyn-deficient mice are

found to have reduced numbers of peripheral B cells and alterations in phosphorylation patterns on receptor crosslinking support the proposed role for Lyn in receptor-proximal signalling events (Nishizumi et al., 1995). However, these mice also appear to have autoimmune responses in that they produce anti-DNA antibodies, have elevated serum IgM and IgA and display splenomegaly (Hibbs et al., 1995). In addition Lyn-deficient B cells are more sensitive to BCR-driven proliferation suggesting a role for Lyn in the negative regulation of signals from the BCR (Chan et al., 1997). Recent studies on B cells from Lyn-deficient mice have shown that tyrosine phosphorylation of both Fc $\gamma$ RIIB and CD22 coreceptors are impaired and that this was associated with their failure to recruit SHP-1 and SHIP explaining the hyper-responsiveness of the Lyn-deficient B cells (Chan et al., 1998; Nishizumi et al., 1998). The loss of these negative regulatory functions would lead to increased signalling. In contrast to Lyn-deficient mice, Fyn-deficient mice appear to be unaffected in terms of BCR signal transduction as they have unaltered tyrosine phosphorylation, inositol hydrolysis and Ca<sup>2+</sup> flux compared to control cells (Sillman and Monroe, 1994). This suggests that either Fyn plays a minor role in the signalling events from the BCR or that in the absence of Fyn other protein kinases can substitute for Fyn activity. Mice deficient in the Src-family tyrosine kinase Blk have not been as yet been reported in the literature.

Bruton's tyrosine kinase (Btk) is a member of the Tec-family of kinases and has been shown to have an important role in B cell signal transduction. Both humans with X-linked agammaglobulinemia (XLA) and mice with X-linked immunodeficiency (*xid*) have severe deficiencies in B cell function caused by mutations in Btk. The activation of Btk has been shown to require both PI-3K and a Src-family kinase (Aoki et al., 1994; Cheng et al., 1994; Kawakami et al., 1997; Rawlings et al., 1996; Satterthwaite et al., 1998; Yao et al., 1994) and its activation results in the phosphorylation and activation PLC $\gamma$  leading to downstream signalling events including calcium flux (Fluckiger et al., 1998; Scharenberg et al., 1998).

Syk-deficient mice die perinatally (Cheng et al., 1995; Turner et al., 1995). However, Syk-deficient B cell development has been studied by making radiation

chimeras reconstituted with foetal liver from these mice and has shown that B cell development is impaired with a profound block in the transition from immature to mature B cells as well as a partial block at the pro- to pre-B cell transition (Turner et al., 1997; Turner et al., 1995). These results are consistent with Syk playing a crucial role in signalling at both the pre-BCR and BCR during these developmental checkpoints.

## 1.3 T cell development and function

### **The T cell receptor structure and function**

The TCR, like the BCR, is an oligomeric complex consisting of antigen binding and signal transduction units. The antigen binding domain of the receptor is a heterodimer of two transmembrane glycoproteins  $\alpha$  and  $\beta$  (there are also  $\gamma\delta$  T cells which have a distinct function and will not be discussed here). The TCR  $\alpha$  and  $\beta$  subunits have constant and variable regions that are similar to those of the BCR (von Boehmer and Fehling, 1997). The variable region of  $\beta$  subunit undergoes RAG-dependent somatic recombination events which join V, D and J segments whilst the  $\alpha$  subunit is assembled by recombination of V and J segments which together generate diversity in TCR antigen specificity. In a similar manner to the BCR, the TCR is also found as a complex with molecules required for signalling, namely CD3 $\gamma$ ,  $\delta$ ,  $\epsilon$  chains and  $\zeta$  and  $\eta$  that allow the T cell to become an armed effector cell (Cantrell, 1996).

### **T cell accessory molecules**

T cells, like B cells, have molecules associated with their receptor that modulate its activity. The TCR coreceptor molecules are CD4 and CD8. These transmembrane glycoproteins aid ligand recognition by interacting with the MHC molecules during peptide presentation (Engelhard, 1994). Specifically CD4 binds to the  $\beta 2$  domain of MHC class II, whereas CD8 binds to the  $\alpha 3$  domain of MHC

class I. CD4 is a single chain molecule composed of four extracellular immunoglobulin-like domains and a short intracellular domain involved in signalling. The CD8 coreceptor is structurally very different from the CD4 molecule. It consists of a disulphide-linked heterodimer comprising  $\alpha$  and  $\beta$  chains which has an extended extracellular structure and a short cytoplasmic tail. The presence of either CD4 or CD8 increases the sensitivity of a T cell to MHC-presented antigens by up to 100-fold (Zamoyska, 1998). These coreceptor and MHC interactions are required for T cell development as well as activation on encounter with antigen. Mice deficient in MHC class I and class II have severely diminished T cell development (Grusby et al., 1993; Zijlstra et al., 1990), whilst mice deficient in either CD4 or CD8 molecules are found to have severely deficient helper or cytotoxic function, respectively (Fung-Leung et al., 1991; Killcen and Littman, 1993; Rahemtulla et al., 1993).

### $\alpha\beta$ T cell development

T cell precursors migrate from the bone marrow to the thymus where they develop into mature cells which enter the blood stream and migrate to the peripheral lymphoid organs such as lymph node and spleen. T cell maturation in the thymus is distinguished by distinct stages of development characterised by the expression of CD4 and CD8 (Roth and DeFranco, 1995)). Thymocytes develop from the least mature CD4<sup>-</sup>CD8<sup>-</sup> double negative (DN) thymocytes through an intermediate CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) stage before maturing to either CD4<sup>+</sup> single positive (SP) or CD8<sup>+</sup> SP cells prior to leaving the thymus to become mature recirculating T cells. Levels of expression of the adhesion molecule CD44 and CD25 on DN cells have been used to further define these immature thymocytes (Godfrey and Zlotnik, 1993; Nikolic-Zugic, 1991). The earliest T cell progenitors that enter the thymus are CD44<sup>+</sup>CD25<sup>-</sup> (DN1) cells. These cells are thought to come directly from the bone marrow and are no longer pluripotent but committed to lymphoid development. At this stage these cells still have their TCR genes in germline configuration. These cells next develop into CD44<sup>+</sup>CD25<sup>+</sup> (DN2) cells

which have started transcription of the TCR $\beta$  chain and rearrangement of D to J gene segments (Dudley et al., 1994). T lymphocytes now become CD44<sup>+</sup>CD25<sup>+</sup> (DN3) and finally CD44<sup>+</sup>CD25<sup>-</sup> (DN4) thymocytes prior to leaving the DN compartment. DN3 thymocytes have undergone V-D-J recombination at the TCR $\beta$  locus which is expressed on the cell surface with the pre-T $\alpha$  chain (pT $\alpha$ ) to form the pre-T cell receptor (pre-TCR) (Saint-Ruf et al., 1994) this provides a signal for the cell to start rearrangement of the TCR $\alpha$  chain. In this way pT $\alpha$  is thought to act in a similar manner to SL chain in B cell development. The role of CD25 and therefore IL-2 during progression through the DN compartment of T cell development is not clear as IL-2 deficient mice have normal thymus development (Sadlack et al., 1993). The IL-2 receptor  $\gamma$  chain, however, is shared by a number of cytokine receptors (IL-4R, IL-7R, IL-9R and IL-15R) and is usually referred to as the common  $\gamma$  chain ( $\gamma$ c). Mice expressing a truncated  $\gamma$ c chain have a profound block in T cell development at the DN stage (Crompton et al., 1997; Ohbo et al., 1996). Moreover mice deficient in IL-7 expression also display a similar block in development (Peschon et al., 1994). This may suggest that IL-7 signalling through  $\gamma$ c may be an important aspect of T cell development.

The earliest known checkpoint in thymic development occurs in the DN thymocyte population at the DN3 stage of development. As described earlier, these thymocytes have rearranged one of their TCR  $\beta$  alleles and expressed it on the cell surface with the pT $\alpha$  subunit to form the pre-TCR. Normally, formation of a functional pre-TCR leads to the prevention of rearrangement of the second TCR  $\beta$  chain alleles leading to allelic exclusion in a similar manner to that seen with pre-BCR (Aifantis et al., 1997). Production of a functional pre-TCR is required for further T cell development. RAG-1 or 2-deficient mice which are unable to rearrange their TCR  $\beta$  alleles, and mice deficient in TCR $\beta$  fail to make a functional pre-TCR and show a block at this stage of development. Mice deficient in pT $\alpha$  also show a profound block at the DN3 stage of thymocytes development as well as a breakdown in the process of allelic exclusion (Aifantis et al., 1997; Fehling et al., 1995), again demonstrating a necessity for the production of a functional pre-TCR in these processes. In a similar manner to B cell studies, mice deficient in TCR

coreceptor molecules have been assessed for T cell development and function. CD3 $\epsilon$ -deficient mice are found to have a complete block in T cell development at the DN3 stage suggesting this molecule is required for the signal transduction from the pre-TCR (Malissen et al., 1995).

Further development from DN thymocytes into DP cells is associated with migration of thymocytes to the thymic cortex and rearrangement of the TCR $\alpha$  genes. As these DP thymocytes mature, TCR $\alpha\beta$  gene expression increases and these cells undergo a positive selection process that only allows further development of those thymocytes expressing functional and non-autoreactive receptors into CD4<sup>+</sup> SP or CD8<sup>+</sup> SP cells (Jameson et al., 1995; von Boehmer, 1991). Positive selection and commitment to the CD4 and CD8 lineage is determined by the affinity of interactions between the TCR and peptides presented by MHC class I and class II molecules on thymic stromal cells (Ashton-Rickardt and Tonegawa, 1994; Williams et al., 1997). This selects those cells that have some affinity for peptide presented by self-MHC. As will be discussed later, the affinity of these interactions is important in determining the outcome of these selection events. It is thought that during this process thymocytes randomly down-regulate either CD4 or CD8 and that this is followed by a step that requires participation by the appropriate coreceptor that permits end stage differentiation (Chan et al., 1993; von Boehmer, 1996). Mice deficient in TCR $\alpha$  chain fail to develop CD4<sup>+</sup> SP or CD8<sup>+</sup> SP thymocytes demonstrating the necessity for a functional TCR for transition between DP and SP stage of thymic development (Fehling et al., 1995; Mombaerts et al., 1992). This is a developmental checkpoint akin to the need for functional L chain rearrangement during B cell development. TCR $\alpha$  chain rearrangement from one allele and the production of a functional TCR is thought to suppress recombination at the second allele in a similar manner to L chain allelic exclusion in B cells. However, it has been found that 30% of mature T cells have two functional rearrangements and therefore have two receptors suggesting that TCR $\alpha$  allelic exclusion may not be complete (Kisielow and Mizsek, 1995; Malissen et al., 1992).

## T cells and autoantigens

T cell development, like B cell development, requires the depletion of those cells that recognise self antigen to avoid autoimmune disease. T cells undergo these negative selection events predominantly by clonal deletion of immature thymocytes. These events have best been studied by the use of TCR transgenic mice. A number of transgenic mouse models exist that express either MHC class I- or class II-restricted rearranged TCRs and their cognate ligands. T cell clonal deletion occurs in these mice at the DP stage of T cell development by apoptosis (Arnold et al., 1990; Mamalaki et al., 1993; Nakayama et al., 1992; Page et al., 1993; Shinkai et al., 1993). As signalling through the same TCR can drive either positive or negative selection at the this stage of development, a model has been proposed to explain this process based on the avidity of the receptor for peptide ligands presented to the thymocyte by APCs in the thymic stroma (Ashton-Rickardt and Tonegawa, 1994). The avidity of the interaction is determined by the affinity of the interaction of the receptor and the peptide as well as the concentration of the peptide. In the avidity model, thymocytes with TCRs interacting with a low avidity to the selecting peptide/MHC (S/M) complex are positively selected and rescued from the default fate of death by neglect whilst TCRs that have a strong avidity with the S/M complex (because of a high affinity interaction and/or high concentration of selecting peptide) are induced to delete. Foetal thymic organ cultures from mice expressing a transgenic receptor (P14) with specificity for lymphocytic choriomeningitis (LCMV) glycoprotein in association with MHC class I H-2D<sup>b</sup> molecule, positively select CD8<sup>+</sup> cells when the level of peptide expression is relatively low whilst expression at higher density causes negative selection to occur (Ashton-Rickardt and Tonegawa, 1994). More recently a transgenic mouse expressing a TCR that has different affinities to peptides derived from different MHC class I molecules has been studied. Here, peptides with low affinity for the receptor could induce negative selection if the ligand was expressed at high concentration, supporting the avidity model (Motyka and Teh, 1998). T cells differ from B cells in that they must recognise antigenic peptides in the context of MHC.

molecules, and the TCR accessory molecules CD4 and CD8 are crucial in this interaction. Both CD4 and CD8-deficient mice have been found to be defective in negative as well as positive selection demonstrating a crucial role for these molecules in thymic selection events (Fung-Leung et al., 1991; Wallace et al., 1992). Moreover transgenic mice expressing MHC class I that has a mutation in the CD8 binding domain, bred to express a class I-restricted TCR, fail to undergo negative as well as positive selection demonstrating a crucial role for MHC and accessory molecule interactions during negative as well as positive selection (Fung-Leung et al., 1991).

T cells also undergo clonal deletion in response to superantigens (Simpson et al., 1993). There are two categories of superantigens, the viral superantigens encoded by the long terminal repeats (LTR) of endogenous mouse mammary tumour viruses (MMTV), and the soluble endotoxins produced by gram-positive bacteria such as *Staphylococcus aureus*. TCR recognition of conventional peptides involves presentation by MHC molecules and their recognition by the variable regions of both the  $\alpha$  and  $\beta$  chains of the TCR (Webb and Gascoigne, 1994). Superantigen recognition, on the other hand depends primarily on binding to V $\beta$  and MHC class II molecules outside the peptide binding domains (Webb and Gascoigne, 1994). In this way, expression of superantigens can lead to the stimulation of antigen receptors bearing particular V $\beta$  chains. Originally called minor lymphocyte stimulating (Mls) antigens, different inbred strains of mice are known to have different MMTV proviral expression patterns (Webb and Gascoigne, 1994). As these MMTV superantigens are essentially autoantigens in these mice, they lead to the negative selection and clonal deletion of particular V $\beta$ -expressing cells during their development, depending on which V $\beta$  a given MMTV superantigen binds. For example the BALB/c mouse strain expresses endogenous MMTV 6, 8 and 9, which causes the deletion in the thymus of T cells expressing V $\beta$  3, 5, 11 and 12 (Simpson et al., 1993). Bacterial endotoxin superantigens act in a similar manner to endogenous proviral superantigens in their interactions with the receptor (Marrack and Kappler, 1990). A number of different endotoxin antigens exist that differ only slightly in structure but have distinct specificities for different

V $\beta$  domains. Treatment of mice with these exogenous superantigens results in MHC class II-dependent V $\beta$ -specific deletion of thymocytes (Marrack and Kappler, 1990). The deletion of these V $\beta$  expressing T cells occurs at the SP stage of thymocyte development rather than at the DP stage where T cells are deleted on encounter with conventional autoantigens (Marrack and Kappler, 1990).

Like B cells, mature T cells that encounter autoantigen in the periphery have mechanisms to prevent the development of autoimmune disease. Indeed all the phenomena associated with B cell encounter with peripheral autoantigen have been demonstrated in T cells. Transgenic mice expressing TCRs of known specificity crossed to mice expressing cognate ligand under the control of a tissue-specific promoter have been used to study this process. Mice expressing a transgenic TCR specific for H-2K<sup>b</sup> crossed with mice in which H-2K<sup>b</sup> is expressed exclusively on hepatocytes demonstrates that the expression of autoantigen can lead to peripheral antigen tolerance of T cells by clonal anergy (Motyka and Teh, 1998). Other transgenic mouse models have demonstrated that T cells can develop clonal ignorance to the antigen (Ferber et al., 1994) or demonstrate clonal deletion of autoreactive T cells (Carlow et al., 1992).

### **Signal transduction from the TCR**

Signal transduction events from the TCR to the nucleus are necessary for both responses to foreign antigen and for passage through checkpoints during thymocyte development (Owen and Venkitaraman, 1996). An early event on TCR activation is a rapid increase in tyrosine kinase activity that is essential for downstream signalling events (Weiss and Littman, 1994; Zenner et al., 1995). The TCR itself does not have any intrinsic enzymatic activity but rather, when activated, recruits specific PTKs to the receptor complex via the conserved ITAMs present in the CD3  $\gamma$ ,  $\delta$  and  $\epsilon$  subunits and  $\zeta$  chain of the TCR complex (Qian and Weiss, 1997). Following TCR activation, Src-family tyrosine kinases Lck and Fyn have been shown to phosphorylate the ITAMs present in CD3 and  $\zeta$  (Zenner et al.,

1995). Once phosphorylated, the ITAMs allow the binding of the Syk-family tyrosine kinase ZAP-70, via its tandem SH2 domains to twin phosphorylated tyrosines in the  $\zeta$  chain of the activated receptor. This binding in turn allows for the subsequent tyrosine phosphorylation of ZAP-70 by Lck and its activation. In turn ZAP-70 may phosphorylate and activate a number of other proteins involved in the signalling pathway including Vav, an exchange factor for the Rho-family GTPases that has been shown to interact with multiple other components of signal transduction pathways (Zhang et al., 1998). Activation of the TCR leads to a number of downstream signalling events that are similar to those discussed earlier for BCR signalling and include hydrolysis of inositol-containing phospholipids,  $\text{Ca}^{2+}$  mobilisation and activation of the Ras/MAPK pathways (Qian and Weiss, 1997). These pathways ultimately lead to alterations in the activities of transcription factors, changes in gene expression patterns and altered cellular function.

The role of the Src-family kinases in membrane-proximal signal transduction has been further characterised in mice deficient in these molecules. Lck is known to associate with the cytoplasmic domains of both CD4 and CD8 as well as the  $\beta$  chain of the IL-2 receptor (Barber et al., 1989). Mice deficient in Lck show a deficient in T cell development (van Oers et al., 1996). They have a partial block at the DN3 compartment of T cell development, suggesting that Lck transduces some of the signals from the pre-TCR. This block is not complete and some DP cells develop, however very few SP T cells are detectable in these mice suggesting that Lck is also involved in the transduction of signals during the positive selection events that lead SP T cell development. Fyn-deficient mice on the other hand do not display any defect in T cell development although thymocytes from these mice do have signal transduction deficits such as reduced  $\text{Ca}^{2+}$  flux and proliferation (Appleby et al., 1992; Stein et al., 1992). This is not the case in peripheral mature Fyn-deficient T cells (Appleby et al., 1992) suggesting that a different signal transduction mechanism may be used during T cell development. Mice deficient in both Lck and Fyn show a more profound block than that seen in Lck-deficient mice alone (Groves et al., 1996). Here thymocyte development is completely blocked at the DN3 compartment of thymocyte development such that

now the thymus of these mice are identical to RAG-deficient mice (Groves et al., 1996; van Oers et al., 1996). These results indicate that Lck plays an important role in the signal transduction events from the pre-TCR and that Fyn can partially substitute for Lck in its absence, but that at least one of these kinases must be present in order to generate a pre-TCR signal.

T cell development in mice deficient in ZAP-70 is blocked at the DP stage demonstrating a need for ZAP-70 signalling during thymic positive selection of CD4<sup>+</sup>SP and CD8<sup>+</sup> SP but not at earlier stages in development (Gong et al., 1997; Negishi et al., 1995). In addition thymocytes from ZAP-70 deficient mice are also deficient in negative selection, a result consistent with a role for ZAP-70 in signal transduction during both positive and negative selection events (Negishi et al., 1995). Syk, the other Syk-family member is also expressed in the T cell lineage, however Syk-deficient mice show no abnormality in T cell development or function (Turner et al., 1995). A role for Syk in T cell development has been resolved by the analysis of mice deficient in both Syk and ZAP-70 (Cheng et al., 1997). These mice have a complete block at the DN3 stage of thymocyte development. Moreover over-expression of Syk in ZAP-70 deficient mice restores thymocyte development (Gong et al., 1997). Together this suggests that Syk and ZAP-70 have overlapping redundant roles in pre-TCR signalling.

T cell development in mice deficient in Vav also demonstrate deficiencies in TCR signalling-dependent thymic selection events (Turner et al., 1997). Thymocyte development is partially blocked at the DN3 stage of development and there is a more profound block at the transition from DP to CD4<sup>+</sup> SP and CD8<sup>+</sup> SP cells. Moreover there is a defect in both positive and negative selection of thymocytes. Thus Vav has a role in the signal transduction events from both the pre-TCR and the TCR that are required during T cell development.

In a similar manner to regulation of signalling through the BCR, TCR signalling is also subject to negative regulation. The best studied of this is the negative regulation of TCR signalling by cytotoxic T-lymphocyte antigen-4 (CTLA-4). CTLA-4 shares with CD28 the ligands B7-1 and B7-2, and indeed

these molecules may compete to bind to these ligands (Linsley et al., 1994; Thompson, 1995). CTLA-4 plays an inhibitory role in the signal transduction events by recruitment of the SHP-2 phosphatase to the receptor after phosphorylation of a conserved YVKM (single-letter amino acid code) SH2 binding site (Thompson and Allison, 1997). Mice deficient in CTLA-4 have a lymphoproliferative disorder, moreover T cells from these mice have been shown to have constitutively active Fyn, Lck and ZAP-70 kinases (Chambers et al., 1997). Nevertheless exactly how CTLA-4 mediates its biological effect and its role in the control of signal transduction during antigen encounter is still controversial (Thompson and Allison, 1997). T cells are also known to express ITIM containing transmembrane proteins such as the killer cell inhibitory receptors (KIRs) (Isakov, 1997). In humans these molecules, which belong to the Ig superfamily, are expressed on both NK and T cells and bind specifically to MHC class I molecules. In a similar manner to the negative regulation of the BCR by Fc $\gamma$ RIIB-1, KIR ITIMs become phosphorylated when the KIR is co-ligated with the receptor and this leads to the recruitment of the SHP-1 phosphatase. *Motheaten* mice, which have a deficiency in SHP-1, exhibit increased proliferative response to TCR stimulation, and have enhanced and prolonged TCR-induced tyrosine phosphorylation of both the TCR complex and cytosolic signalling proteins (Pani et al., 1996), demonstrating the importance of this protein in modulating signalling from the receptor.

## 1.4 The role of CD45 in the immune response

### **CD45 structure: the extracellular domain**

CD45 (also called T200, B220, Ly-5 or leukocyte common antigen; Figure 1) is an abundant 180-220kD transmembrane protein tyrosine phosphatase that is expressed on all nucleated haemopoietic cells where it can comprise of up to 10% of the cell surface membrane (Alexander, 1997). It has an elongated receptor-like

extracellular domain that is highly glycosylated and sulphated (Giordanengo et al., 1995; Sato et al., 1993). At least eight different isoforms of CD45 exist due to the alternative splicing of three variable exons (exons 4, 5 and 6) encoding parts of the extracellular domain of the protein (Chang et al., 1991; Saga et al., 1990). Isoforms recognised by monoclonal antibodies specific for epitopes within exons 4, 5 and 6 are designated CD45RA, RB and RC respectively (Alexander, 1997). A further antibody specificity exists that recognises an epitope in human CD45 that is created only in the absence of these three epitopes; the isoform recognised by this antibody is called CD45RO. No equivalent antibody exists for mouse CD45. Different CD45 isoforms are known to be expressed on different T cell subsets. In mice, for example, expression of the CD45RB isoform is associated with previously activated/memory T cells whilst the CD45RA isoform is expressed by naive T cells (Thomas and Lefrancois, 1988). B cells in contrast only express a high molecular weight isoform of CD45 known as B220, which is antigenically distinguishable from isoforms expressed on T cells ((Thomas and Lefrancois, 1988). The true biological significance of these different isoforms is not understood as, despite its resemblance to known receptors, no specific ligands for CD45 isoforms have been identified. The only CD45 ligand that has been proposed is the B cell specific molecule CD22 which was initially shown to interact specifically with the CD45RO isoform (Stamenkovic et al., 1991). Later experiments however, have shown that CD22 is a lectin that interacts with a wide range of glycosylated proteins including all isoforms of CD45, not just with CD45RO (Powell et al., 1993; Sgroi et al., 1993).

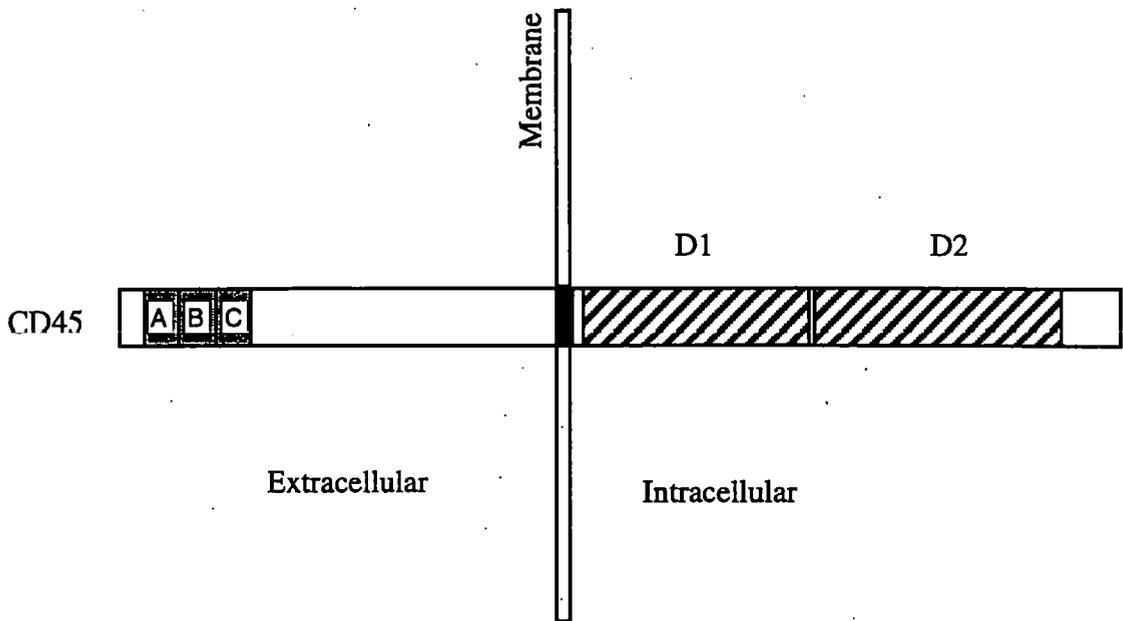
One possible role for the extracellular domain of CD45 is that it may interact with cell membrane molecules in cis (i.e. on the same cell). There is evidence that CD45 interacts with the TCR (Volarevic et al., 1990), the coreceptors CD4 and CD8 (Mittler RS, 1991), the B cell receptor (Brown et al., 1994), CD16 (Altin et al., 1994) and CD26 (Torimoto et al., 1991). However, given the abundance of CD45 on the cell surface it is unclear whether these interactions are of physiological significance.

In the absence of a convincing demonstration of a physiological ligand for CD45, monoclonal antibodies (mAb) reactive to the extracellular domain of CD45 have been used to study the consequences of an extracellular stimulus. The results of many of these studies have been confusing since the effects of the mAbs are epitope specific. For example certain CD45 mAbs that recognise all isoforms of CD45 (anti-panCD45 mAb) exert either no effect or a negative effect on TCR mediated signalling (Goldman et al., 1992), whilst a monoclonal that recognises a T cell specific CD45 isoform can exert a positive effect (Torimoto et al., 1992). Moreover, certain anti-panCD45 mAb have been found to exert a positive effect on TCR signalling in CD45RA<sup>hi</sup> but not CD45RO<sup>hi</sup> cells (Welge et al., 1993), whilst other studies have shown CD45 mAbs to have an inhibitory effect on CD4<sup>+</sup> but not CD8<sup>+</sup> T cell lines (Maroun and Julius, 1994). This suggests that responses to these mAbs may be different depending on the differentiation and activation state of the T cells.

CD45-deficient T cell lines are defective in many receptor-mediated signalling events (Alexander, 1997). Transfection of full length CD45 back into T cell lines have been shown to restore the signalling capability of these cells suggesting CD45 plays a positive role in signal transduction events (Koretzky et al., 1992; Shiroo et al., 1992). Transfection using mutant forms of CD45 that lack the extracellular domain of CD45 was also found to restore receptor-mediated signalling suggesting that the extracellular domain of CD45 is not required for its function (Desai et al., 1993; Donovan et al., 1994; Hovis et al., 1993; Volarevic et al., 1993). Moreover when a chimeric molecule was made joining the intracellular portion of CD45 to the extracellular domain of the epidermal growth factor receptor (EGFR) whose ligand (EGF) is known, addition of the ligand inhibits TCR signal transduction (Desai et al., 1993), suggesting that if a physiological ligand for CD45 exists then its role may be inhibitory. EGFR binding to its ligand causes dimerisation of the receptor and its activation. The inhibition of CD45 activity on EGF binding is due to steric inhibition of these molecules on ligation. As will be discussed later the tertiary structure of the phosphatase domains of CD45 are known and this has aided our understanding of the nature of this inhibition (Bilwes et al.,

1996). Some CD45 has been detected in a dimeric form on the surface of cell lines, suggesting that dimerisation may be an important event for CD45 regulation (Takeda et al., 1992). Whether or not this is a physiologically important mechanism for CD45 regulation will only be solved if a physiological ligand for CD45 is identified.

Despite our lack of knowledge of the function of these different isoforms of CD45 in extracellular interactions, they clearly play different roles. Expression studies in CD45-deficient cell lines have been used to demonstrate that different CD45 isoforms can differentially control levels of receptor signalling. One of the best studies of this issue used transfection of different CD45 isoforms (CD45RO, CD45RABC, CD45RBC and CD45RC) together with CD4 and a TCR that recognises HEL into a CD4TCR-CD45<sup>-</sup> T cell line (Novak et al., 1994). The ability of these cell lines to respond to TCR stimulation was evaluated. It was found that those isoforms that have the lowest molecular weight (CD45RO and CD45RC) are more efficient at promoting antigen recognition via the TCR than higher molecular weight isoforms. This is in agreement with a proposed role for the extracellular domain of CD45 in the physical interactions of T cells during antigen recognition and binding (Shaw and Dustin, 1997). The extracellular domain of CD45 extends 28-51 nm from the surface of a T cell which is considerably further than the TCR. Therefore the physical presence of CD45 would interfere with TCR and MHC/peptide interactions and it must therefore be excluded to allow these interactions. As will be discussed later this physical exclusion of CD45 during TCR interactions has implications on the role of CD45 during TCR signal transduction events.



**Figure 1: Schematic diagram of the CD45 protein.** CD45 is a transmembrane molecule. The extracellular domain is large and highly glycosylated. Different isoforms of CD45 exist due to the differential splicing of three exons (exons 4, 5 and 6) which allows the presence or absence of the CD45RA, B or C epitopes (shaded boxes). After the membrane spanning region (black box) is the intracellular portion which contains the phosphatase domains of which domain 1 (D1) catalyses the phosphatase reaction whilst domain 2 (D2) is important in providing the correct tertiary structure for this reaction.

### **CD45 structure: the intracellular phosphatase domains**

The intracellular portion of CD45 is composed of two homologous protein tyrosine phosphatase (PTP)-like domains called Domain 1 and 2 (D1 and D2, Figure 1) separated by a short spacer region and a C-terminal tail (Alexander, 1997). D1 contains the PTP active site signature motif [V/I]HCXAGXGR[S/T]G (single letter amino acid code where X is any amino acid) found to be present in all PTPs. It contains a critical cysteine residue (Cys<sup>828</sup>) necessary for the dephosphorylation reaction (Streuli et al., 1990). D2, although sharing much homology with D1, does not contain the PTPase active site motif and does not itself have intrinsic phosphatase activity (Johnson et al., 1992; Streuli et al., 1990). There is a lot of evidence that argues for a structural requirement of D2 to provide the correct structure to allow phosphatase activity from D1. Firstly, in experiments where D2 has been removed, this deletion severely reduces the phosphatase activity

of CD45 (Streuli et al., 1990). Secondly, mutation of various conserved residues in D2 either severely inhibits or completely abrogates CD45 activity (Johnson et al., 1992). Finally, deletion of the spacer region between the domains ablates CD45 activity (Ng et al., 1995). Together this suggests the importance of the spatial and structural integrity of D2 is required for optimal phosphatase activity of D1.

Recently the tertiary structure of a dimer of CD45 phosphatase D1 has been solved (Bilwes et al., 1996). The structure of this dimer showed that each domain formed a wedge-like structure that protruded into the phosphatase catalytic site of the other domain. This is particularly interesting as it could explain the inhibition of CD45 activity by ligand-driven dimerisation, as seen in the EGFR/CD45 chimera studies discussed above. Two acidic residues within the wedge domain are found to be highly conserved. A cell line has been created containing an EGFR/CD45 chimeric molecule with a mutation of one of these conserved residues from a glutamate residue at position 624 of CD45 to alanine or arginine (Majeti et al., 1998). These mutated molecules did not show reduction in phosphatase activity on EGF dimerisation. This is presumably due to these mutated molecules having an altered tertiary structure that prevents the interaction of the inhibitory wedge with the phosphatase domain. This supports the idea of ligand induced inhibition of phosphatase activity by dimerisation and could be a general mechanism of regulation of all PTP family members.

### **CD45 genomic structure**

CD45 is encoded by a single gene (*Cd45*) on chromosome 1 in both mouse and man. It is composed of 34 exons stretching across approximately 110kb of DNA (Saga et al., 1988). Transcription is initiated from either of two exons, 1a and 1b which together with exon 2 encode the 5' untranslated region of the gene. The functional significance of the alternative initiation sites is not clear but they are not used in a cell-type specific manner. Exons 3 to 15 encode the extracellular domain of CD45 and includes the alternatively-spliced exons 4, 5 and 6. Exons 3-8

encodes for amino-acid sequences rich in serine, threonine and proline amino acids characteristic of O-linked glycosylation sites. Exon 16 encodes the membrane spanning region whilst exons 17-33 encode the cytoplasmic domain and 3' untranslated region.

### **CD45 and signal transduction in T cells**

As discussed earlier, *in vitro* studies of CD45 mutant cell lines has, in general, indicated a positive role for CD45 in TCR signalling. Both CD4<sup>+</sup> and CD8<sup>+</sup> CD45-deficient murine T cell clones are defective in both antigen-induced proliferation and IL-2 secretion while CD45-expressing revertants regained these abilities (Pingel and Thomas, 1989; Weaver et al., 1991). Moreover, CD45-deficient leukemic T cell lines have been found to be deficient in TCR-induced tyrosine phosphorylation, Ca<sup>2+</sup> mobilisation, and PKC activation (Koretzky et al., 1992; Koretzky et al., 1990; Shiroo et al., 1992). All these features of signal transduction were restored on transfection of a cDNA encoding a phosphatase active CD45 (Desai et al., 1993; Hovis et al., 1993; Volarevic et al., 1993). Together these results clearly point to an absolute requirement for CD45 to maintain functional TCR signal transduction pathways in these cells.

A possible explanation for the regulation of TCR signal transduction by CD45 was found by studying the phosphorylation events that control the activity of the Src-family tyrosine kinases Lck and Fyn. Lck and Fyn are inactive when phosphorylated on a COOH-terminal tyrosine residue (Tyr<sup>505</sup> in Lck and Tyr<sup>528</sup> in Fyn) because of an intramolecular binding of this phosphotyrosine to the SH2 domain of the kinase (Qian and Weiss, 1997). When these negative regulatory tyrosines are dephosphorylated, the conformation of the kinases is altered and they autophosphorylate another regulatory tyrosine (Tyr<sup>394</sup> for Lck and Tyr<sup>417</sup> of Fyn) leading to the full activation of the kinase activity (Qian and Weiss, 1997). Studies in CD45-deficient murine lymphoma as well as human cell lines have demonstrated that Lck and Fyn are both hyperphosphorylated at the negative regulatory tyrosine

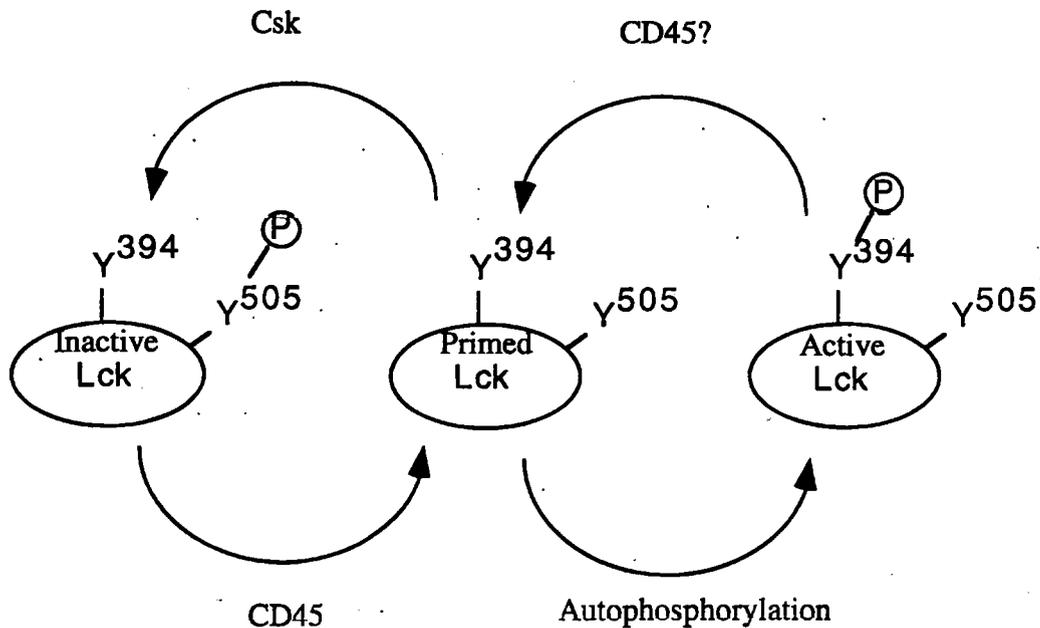
residues and therefore could suggest they are inactive (Hurley et al., 1993; Ostergaard et al., 1989; Sieh et al., 1993). Thus the failure of TCR signal transduction in CD45-deficient T cells could be due to the failure to activate these Src family kinases. When the kinase activity of Lck and Fyn has been assayed directly from CD45-deficient cell lines and compared to wildtype cells, conflicting results have been obtained depending on the cell lines studied. For example, one study found no differences in Lck or Fyn activity in a CD45-deficient murine T cell lymphoma compared to controls (Hurley et al., 1993), others found either an increase in Fyn activity but no change in Lck activity when CD45-deficient HPB-ALL cells were transfected with a cDNA for CD45 (Shiroo et al., 1992) and finally it has been reported that in a CD45-deficient T-leukaemia cell line (CB-1) there are increases in Lck activity without changes in Fyn activity (Biffen et al., 1994). The reason for this confusion could arise from the fact that there may be different pools of these Src-family members, with only those proximal to the receptor being responsible for transduction of signals from it (Biffen et al., 1994). Thus the activity of the receptor associated pool of Src-family kinase activity may be masked when total cellular activity is measured. As discussed earlier, phosphorylation of the negative regulatory tyrosine of Lck and Fyn allows an intramolecular interaction between the SH2 domains and this phosphorylated residue which inhibits their kinase activity (Sieh et al., 1993). These changes in conformation have been utilised to study the activity states of Lck and Fyn by using a synthetic peptide that can bind to the SH2 domain of Src family kinases (Sieh et al., 1993). The SH2 domain becomes inaccessible to the peptide when these kinases are in an inactive conformation as the SH2 domain is bound to the negative regulatory phosphotyrosine. This peptide was used to probe the molecular structure (and therefore the activity state) of Src family kinases in CD45-deficient cell lines and compared to control cells. These studies showed that the amount of active Lck, but not Fyn, is dependent on CD45 (Sieh et al., 1993). Another study was designed to look at receptor-associated pools of Src-family kinase activity (Biffen et al., 1994). This study was conducted in a CD45-deficient sub-clone of the CB-1 T cell line where the total Lck and Fyn kinase activity were similar or higher than

control levels. When the activity of Lck and Fyn associated either with the receptor or coreceptor was assayed it was considerably reduced compared to controls, suggesting that the role of CD45 is to selectively regulate the pools of these kinases which are in close proximity to the receptor.

Although most CD45-deficient cell lines cannot undergo receptor-mediated signalling, intriguingly there is one example of a CD45-deficient T cell line that is still able to signal through its antigen receptor (Chu et al., 1996). This line was found to be unusual in that it expressed the PTK Syk, whereas signalling-incompetent CD45-deficient lines do not (Chu et al., 1996). Comparison of both signalling-competent and incompetent CD45-deficient cell lines found that in both of these lines Lck was in an inactive conformation, Csk activity was equivalent, as was TCR- $\zeta$  chain phosphorylation. Subsequent transfection of Syk into the signalling-incompetent CD45-deficient cell line restored TCR signalling (Chu et al., 1996). This suggests that TCR signalling can proceed in a CD45- and Lck-independent manner through Syk.

More recently, a role for CD45 as both a positive and negative regulator of Src-family kinase activity has been proposed (Shaw and Dustin, 1997). As discussed earlier, the TCR (and indeed the MHC) is a relatively small molecule compared to CD45 extending only 15nm from the cell surface compared to CD45 whose span is 28-51 nm. Removal of CD45 from the vicinity of the receptor could therefore be necessary for TCR/MHC interactions to occur. In resting T cells most Lck molecules are in fact dephosphorylated at both the negatively regulating residue Tyr<sup>505</sup> and the activating residue Tyr<sup>394</sup> (Alexander, 1997). CD45 may be responsible for maintaining the dephosphorylated state of Src-family kinases. Figure 2 shows this model of Src-family kinase regulation using Lck as an example. Dephosphorylation of Lck at Tyr<sup>505</sup> by CD45 allows the kinase to autophosphorylate Tyr<sup>394</sup> and become kinase active. CD45 may also be responsible for the dephosphorylation of Tyr<sup>394</sup> returning the kinase to a "primed" dephosphorylated state. Indeed Csk, has the ability to phosphorylate the negative regulatory tyrosine of Lck and therefore to inactivate it. Thus the activity of

Src-family kinases, and therefore downstream signalling events, could be controlled by the relative activities of CD45 and Csk (Okada et al., 1991). Exclusion of CD45 from the receptor complex during antigen recognition would mean that both the recruitment of inactivated kinases to an active state and inactivation of kinase in an active state would fail to occur (Rodgers and Rose, 1996). As most Lck in a cell is in the “primed” state this could favour the accumulation of active Lck



**Figure 2: Proposed model for the regulation of Lck activity.** Lck is inactive when phosphorylated on a negative regulatory tyrosine Y<sup>505</sup>. CD45 can dephosphorylate this tyrosine residue allowing autophosphorylation on a second activating tyrosine (on Y<sup>394</sup>). Once activated Lck could phosphorylate ITAMs present in the TCR/CD3 complex allowing the binding and activation of ZAP-70 and downstream signal transduction events. CD45 may also be responsible for the dephosphorylation of Y<sup>394</sup> such that CD45 is in a primed state whereby the tyrosine kinase Csk can phosphorylate Lck on its negative regulatory tyrosine and therefore inactivate it. A similar mechanism may apply to other Src-family kinases.

### CD45 and signalling in B cells

Less is known about the role of CD45 in BCR signalling compared to T cell signalling although some studies have been conducted using CD45-deficient B cell lines. In a CD45-deficient subclone of the plasmacytoma cell line J558L $\mu$ m3, BCR ligation no longer triggered Ca<sup>2+</sup> influx, Ras activation, PLC activation and ERK

MAP kinase activation (Justement et al., 1991; Kawauchi et al., 1994), clearly demonstrating a positive role for CD45 in these events. In contrast BCR ligation in a CD45-deficient WEHI-231 derived immature B cell line resulted in prolonged  $Ca^{2+}$  signalling and increased receptor mediated apoptosis suggesting that in this cell line CD45 maybe acts as a negative regulator of BCR signalling (Ogimoto et al., 1994). These difference may be dependent on the stage of maturation of the B cell or reflect peculiarities of transformed cell lines.

CD45 is thought to play a similar role in B cells in regulating Src-family kinases in B cells as it does in T cell activation. Of the Src-family tyrosine kinases associated with signalling from the BCR, CD45 has been shown to be physically associated with Lyn but not Blk or Fyn (Brown et al., 1994). Moreover, Lyn activity is dysregulated in CD45-deficient B cell lines (Katagiri et al., 1995), suggesting a requirement for CD45 for the functional activity of this Src-family kinase. In a similar manner to studies on the phosphorylation patterns of Lck in CD45-deficient T cell lines, Lyn has been found to be hyperphosphorylated on both the negative regulatory tyrosine and on the autophosphorylated tyrosine associated with Lyn activation (Yanagi et al., 1996).

### **CD45 Associated protein**

Most of the CD45 in lymphocytes exists in a complex with a lymphocyte-specific protein called CD45 associated protein (CD45-AP). CD45 and CD45-AP have been shown to specifically bind each other via their transmembrane domains. A role for CD45-AP in signal transduction has been recently revealed by the creation of mice deficient in CD45-AP (Matsuda et al., 1998). These mice have normal lymphocyte development however they have deficiencies in both lymphocyte proliferation and signalling. When the amount of Lck physically associated with CD45 was assayed using immunoprecipitation and Western blotting using control T cells the Lck/CD45 ratio was found to rise after receptor cross-linking. In CD45-AP-deficient T cells the basal Lck/CD45 ratio was much

lower than in control T cells and it did not increase on receptor stimulation. This has led to the suggestion that CD45-AP directly or indirectly mediates the CD45/Lck interaction and it is a defect in this interaction that explains the failure of signal transduction events in the absence of CD45-AP. As yet no information has been published on the phosphorylation status of the Src-family kinases in these mice.

### CD45-deficient mice

The first published CD45-deficient mouse strain created by homologous recombination in ES cells was mutated in exon 6 of the *Cd45* gene (*Cd45<sup>ex6/ex6</sup>*) (Kishihara et al., 1993). Exon 6 is one of the differentially expressed exons of the *Cd45* gene encoding for the epitope recognised by anti-CD45RC monoclonal antibodies. A mutation in this region of the gene was created with the intention of making mice deficient in all isoforms containing this exon but retaining all other CD45 isoforms. However in view of the fact that the targeting left behind a *neo* gene within exon 6, it was not surprising that these mice have virtually no expression of CD45 on most lymphocytes except for a subset of around 20% of T cells. These cells have levels of expression of all isoforms of CD45 (except those containing the targeted CD45RC epitope) equivalent to controls. On the other hand B cells within these animals do not express CD45 and therefore these mice have been particularly useful in studying CD45-deficient B cell development and function. B cell development was found to be normal (Kishihara et al., 1993) and *in vivo* *Cd45<sup>ex6/ex6</sup>* B cells displayed normal T-dependent and T-independent responses (Bachmann et al., 1997; Kong et al., 1995). However *Cd45<sup>ex6/ex6</sup>* B cell proliferation after BCR crosslinking *in vitro* was found to be greatly reduced, confirming the earlier studies of CD45-deficient B cell lines which had concluded that CD45 is a positive regulator of BCR signalling (Benatar et al., 1996; Kishihara et al., 1993).

A second CD45-deficient mouse strain has been made using homologous recombination to target exon 9 of the *Cd45* gene (*Cd45<sup>ex9/ex9</sup>*), an exon common to

all isoforms of CD45 (Byth et al., 1996). These mice were found to have no expression of any isoform of CD45 in either their B or T cells. Their B cell development was found to be identical to that found in *Cd45<sup>ex6/ex6</sup>* in both cell surface phenotype and lack of proliferation following BCR crosslinking. *Cd45<sup>ex9/ex9</sup>* T cell development was found to be deficient particularly in the transition from DP to SP CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes, a feature similar to that found in *Cd45<sup>ex6/ex6</sup>* mice, despite the expression of some CD45 in T cells from these mice.

## 1.5 Aims of the work in this thesis

The aim of the work in this thesis was to gain a greater understanding of the role of CD45 in lymphocyte development and in particular to assess its role in the receptor-dependent processes of positive and negative selection of lymphocytes. This project involved the creation of a mouse strain deficient in CD45 by targeted mutation of exon 12 of the *Cd45* gene, an exon used in all isoforms of CD45. This project was originally started prior to the publication of the *Cd45<sup>ex6/ex6</sup>* mice (Kishihara et al., 1993) and as these mice had expression of CD45 in a proportion of their T cells we decided to create mice with a deficiency in a conserved exon of the CD45 gene. During the course of this project the second CD45-deficient (*CD45<sup>ex9/ex9</sup>*) mice was reported (Byth et al., 1996). In this thesis I describe the generation of mice targeted in exon 12 of *Cd45* (*Cd45<sup>-/-</sup>*) and present the analysis of B and T cell development in the absence of CD45, with particular emphasis on developmental checkpoints known to require antigen receptor signals.

# Chapter Two

## MATERIALS AND METHODS

### 2.1 Solutions

TAE: 0.04M Tris-HCl, 0.02M glacial acetic acid, 1mM EDTA (pH 8.0).

SSC: 0.15M NaCl, 0.015M tri-sodium citrate (pH 7.2).

TE: 10mM Tris-HCl pH 8.0, 0.1mM EDTA.

PBS: 150mM NaCl, 2mM KCl, 8mM Na<sub>2</sub>HPO<sub>4</sub>.

PBS-Tween: PBS containing 0.05% Tween-20.

CAPS: 10mM 3-cyclohexylamino-1-propanesulphonic acid, pH 11.0.

TNES: 10mM Tris-HCl, pH 8.0, 100mM NaCl, 1mM EDTA, 1% SDS, 0.25mg/ml Proteinase K.

Tail Mix: 50mM Tris-HCl, pH 8.0, 100mM NaCl, 100mM EDTA, 1% SDS, 0.25mg/ml Proteinase K.

Hybridisation Mix: 0.2M NaPO<sub>4</sub>, 1mM EDTA, 1% BSA, 7% SDS, 15% formamide.

TBS: 25mM Tris-HCl pH 7.4, 140mM NaCl, 5mM KCl.

SDS-PAGE upper buffer: 0.125M Tris-HCl, 0.1% SDS, pH 6.8.

SDS-PAGE lower buffer: 0.375M Tris-HCl, 0.1% SDS, pH 8.8.

SDS-PAGE running buffer: 0.025M Tris base, 0.19M glycine, 0.1% SDS.

DNA sample buffer: 3% glycerol and 0.02% bromophenol blue and xylene cyanol FF.

NaI solution: 6M NaI, 0.1M Na<sub>2</sub>SO<sub>3</sub>.

Denaturing Buffer : 0.5 M NaOH, 1.5M NaCl.

Stop Buffer: 0.025M EDTA, 1% SDS in TE.

High Salt Buffer: 2x SSC, 0.5% SDS.

Low Salt Buffer: 0.1x SSC, 0.5% SDS.

HBS/2-ME: HBS with  $7 \times 10^{-4}$ M  $\beta$ -mercaptoethanol.

NP40 lysis buffer: 1% NP40, 150mM NaCl, 20mM Tris pH 7.0, 10mM iodoacetamide and 1mg/ml of each of the peptide inhibitors chymostatin, leupeptin and pepstatin.

Laemmli reducing sample buffer: 10% glycerol, 3% SDS, 0.5x SDS-PAGE upper buffer, 500mM  $\beta$ -mercaptoethanol, 0.005% bromophenol blue.

Na Citrate buffer: 50mM sodium Citrate, pH 5.5.

DNA sample buffer: 20% glycerol, 100mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol.

## 2.2 Tissue Culture Media

ES cell medium: Dulbecco's Modified Eagles Media (DMEM), 15% foetal calf serum, 50 units/ml penicillin, 2mM glutamine, 50 $\mu$ g/ml streptomycin,  $5 \times 10^{-5}$ M  $\beta$ -mercaptoethanol, non-essential amino acids (Gibco BRL) and  $1 \times 10^3$  units/ml LIF (Gibco BRL).

EF medium: DMEM with 10% Foetal calf serum (FCS) and 50 units/ml penicillin, 2mM glutamine, and 50 $\mu$ g/ml streptomycin.

Blastocyst media: Dulbecco's Modified Eagles Media with 10% FCS and 50 units/ml penicillin, 2mM glutamine and 50 $\mu$ g/ml streptomycin.

Injection Media: As above but with 20mM Hepes.

Mast cell medium: RPMI 1640, 10% foetal calf serum, 2mM glutamine, 1mM sodium pyruvate, 50 $\mu$ M  $\beta$ -mercaptoethanol, 1mM sodium pyruvate, non-essential amino acids (Gibco BRL) and 10% conditioned medium from WEHI-3B cells as a source of IL-3.

NTOC medium: Iscove's modified Dulbecco's medium, 10% FCS, 50 $\mu$ M  $\beta$ -mercaptoethanol, 50 units/ml penicillin, 2mM glutamine and 50 $\mu$ g/ml streptomycin.

RPMI-COM medium: RPMI1640 (Gibco), 50 units/ml penicillin, 2mM glutamine and 50 $\mu$ g/ml streptomycin.

Trypsin solution: 0.25% trypsin (Gibco), 1.4mM EDTA, 120mM NaCl, 0.8mM Na<sub>2</sub>PO<sub>4</sub>, 1.8mM KH<sub>2</sub>PO<sub>4</sub>, 4.0mM KCl, 5.6mM D-glucose, 2.5mM EDTA, 0.005% Phenol Red, pH 7.6.

## 2.3 Bacterial Media

Luria broth: Bacto-tryptone 10g/l, Yeast extract 5g/l, NaCl 10g/l adjusted to pH 7.2.

Luria agar: as above but with 15g/l Difco agar.

Antibiotics: Ampicillin 100 $\mu$ g/ml when needed.

## 2.4 Embryonic Stem Cells

### Preparation of Embryonic Fibroblast Feeder Cells

G418-resistant embryonic fibroblast feeder cells (EFneo) were prepared by mating homozygous IL3-deficient mice which contain the *neo* gene (Nishinakamura et al., 1996) with wildtype female BALB/c mice. Pregnant females were sacrificed at day 13 of gestation. Soft organs and viscera were dissected from the embryos and the remaining carcass minced finely in Trypsin solution (2ml/embryo). The embryos were then placed at 30°C for 30min with disruption by pipetting every 5-10min. An equal volume of EF medium was added, mixed and allowed to settle for 1-2min. The supernatant was removed and each embryo was plated out into a T175 flask which was incubated at 37°C, 5% CO<sub>2</sub> overnight. The following day the medium was changed and the cells incubated again until they reached extreme confluency. Each flask was then split into five T175 flasks and again grown to extreme confluency. These confluent flasks were again split into five T175 flasks. The cells were then trypsinised, resuspended in medium and irradiated with 3500 rads.

and frozen at  $4 \times 10^6$  cells/vial or  $20 \times 10^6$  cells/vial in EF medium containing 10% DMSO.

### **Routine Maintenance of ES Cells**

All plates for ES cell growth were gelatinised using 0.1% gelatin for one hour at room temperature. Irradiated EFneo feeders were thawed out and spun through medium and resuspended in ES medium prior to use. EFneo cells were used at the following densities:  $4 \times 10^6$  cells/10cm dish,  $2 \times 10^6$  cells/6cm dish,  $1.3 \times 10^5$  cells/24 well plate and  $2 \times 10^4$  cells/96 well plate. ES cells were fed 1-4hr before passage, washed with PBS and trypsinised as described elsewhere. ES cells were normally fed every day when routinely passaging. Cells were normally split 1/10 every 3 days onto new plates with feeders.

### **Electroporation of ES Cells**

D3 ES cells (Tybulewicz et al., 1991) were grown to 50-80% confluency on EFneo feeders in a 10cm tissue culture plate. The DNA targeting construct, that was previously prepared by CsCl<sub>2</sub> purification and was linearised by *NotI* restriction digestion, was ethanol precipitated by standard procedures and dissolved in sterile TE at 2mg/ml. 10cm plates to receive the electroporation were seeded with  $4 \times 10^6$  EFneo cells. The ES cells were trypsinised and disrupted to produce a single cell suspension and resuspended in HBS/2-ME at  $10^7$  cells/ml with 30-50µg/ml DNA. For each electroporation 0.8ml of the cell/DNA suspension was placed in an electroporation curvette (0.4cm electrodes, Biorad). Electroporations were carried out at 400V and 25µF using a Biorad Gene Pulser. The cells were then resuspended in 3.3ml of media and 10% of the cells plated out onto one 6cm plate with G418 alone (to allow the calculation of enrichment) and the remaining cells were plated onto four 10cm plates containing G418 and gancyclovir. G418 was used at 300µg/ml and gancyclovir at 2µM, unless otherwise stated, and was added

to the cells 24hrs after plating out the electroporated cells. Plates were then incubated at 37°C for approximately 10 days prior to picking of ES cell colonies.

### **Picking and Expanding ES Clones**

The day before picking of the ES cell colonies, a 96 well plate (Costar) was seeded with  $2 \times 10^4$  irradiated EFneo feeders per well. On the day of picking the colonies a further 96 well plate was prepared with 40 $\mu$ l trypsin per well. The 10cm plates containing the colonies were washed with PBS and 10ml of PBS was added. The colonies were picked under the microscope using a P200 Gilson pipette set to 20 $\mu$ l. Individual colonies were then placed in the 96 well plate containing the trypsin. After incubation at 37°C for 3min the colonies were disrupted by vigorous pipetting. The dispersed clones were then placed into the 96 well plate that contained the feeders. The colonies were fed the next day and allowed to grow for six days prior to trypsinisation and transfer into 24 well plates containing EFneo feeders. Four days later the confluent ES clones were used to make DNA and were frozen as described below.

### **Freezing of ES Clones**

After the ES cell clones reached confluence in the 24 well plates, as described above, half the cells were frozen and the other half was used to make DNA for Southern analysis. Each well was washed in PBS and 80 $\mu$ l trypsin was added. Plates were incubated at 37°C for 3min and the clones disrupted using a P200. 40 $\mu$ l of the cells were added to 400 $\mu$ l of ES medium containing 12% DMSO in a 1.5ml freezing vial. These vials were then placed at -70°C overnight prior to placing them into liquid nitrogen. The remaining 40 $\mu$ l of cells were used to make DNA as described below.

## **Preparation of Genomic DNA from ES Cells**

Half the ES cell clones grown to confluence in the 24 well dishes were removed for freezing as described above, whilst the other half were placed in a 1.5ml microfuge tube to make DNA. After addition of 400 $\mu$ l of TNES with 0.5mg/ml proteinase K, these cells were incubated at 37°C overnight. Next an equal volume of TE-equilibrated phenol was added and the sample extracted by vortexing. The sample was spun in a microfuge and the aqueous layer was removed this was followed by a further extraction using an equal volume of chloroform. After centrifugation the aqueous layer was again removed and the DNA collected by ethanol precipitation (two volumes EtOH:3M NaOAc in a 25:1 mix) and spooling out with a sealed Pasteur pipette. The DNA was next washed in 70% ethanol and air-dried before being dissolved in 30 $\mu$ l TE. Typically 10 $\mu$ l was used for subsequent restriction digestion and Southern blotting.

## **2.5 Microinjection**

### **Harvesting of Blastocysts for Microinjection**

Four days prior to microinjection, B6 females were mated to B6 males and mice with vaginal plugs were set aside. On the day of microinjection the female mice were sacrificed by cervical dislocation and their uterine horns removed. Fat around the uterine horns was dissected away and the horns flushed with 0.5ml Blastocyst media into a 35mm Petri dish. The blastocysts were removed from the debris with a pipette tubing flamed and pulled to give a diameter of approximately 200 $\mu$ m which was attached to a rubber tube and manipulated by mouth. The recovered blastocysts were then placed in a drop of Blastocyst medium, under oil to prevent evaporation and placed in a 37°C, 5% CO<sub>2</sub> incubator until required.

## **Microinjection of Blastocysts**

Microinjection was carried out using standard techniques (Robertson). A Nikon microscope and Leitz micromanipulators assembled on a hydraulic table were used. Injections were conducted in Injection Medium in a solid state cooling chamber at 10°C (Robertson). Approximately 15 ES cells were injected into each blastocyst and the injected blastocysts were stored in a drop of Blastocyst medium under oil in a incubator at 37°C, 5% CO<sub>2</sub> until ready to transfer to the foster mothers.

## **Transfer of Embryos to Foster Mothers**

Embryos were transplanted into (C57/BL/6 x CBA) F<sub>1</sub> pseudopregnant foster mothers on the same day of injection. Three days prior to the day of transfer the foster mothers were mated to vasectomised males and those females with vaginal plugs were set aside for use. Just prior to the transfer the female fosters were anaesthetised using 17µl of 2.5% Avertin per gram of body weight. 10-15 embryos were transferred into one of the uterine horns of each mouse. Chimaeras that were born were identified by their agouti coat colour chimaerism against the black of the host. Male chimaeras were then initially bred to C57/BL6 (B6) females. This allows the identification of chimaeras that undergo germline transmission of the ES cell line by the presence of agouti pups from these matings. Transmission of the mutation was confirmed by Southern blotting of tail DNA from these pups.

## **Preparation of genomic DNA from mice**

For genotyping, a tail biopsy of approximately 1cm in length was taken from each mouse at the time of weaning and placed in 700µl of Tail Mix. The samples were rotated at 55°C overnight before being extracted twice with an equal volume of phenol:chloroform (1:1) and once with chloroform. After the final extraction, 0.6 volumes of isopropanol was added to the aqueous layer and the sample vortexed to

generate a pellet of DNA, which was then spooled out using a flame-sealed Pasteur pipette. After washing in 70% ethanol, the DNA was left to dissolve overnight at 4°C in 100µl TE.

## 2.6 Molecular Biology

### Endonuclease Restriction Digestion of DNA

DNAs were digested using restriction enzymes in the buffer recommended by the manufacturer (New England Biolabs). Typically 1-2µg of plasmid DNA was digested in a volume of 10µl for 1hr whilst 10µg of genomic DNA was digested in a 25µl volume overnight. In both cases the enzyme constituted no more than 5% of the volume of the reaction. Digests were terminated by the addition of 1/10 volume of DNA sample buffer.

### Agarose Gel Electrophoresis of DNA

For routine analysis, preparative gels and Southern blotting, DNA was separated in 1% w/v molecular biology grade agarose gels (SeaKem™, FMC Bioproducts) whilst fragments smaller than 0.5kb were analysed using 1% SeaKem™, 3% NuSieve™ (FMC Bioproducts) gels made up in TAE with 1µg/ml ethidium bromide (EtBr) added to both gel and buffer to visualise the DNA under ultra-violet light (UV). DNA sample buffer was added to the samples prior to loading. All agarose gels were powered in horizontal perspex electrophoresis tanks (Owl Scientific). Running buffer was TAE with 1µg/ml EtBr and gels were run at 10Vcm<sup>-1</sup>. UV analysis was performed using a UVP<sup>INC</sup> CHROMATO-VUE<sup>R</sup> transilluminator. *Bst*EII digested λ DNA was used routinely as size markers (NEB).

## **Isolation of DNA Fragments from Agarose Gels**

Purification of DNA restriction fragments from agarose gels was routinely used prior to using these fragments in ligation reactions in cloning experiments. After agarose gel electrophoresis the band of interest was cut out using a clean razor blade. 2-3 volumes of NaI solution was added to the gel slice and the sample incubated at 50°C with occasional vortexing until the gel was completely dissolved (around 10min). Between 2-5µl of a silica/water slurry (1:1) was added and the mixture incubated on ice for 17min with occasional vortexing. The glass, to which the DNA adheres, was spun out in a microfuge for 15s and the pellet washed once with 300µl NaI solution and twice with ice-cold ethanol wash solution (50% ethanol, 0.1M NaCl, 10mM Tris pH 7.5). After the last wash, the sample was spun briefly in a microfuge and the remaining last drops of ethanol wash solution removed with a microcapillary. The glass was then resuspended in the desired amount of TE and incubated at 50°C for a further 17min to allow the DNA to elute, after which the glass was spun out (15s in a microfuge) and the DNA solution recovered from the liquid phase.

## **Transfer of DNA from Agarose Gels to Membranes**

Genomic DNA was digested with the appropriate restriction enzyme as described earlier and separated according to size on a 1% agarose gel with 1µg/ml EtBr in TAE. Gels were run at 1-2 Vcm<sup>-1</sup> for 14-16 hours and photographed. The DNA was denatured by twice soaking in Denaturing buffer for 20min with gently rocking. The DNA was then transferred to Hybond N+ nylon membrane (Amersham) by capillary action using 20x SSC as transfer buffer according to the manufacturer's instructions and the DNA was bound to the membrane by placing the filter onto 2 sheets of Whatmann paper soaked in 0.4M NaOH for 20min.

## **Labelling of DNA using Random Priming**

Labelling of DNA with which to probe Southern blots was achieved using the random priming kit from Boehringer and according to their instructions using  $\alpha$ -<sup>32</sup>P dCTP (~ 6000Ci mmol<sup>-1</sup>, Amersham). Briefly 25-50ng of the probe fragment in a volume of 9 $\mu$ l was boiled for 5min to generate single stranded DNA. The reaction was then set up according to the manufacturer's instructions by adding 3 $\mu$ l of nucleotide mixture (without dCTP), 2 $\mu$ l of the hexanucleotide reaction mixture and 5 $\mu$ l of the  $\alpha$ -<sup>32</sup>P dCTP. To this reaction 2 $\mu$ l of Klenow enzyme was added, vortexed, spun briefly (15s) in a microfuge, and incubated at 37°C for 30min. The reaction was stopped by adding 100 $\mu$ l of Stop Buffer. Unincorporated nucleotides were removed using a Sephadex G-50 column (Pharmacia) according to the manufacturer's instructions. The probe was eluted from the column in a 400 $\mu$ l volume. 2 $\mu$ l of the purified labeled probe was removed and the amount of radioactivity incorporated was measured in a beta counter (Pharmacia). A typical specific activity was around 10<sup>8</sup>cpm/ $\mu$ g.

## **Hybridisation of Southern Blots**

Filters carrying the target DNA were wetted in 2x SSC and inserted into a hybridisation bottle (Hybaid). 10ml of Hybridisation mix was added and the bottle rotated in a hybridisation oven at 65°C for a minimum of 15min. After the prehybridisation, the probe, along with 0.1mg/ml herring sperm DNA, was boiled for 5min to denature the DNA and this mix was added to the hybridisation bottle. The membranes were then washed to remove non-specifically bound probe. Firstly the membranes were washed three times in High Salt Buffer for 20min each wash at 65°C, followed by two further 20min washes in Low Salt Buffer. After the final washes, the membranes were placed between two sheets of Saran Wrap (Dow) and exposed to film at -70°C using intensifying screens to strengthen the signal. Probed filters could be stripped to remove the probe to allow reprobing with a different

fragment. This was achieved by adding boiling 0.5% (w/v) SDS to the membrane and allowing to cool to room temperature.

### **Ligation of DNA Fragments**

Vector and insert that had been digested with the appropriate restriction enzyme and dephosphorylated or blunted as required were purified after gel electrophoresis as described previously. Typically 300ng of purified insert DNA was ligated in a reaction with a vector to insert ratio of 1:3 moles. Ligations were carried out in a volume of 10 $\mu$ l using the protocol and buffer supplied with T4 DNA ligase (New England Biolabs). The reaction was routinely incubated at 15°C overnight and ligation products used to transform *E. coli*. Some ligation reactions required the filling in of 5' overhanging DNA termini prior to the ligation reaction. Typically 1 $\mu$ g of insert in 20 $\mu$ l of distilled water was incubated with 4 units of Klenow fragment in the appropriate buffer supplied with the enzyme (New England Biolabs). The reaction was incubated for 20min at room temperature and stopped by the addition of EDTA to 5mM, followed by heating to 75°C for 10min.

### **Transformation and Culture of *E. coli***

Competent *E. coli* (Subcloning Efficiency DH5 $\alpha$ ) were purchased from Gibco BRL and the transformation was carried out according to manufacturer's instructions. *E. coli* were grown in liquid culture in Luria broth containing ampicillin. 2ml small scale cultures (minipreps) were grown in sterile universals and 500ml cultures (maxipreps) were grown in 2l conical flasks, overnight at 37°C with shaking at 225rpm to aerate the media. Long term storage of *E. coli* strains was by storage at -70°C in 12% sterile glycerol.

## Harvesting of Plasmid DNA

Both small scale and large scale cultures were harvested and the plasmid DNAs recovered using standard techniques. Plasmid DNA from maxipreps was purified by centrifugation in a caesium chloride gradient by standard procedures. All DNA preparations were stored in TE at -20°C. Quantification of DNA was accomplished by measuring the optical density of a 1 in 200 dilution of the sample at a wavelength of 260nm using a spectrophotometer and assuming that an  $OD_{260nm}$  of 1.0 represents a concentration of 50µg/ml.

## 2.7 Mice

All strains were bred at the National Institute for Medical Research in a conventional facility. The B and T cell transgenics have been described previously: BM3.6 (Sponaas et al., 1994), A1(m-2) (Douek et al., 1996) F5 (Mamalaki et al., 1993) and 3-83µδ (Nemazee et al., 1991). The presence of the transgenes as well as mutations in endogenous genes were screened for by Southern analysis of mouse tail DNA using standard techniques described above. All mice were between 6 and 16 weeks of age when analysed. The *Cd45<sup>-/-</sup>* BALB/c line was created by backcrossing the mutation 10 generations onto BALB/c mice. All other mice were outbred being mixtures of 129/Sv, C57BL/6 (B6), DBA/2 and CBA. Littermates were used as controls in all cases.

## 2.8 Flow cytometric analysis

Expression of cell surface antigens was determined by cytofluorometric analysis. Typically  $1 \times 10^6$  cells were stained with biotinylated, FITC- or PE- conjugated mAbs (see below) in PBS containing 0.1% sodium azide and 1% BSA for 45min at 4°C in the presence of 10% heat-inactivated normal rat serum. Biotinylated antibodies were revealed with streptavidin-PE (Biogenesis, Poole),

streptavidin-RED613, or streptavidin-RED670 (Gibco-BRL). Fluorescence was acquired and analysed with Cellquest software on a Facs Vantage flurocytometer (Becton-Dickinson). Dead cells were excluded on the basis of low forward light scatter and only live cells falling within the lymphocyte scatter gate are shown.

## Antibodies

The Table below lists the source of monoclonal antibodies used.

<b>Specificity</b>	<b>Antibody</b>	<b>Species</b>	<b>Conjugate</b>	<b>Source</b>
3-83 idiotype	54.1	Rat IgG	BIO and FITC	(Nemazee et al, 1991)
BM3 clonotype	Ti98	Rat IgG	BIO and FITC	(Sponaas et al., 1994)
CD11b	M1/70	Rat IgG2b	BIO	Pharmingen
CD19	1D3	Rat IgG2a	FITC	Pharmingen
CD3	2C11	Hamster IgG	BIO	Pharmingen
CD4	H129.19	Rat IgG2a	RED613	GibcoBRL
CD4	GK1.5	Rat IgG2b	FITC	ATCC
CD44	IM7	Rat IgG2b	PE	Pharmingen
CD5	53-7.3	Rat IgG2a	APC	Pharmingen
CD69	H1.2F3	Hamster	BIO	Pharmingen
CD8	53-6.7	Rat IgG2a	APC	Pharmingen
CD8	YTS169vF		FITC	(Cobbold, 1984)
HSA	M1/69	Rat IgG2b	PE	Pharmingen
IgD	1.19	Rat IgG2a	BIO	Pharmingen
IgM	b-7-6	Rat IgG1	FITC	ATCC
L-Selectin	MEL-14	Rat IgG2a	FITC	Pharmingen
pan-V $\beta$ 8	F23.1	Mouse IgG2a	BIO	(Staerz, 1985)

TCR $\alpha\beta$	H57-597	Hamster IgG	FITC	Pharmingen
VB-11	RR-3-15	Rat IgG2b	BIO	Pharmingen
V $\beta$ 3	KJ25	Hamster IgG	BIO	Pharmingen
V $\beta$ 5	MR9-4	Mouse IgG1	BIO	Pharmingen
V $\beta$ 8.2	F23.2	Mouse IgG2a	BIO	(Staerz, 1985)

### **Intracellular calcium analysis**

Thymocytes were incubated with Indo-1 acetoxy-methyl ester (Indo-1; 1mM; Calbiochem) for 45min at 37°C in RPMI1640 (Gibco), 1% bovine serum albumin, washed and stained with anti-CD8-FITC, anti-CD4-PE and anti-CD3. Four colour cytometric analysis was performed on a FACSVantage dual laser flow cytometer (Becton-Dickinson). FITC and PE were excited by an argon ion laser (488nm, 100mW) and Indo-1 by a UV argon ion laser (320nm, 50mW). Indo-1 emission was detected using 405/40nm (violet) and 495/20nm (blue) bandpass filters. To determine relative calcium concentration, cells were warmed to 37°C, analysed for 30-45s to establish baseline calcium levels and CD3 was crosslinked by the addition of up to 100 $\mu$ g/ml goat anti-hamster-IgG (Jackson Immunoresearch). Acquisition was continued in real time for up to six minutes.

## **2.9 B cell Purification and Proliferation Assay**

Spleens were dissected out aseptically and cells disrupted using a sterile cell strainer (Falcon) and resuspended in 1.8ml of RPMI COM/ABS medium (Gibco). To remove T cells rat anti-mouse Thy-1 antibody (NIMR-1) was added at 1/900 final concentration with 1/9 dilution of guinea pig complement pre-absorbed with agarose (Harlan Seralab). Cells were incubated for 30-45min at 37°C. Cells were spun at 2000rpm for 5min to remove cell debris. The supernatant was discarded and the cells resuspended in 500ml of RPMI-COM and added to a Percoll gradient. The Percoll gradient was a three step gradient with 85% Percoll as the bottom layer, 75% in the middle, and 50% Percoll at the top. The cells were carefully layered on

top of the gradient and the cells spun for 20min at 2,500rpm. Small resting B cells which form the a layer between the 85% and 75% Percoll gradient were removed and washed in media to remove any Percoll. Cells were then seeded into a 96 well flat bottom dish (Falcon) at  $10^6$  cells per well in 100 $\mu$ l. The b-7-6 anti-mouse IgM antibody was then added at the stated concentrations in media with or without IL-4 (100U/ml) and the plates incubated at 37°C, 5% CO<sub>2</sub> for two days. Next 0.5 $\mu$ Ci (5.0Ci/mmol, Amersham) <sup>3</sup>H-thymidine was added per well and the plate incubated for 4hrs. Supernatants were harvested on a Wallac Betaplate cell harvester and incorporation of <sup>3</sup>H-thymidine was measured using a betaplate counter.

## 2.10 Culture of Mast Cells

Mast cell cultures were generated by culturing  $2 \times 10^5$  bone marrow cells from 6 week old *Cd45<sup>+/+</sup>* or *Cd45<sup>-/-</sup>* mice in Mast cell medium as described previously (Costello et al, 1997). After 7 days in culture the adherent cells were scraped off and combined with the suspension cells and reseeded at  $2 \times 10^5$ . This was repeated every week. After 4 weeks of culture when the cells were predominately mast cells, only the suspension cells were propagated.

## 2.11 Western blotting

### Total Cell Lysates

$2 \times 10^6$  primary murine cells or cultured mast cells were pelleted at 1200rpm for 3min in a benchtop centrifuge and washed twice with PBS. 50 $\mu$ l of NP40 lysis buffer was added and the samples rotated at 4°C for 15min. After the lysis the samples were spun in a microcentrifuge for 15min at 4°C to remove the nuclei and membranous debris. The supernatants were transferred to a fresh tube and an equal volume of Laemmli reducing sample buffer was added. The samples were then heated to 100°C for 5min prior to loading on to the gel.

## **Immunoprecipitation**

$10^7$  cells were pelleted, washed and lysed in 1ml of lysis buffer as described for total cell lysates. After spinning out the nuclei,  $3\mu\text{l}$  of antibody specific for the cytoplasmic domain of CD45 was added and the samples incubated rotating for 1hr at  $4^\circ\text{C}$ . The antibody and protein were precipitated by adding  $20\mu\text{l}$  of Protein-A-Sepharose beads (Pharmacia) and the samples were incubated rotating for a further 2hr at  $4^\circ\text{C}$ . Protein was recovered by spinning samples in a microfuge for 15s to pellet the beads and the supernatant was removed by aspiration. Some of the samples were used to confirm by Western analysis that the immunoprecipitation was successful. In these samples the beads were washed 4 times in 1ml of lysis buffer before being resuspended in  $100\mu\text{l}$  of Laemmli sample buffer, boiled for 5min, centrifuged, and the supernatant used to run on a polyacrylamide gel as described below. For samples that were to be used for tyrosine phosphatase assay, the precipitated beads were washed three times in 1ml of lysis buffer, followed by three washes in 1ml Na Citrate buffer and resuspended in  $100\mu\text{l}$  of Na Citrate buffer ready for the tyrosine phosphatase assay. The immunoprecipitation of Lyn, used as a control in these experiments, was conducted using anti-Lyn antibodies previously coupled to beads which were a gift from Dr. C. Cunningham.  $20\mu\text{l}$  of these packed beads were added to the total cell lysates and the samples were then treated the same as those containing anti-CD45 antibodies.

## **SDS-Polyacrylamide gel electrophoresis (PAGE)**

40ml gels were poured using 10ml of Protogel (National Diagnostics), which results in a 10% acrylamide gel, in SDS-PAGE lower buffer.  $100\mu\text{l}$  of 25% ammonium persulphate and  $20\mu\text{l}$  of TEMED were added to polymerise the gel. Stacking gels (4% acrylamide) were cast using SDS-PAGE upper buffer. Gels were run in SDS-PAGE running buffer for either 4 hours at 200V or overnight at

50V. Pre-stained molecular weight protein markers (16kD-175kD) were run alongside the samples (New England Biolabs).

### **Immunoblotting/ECL**

Proteins separated by SDS-PAGE were immobilised on polyvinylidene flouride (PVDF) membrane (Millipore) for immunoblotting. The PVDF membrane was soaked in methanol to pre-wet it before being immersed in CAPS. Transfers were carried out at pH 11.0 as proteins are negatively charged at this pH and this increases the efficiency of the transfer. The blot was then assembled in the transfer tank cassette using filter papers soaked in CAPS and with the gel nearest the cathode of the tank. The tank was filled with CAPS and the buffer recirculated throughout the tank during transfer. Blotting was carried out at 100V and 300mA constant current for between 6-12hr at 4°C. The PVDF membrane was then placed in blocking solution (PBS-Tween with 5% Marvel) for 1hr prior to antibody staining.

After vigorous washing of the blot in PBS-Tween for 10min the blot was firstly stained with rabbit polyclonal antibodies to the intracellular portion of CD45 (gifts of Dr. M Thomas or Dr. P Johnson) diluted a thousand fold in PBS-Tween with 2% Marvel and incubated at room temperature for 1hr. After washing 4 times in PBS-Tween the anti-CD45 antibodies were detected by using horseradish peroxidase (HRP)-protein A (Amersham) for 30min at room temperature at the concentration recommended by the manufacturer. The blots were then washed 6 times for 5min with PBS-Tween to remove any unbound secondary reagent. CD45 was detected on immunoblots using enhanced chemiluminescence (ECL™) reagents (Amersham). This method utilises the oxidation of luminol by horseradish peroxidase, an enzymatic reaction which generates light which is detected by film. 10ml of the ECL reagent was added to the blot for 1min with gentle rocking. Finally the blot was sandwiched between two sheets of Saran Wrap and the excess ECL solution removed using a roller. The blot was placed in a cassette with

X-ograph XB-200 high definition film for approximately 15-60s or until the correct exposure time was achieved.

### Membrane stripping

Blots were stripped by incubating in a solution of 10mM  $\beta$ -mercaptoethanol, 2%SDS and 6.25mM Tris-HCl pH 6.7 at 50°C for 30min. After washing 4 times in PBS-Tween for 10min per wash in a fume hood, the blot was re-blocked in 5% Marvel for up to 12 hours prior to reuse.

## 2.12 Tyrosine phosphatase assay

Tyrosine phosphatase activity was assessed using a kit from Promega and was conducted as described in the instructions for the kit. Total cell lysates were prepared and immunoprecipitations conducted as described earlier. Assays were performed using a mixture of both the tyrosine phosphorylated peptides provided with the kit in Na Citrate buffer. Assays were conducted in the presence of a standard curve using dilution's of a 1.0mM  $\text{KH}_2\text{PO}_4$  phosphate standard provided with the kit. Reactions were incubated at 37°C for 15min and the reaction stopped by the addition of the Molybdate Dye/Additive mix as described in the instructions. Plates were then read at 630nm using a Titertek Multiscan MCC/340 Elisa plate reader.

## 2.13 Neonatal thymic organ cultures

Neonatal (day of birth) thymus lobes were obtained from matings of  $\text{Cd45}^{+/+}$  or  $\text{Cd45}^{-/-}$  mice that were also  $\text{Rag-1}^{-/-}$  and carried the F5 TCR transgene. Mature  $\text{CD4}^+$  single positive thymocytes were generated in neonatal thymic organ cultures (NTOCs) as previously described (Basson et al., 1998; Bommhardt et al., 1997) in

the presence of a genetically engineered bispecific F(ab')<sub>2</sub> reagent recognising both CD3ε and CD4 (Kostelny et al., 1992). Lobes were cultured for three days in culture medium containing antibody, transferred to fresh filters in medium without antibody and cultured for an extra day prior to FACS analysis. Single cell suspensions prepared from the lobes were stained with directly conjugated antibodies to CD4 (APC-conjugated RM4-5, Pharmingen), CD8α (PE-conjugated 53-6.7, Sigma), Vβ-11 (FITC-conjugated KT11.5) and biotinylated PNA (Oxford Glycosystems), followed by streptavidin-RED613 (Life Technologies). Live cells were gated on forward/side scatter profiles and numbers of mature (Vβ-11<sup>hi</sup>/PNA<sup>lo</sup>), CD4<sup>+</sup> cells that differentiated were calculated from three individual lobes for each concentration of anti-CD3/CD4.

# Chapter Three

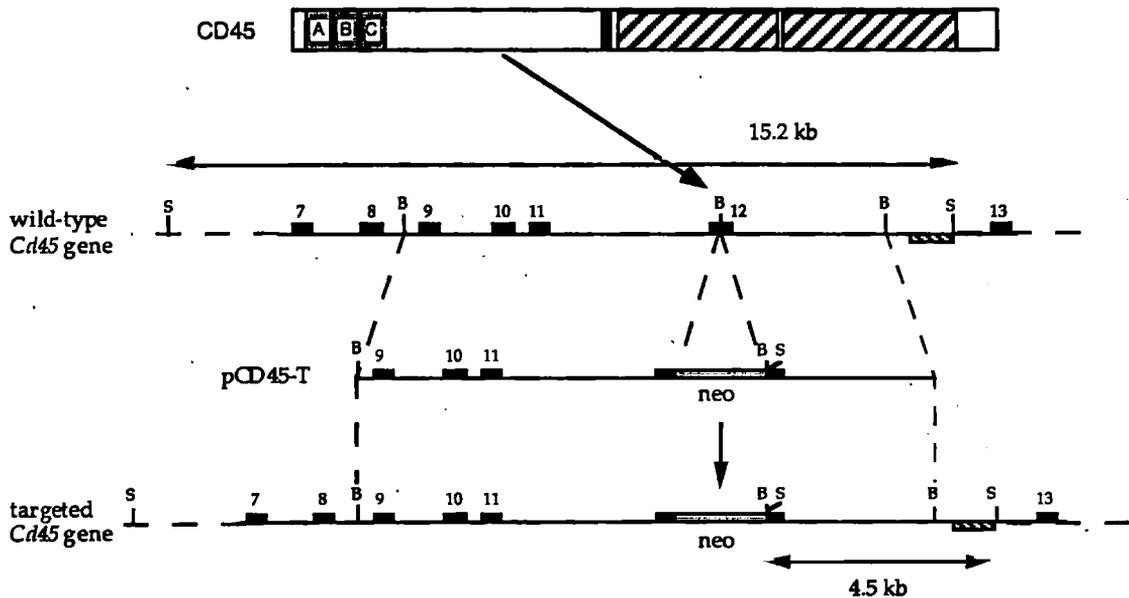
## RESULTS

### 3.1 Creation of CD45-deficient mice

#### Construction of the *Cd45* targeting vector

To create the *Cd45*<sup>-/-</sup> mice I used the technique of gene targeting by homologous recombination to insert a neomycin (*neo*) gene cassette into a *Bam*HI site in exon 12 of the *Cd45* gene in 129/Sv-derived embryonic stem (ES) cells (Figure 3). This exon is located 3' to the differentially spliced *Cd45* exons 4-6 and 5' to the transmembrane domain exon 16. I chose to target exon 12 of the *Cd45* gene as it encodes part of the extracellular domain and thus targeting this exon would separate the signal sequence of CD45 from the phosphatase domain; either part of the *Cd45* gene, 5' or 3' of exon 12 is unlikely to be able to generate a membrane localised tyrosine phosphatase; work in CD45-deficient cell lines demonstrated that in order to function, CD45 needs to be membrane associated. Furthermore, exon 12 is not known to be subject to alternative splicing and is thus common to all CD45 isoforms. Hence I anticipated that mice targeted in exon 12 of *Cd45* would be deficient in all isoforms. My colleague Edmund Siu had previously isolated overlapping  $\lambda$  bacteriophage clones containing *Cd45* genomic sequences by screening a 129/Sv library with an 806 bp *Eco*RV/*Hind*III fragment from the mouse *Cd45* cDNA (Chang et al., 1991) extending from exon 7 to exon 13. He isolated four phage containing overlapping genomic fragments that spanned 16 kb of the 100 kb genomic sequence. In collaboration with Dr.V. Tybulewicz, from one of these bacteriophage I subcloned a 6.7 kb *Bam*HI fragment extending from intron 8 to exon 12 and a 3.1 kb *Bam*HI fragment extending from exon 12 to intron 12 into

the *XhoI* and *EcoRI* sites respectively of pPNT (Tybulewicz et al., 1991) to create the targeting vector p*Cd45*-T (Figure 3). To check for the integrity of the completed targeting construct and its intermediates I conducted extensive restriction enzyme mapping and confirmed the presence of fragments of predicted size and checked the DNA sequence across the junctions between the *Cd45* genomic DNA and the *neo* gene (not shown).



**Figure 3: Targeted disruption of the *Cd45* gene.** Schematic diagram of the targeting strategy used to create the *Cd45*<sup>-/-</sup> mice. The *Cd45* gene (top line) was disrupted by the insertion of the neomycin resistance gene (*neo*-shaded box) at a *Bam*HI site (B) located in exon 12 of the gene (exons are black boxes). A gene replacement targeting vector p*Cd45*-T was constructed to contain 9.8 kb of isogenic genomic DNA cloned as *Bam*HI fragments into the *Eco*RI and *Xho*I sites of pPNT (Tybulewicz et al., 1991) (dotted lines) such that the neomycin gene was located in exon 12. Neomycin-resistant ES cell lines were initially screened using a probe 3' to the region of homology called ES750 (hatched box). Integrity of the targeting event was confirmed using a probe homologous to the *neo* gene (shaded box) isolated as a *Pst*I fragment from pPNT and a 236 bp *Eco*RV/*Xba*I fragment of the *Cd45* cDNA. Restriction enzyme sites are marked; B for *Bam*HI and S for *Sac*I.

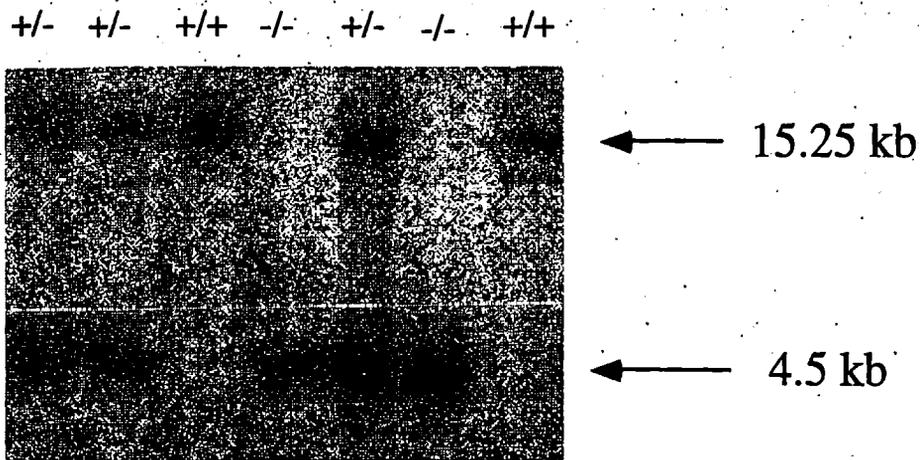
### Electroporation of the *Cd45* targeting construct

The D3 embryonic stem (ES) cell line, which is derived from 129/Sv mice, was electroporated with linearised p*Cd45*-T plasmid and plated the cells into G418-

and gancyclovir-containing medium to select for cells that had stable integration of the *neo* gene into their genomes, but had lost the *hsv-tk* gene. Homologous recombinants were identified by the restriction length polymorphism generated by the presence of a novel *SacI* site in the 5' UTR of the *neo* gene on integration of the vector (Figure 3 and data not shown). This produces a diagnostic 4.5 kb band which is distinguishable from the endogenous 15.2 kb band by Southern blotting of *SacI*-digested genomic DNA probed with the ES750 probe (Figure 3). This probe was constructed by sub-cloning an *EcoRI/SacI* fragment from one of the bacteriophage clones into the pUC19 cloning vector. This probe contains sequences 3' to the targeted region of the *Cd45* gene and I used this probe for both the initial screening of colonies for homozygous recombinants and for genotyping of mice (Figure 3 and 4). Two other probes were used in restriction site mapping experiments to confirm the integrity of the targeting event in recombinant clones. These probes were a *neo* gene specific probe and a probe specific for sequences 5' of the targeted region (Figure 3 and not shown). Three independent electroporations were conducted. Cells from the first electroporation were selected at 300 $\mu$ g/ml G418 and from the 480 colonies screened no homologous recombinants were isolated. This protocol was repeated a second time and only one putative recombinant was isolated after screening 356 colonies. This clone was further mapped by restriction digestion and Southern blotting and was found to have a duplicated insertion of the construct and thus the structure of the targeted *Cd45* locus was incorrect (not shown). To overcome the lack of homologous recombinants we decided to decrease the G418 concentration during selection on the basis that the particular site into which the *neo* gene was being targeted may hinder its expression. A third electroporation was conducted and selected in 250 $\mu$ g/ml G418. 456 colonies were screened for homologous recombination by Southern Blotting using the ES750 probe and 16 homologous recombinant cell lines were isolated.

## Generation of *Cd45*<sup>-/-</sup> mice

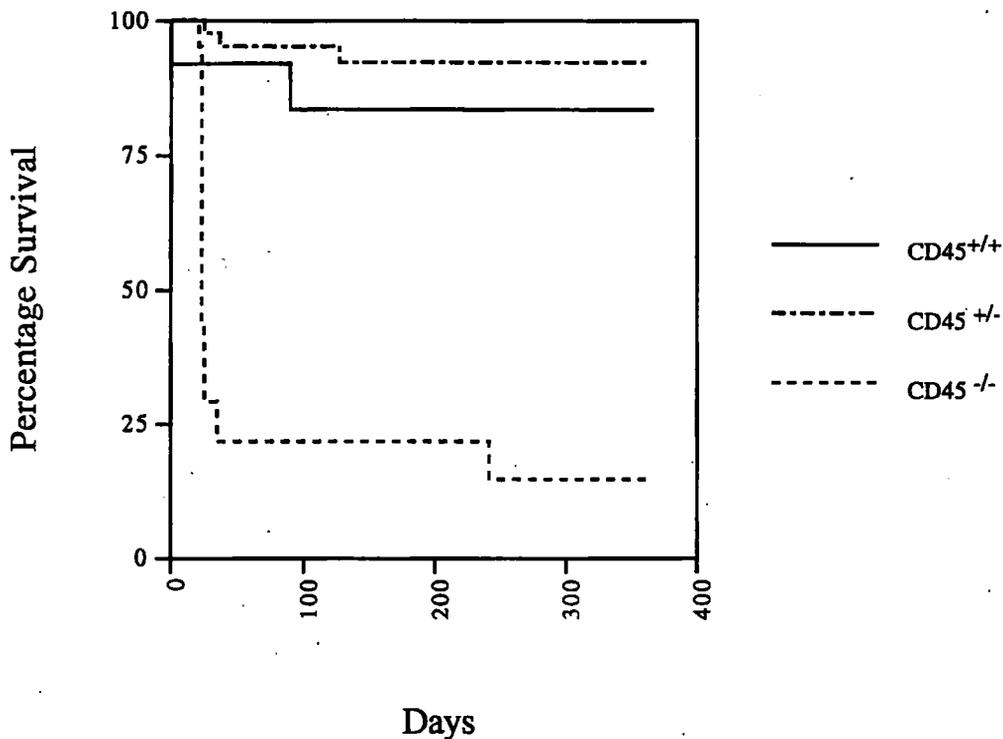
To generate chimaeras I microinjected three of these homologous recombinant ES cell lines into C57 Bl/6 (B6) mouse blastocysts. All three lines generated chimaeric mice with varying degrees of coat colour chimaerism. Initially these chimaeras were bred to (B6xDBA/2)<sub>F<sub>1</sub></sub> mice to determine whether the ES cell line had populated the germline of the mice. Germline transmission from the chimaeras can be determined by the presence of agouti offspring. In addition, these <sub>F<sub>1</sub></sub> mice make better mothers and have larger litters than mice from inbred strains. Germline transmission was achieved from one of the three recombinant cell lines injected. To confirmed germline transmission of the mutation, genomic DNA from mouse tail biopsies from the agouti pups was digested with *Sac*I, Southern blotted and probed with the ES750 probe. This identified mice heterozygous for the *Cd45* exon 12 mutation (*Cd45*<sup>tm1Tyb/+</sup>; *Cd45*<sup>+/-</sup>). Homozygous mutant mice (*Cd45*<sup>-/-</sup>) were generated by intercrossing heterozygous mutants (Figure 4) and were further intercrossed to generate a colony of *Cd45*<sup>-/-</sup> mice on a mixed genetic background (B6 and DBA/2 and 129/Sv) which I will refer to as the "outbred" line. To generate *Cd45*<sup>-/-</sup> mice on a 129/Sv background I bred the chimaeras directly to 129/Sv mice and screened all progeny for the presence of the mutation. As the chimaeras were generated from an ES cell line of 129/Sv origin this allowed the creation of an inbred line in one generation. To generate inbred *Cd45*<sup>-/-</sup> BALB/c backgrounds I backcrossed the mice sequentially for 10 generations against wildtype BALB/c mice and finally intercrossed heterozygous mutants to generate the inbred homozygous mutants.



**Figure 4: Southern blot analysis of the *Cd45* mutation from mouse tail DNA.** Southern blots of *SacI*-digested tail biopsy DNA from mice whose parents were known to be heterozygous for the *Cd45* mutation. The wildtype allele (15.25 kb) can be distinguished from the mutant allele (4.5 kb). The genotype of the mice is shown at the top of each lane. Note the absence of the 15.25 kb band in *Cd45<sup>-/-</sup>* mice.

#### Strain-specific lethality of 129/Sv *Cd45<sup>-/-</sup>* mice

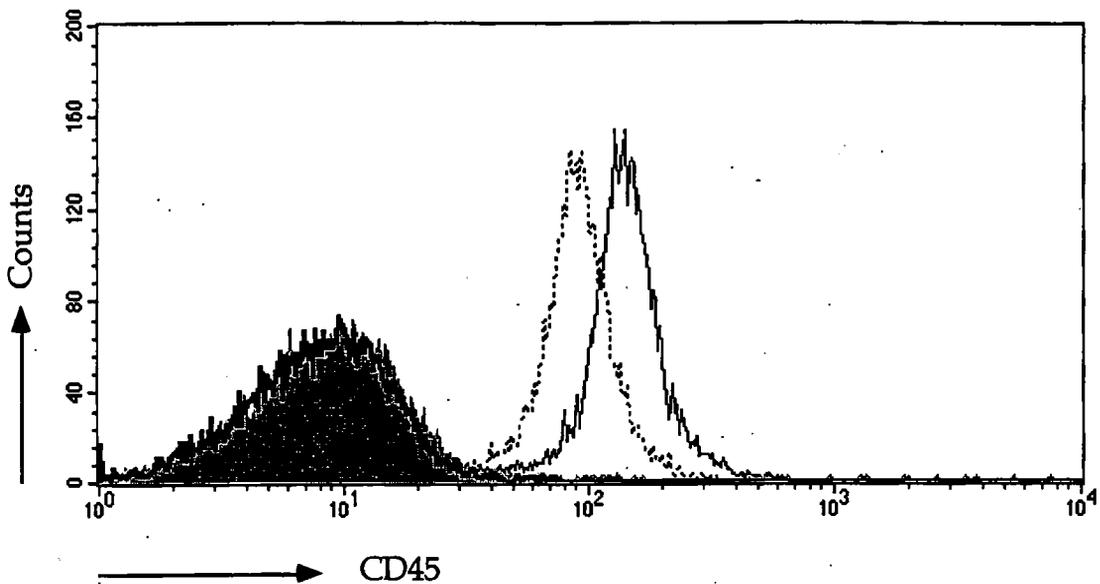
Initial attempts to derive an inbred 129/Sv *Cd45<sup>-/-</sup>* mouse strain were thwarted due to lethality in this strain. As can be seen in Figure 5, a small minority of *Cd45<sup>-/-</sup>* mice reach adulthood, however greater than 75% of the mice die within one month of birth. This lethality was characterised by juvenile diarrhoea, malnutrition and eventual death. This lethality was not seen in *Cd45<sup>+/+</sup>* or *Cd45<sup>+/-</sup>* littermate controls (Figure 5). Increased mortality was also not observed in *Cd45<sup>-/-</sup>* outbred mice or mice on the BALB/c genetic background (not shown), suggesting strain specificity of this lethality. I also observed a strain-specific phenotype in inbred *Cd45<sup>-/-</sup>* BALB/c mice. Although superficially these mice appear healthy, post-mortem examination revealed splenomegaly. Spleens from *Cd45<sup>-/-</sup>* mice were found to be 3-5 times heavier than *Cd45<sup>+/+</sup>* controls mice (not shown). This enlargement of the spleen is not observed in *Cd45<sup>+/-</sup>* BALB/c mice, in *Cd45<sup>-/-</sup>* outbred or 129/Sv mice (not shown).



**Figure 5: Lethality of the *Cd45* mutation on a 129/Sv genetic background.** Line diagram of percentage survival of mice genotyped by Southern blotting of tail DNA to be either *Cd45*<sup>+/+</sup> (solid line), *Cd45*<sup>+/-</sup> (dash and dotted line) or *Cd45*<sup>-/-</sup> (dashed line) plotted from birth in days. All mice are littermates generated from *Cd45*<sup>+/+</sup> mating pairs.

### ***Cd45*<sup>-/-</sup> mice do not express any isoforms of CD45**

To confirm the absence of cell surface expression of the CD45 protein in homozygous animals, flow cytometric analysis was conducted with a panel of monoclonal antibodies to all the different CD45 isoforms (A,B,C and B220) (not shown) and a pan-CD45 antibody that recognises all CD45 isoforms (Figure 6). Using this technique I could not detect any expression of any isoform of CD45 on thymus, lymph node, spleen, and bone marrow cells, and on cultured primary mast cells (Figure 6 and data not shown). *Cd45*<sup>+/-</sup> splenocytes were found to have reduced levels of CD45 on their cell surface (Figure 6) as compared to *Cd45*<sup>+/+</sup> cells, consistent with the disruption of expression from one of the *Cd45* alleles.

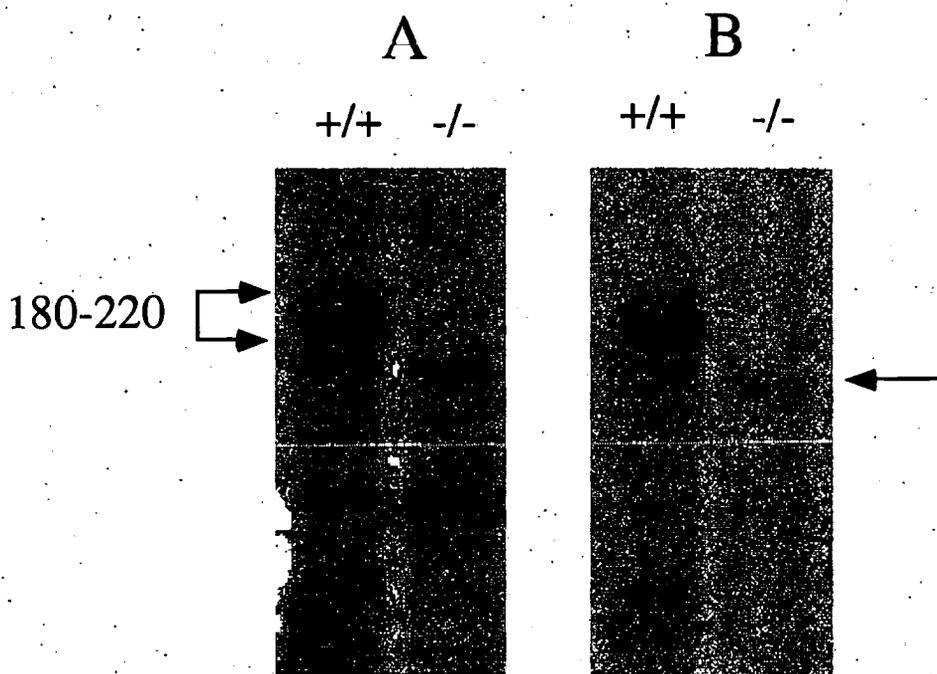


**Figure 6: Flow cytometric analysis of CD45-deficient splenocytes.** CD45 expression levels on splenic lymphocytes from outbred *Cd45<sup>+/+</sup>* (thin line), *Cd45<sup>+/-</sup>* (dotted line) and *Cd45<sup>-/-</sup>* (bold line) mice revealed with a pan-*Cd45* antibody. Staining with an isotype-matched control antibody of irrelevant specificity (rat IgG2a) (shaded) is indistinguishable from staining of *Cd45<sup>-/-</sup>* cells.

### Western analysis of *Cd45<sup>-/-</sup>* cells

I used Western blotting to determine if there was any expression of any truncated form of the protein 3' of the mutation. Figure 7 shows the result of Western blotting of primary mast cell extracts with two independently derived-polyclonal antisera raised against the cytoplasmic domain of CD45. Both of these antisera detect CD45 in cell lines, as seen as a smeared band of 180-220 kD in the *Cd45<sup>+/+</sup>* lanes. One of the antisera (A) failed to detect any additional bands present in the *Cd45<sup>-/-</sup>* cells that could have been a truncated form of CD45. However, this antiserum had a number of background bands that could have masked the presence of such a band. Thus I reprobated this Western blot with a second cytoplasmic tail-specific polyclonal antiserum which gave fewer background bands (B). This antiserum revealed the presence of a crossreacting truncated protein that was not present in the *Cd45<sup>+/+</sup>* cells. Densitometry showed this protein to be present at approximately 5% of the level of the endogenous protein (not

shown). Additional experiments showed that this truncated protein was also present in thymocytes and splenocytes from *Cd45*<sup>-/-</sup> mice and was present at similar levels to those found in mast cells (not shown).

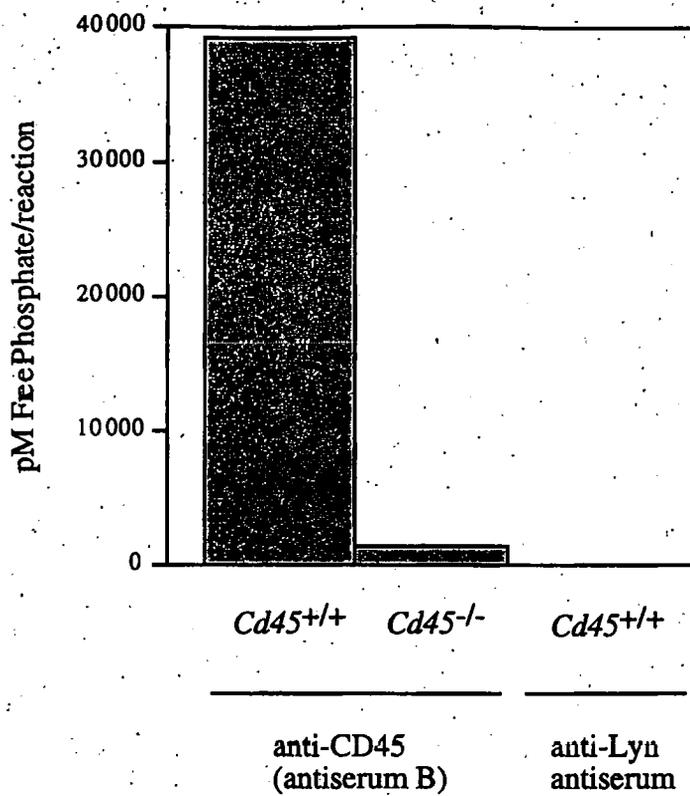


**Figure 7: Western blotting reveals a truncated form of the CD45 protein.** Western blot analysis was conducted on cell lysates prepared from mast cell lines derived from *Cd45*<sup>+/+</sup> and *Cd45*<sup>-/-</sup> mice. Blots were probed with two different polyclonal rabbit anti-mouse antisera specific for the intracellular domain of the CD45 protein. Blots probed with antiserum supplied by Dr. M. Thomas (A) and antiserum supplied by Dr. P. Johnson (B) were visualised using ECL. The arrow in B identifies a truncated form of CD45 in the targeted cells.

### **The truncated form of *Cd45* has phosphatase activity**

To determine if the observed truncated form of the protein had any tyrosine phosphatase activity I used the polyclonal antiserum obtained from Dr. P. Johnson (B in Figure 7) to immunoprecipitate this CD45 fragment and assayed its activity in a tyrosine phosphatase assay. This assay measures the ability of tyrosine phosphatases to liberate free phosphate from tyrosine-phosphorylated synthetic peptides. This free phosphate is detected by a colorimetric assay. Figure 8 shows the results obtained from cell lysates that were prepared from cultured mast cells

from *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* mice. Protein was immunoprecipitated with either antiserum to the cytoplasmic domain of CD45 (antiserum B) or as a control, with an antiserum against the tyrosine kinase Lyn. As expected, *Cd45<sup>+/+</sup>* cells were found to have a high level of tyrosine phosphatase activity in protein immunoprecipitated using the CD45-specific antiserum. Protein immunoprecipitated with antiserum from *Cd45<sup>-/-</sup>* mast cells was found to have low but detectable levels of tyrosine phosphatase activity which are approximately 3% of the levels in *Cd45<sup>+/+</sup>* cells. Protein precipitated using an antiserum to the irrelevant protein, Lyn, did not give any detectable tyrosine phosphatase activity over background suggesting that the observed tyrosine kinase activity in *Cd45<sup>-/-</sup>* mast cells is not an artefact of the immunoprecipitation protocol. It is not possible to determine the specific activity of the immunoprecipitated protein as protein concentrations could not be calculated due to the presence of antibody in the immunoprecipitations. For this reason the results are displayed as pM free phosphate detected per 15min reaction.



**Figure 8: The truncated isoform of CD45 has tyrosine phosphatase activity.** Phosphatase activity as measured by the liberation of free phosphate from synthetic peptides was assessed by the protein immunoprecipitated from total cell lysates from mast cells from either *Cd45*<sup>+/+</sup> and *Cd45*<sup>-/-</sup> mice using either antisera to the cytoplasmic domain of CD45 (antiserum B) or an irrelevant antiserum (against the Lyn kinase).

### Summary

I have created mice which are homozygous for a targeted mutation of exon 12 of the *Cd45* gene. There is a strain specific lethality of the mutation on the 129/Sv genetic background. *Cd45*<sup>-/-</sup> mice do not express any isoform that is recognisable by antibodies to the extracellular domain of CD45. A small amount of a truncated form of CD45 (approximately 5% of wildtype levels) is detected in *Cd45*<sup>-/-</sup> cells and tissues by antiserum to the intracellular domain of CD45. This truncated protein has tyrosine phosphatase activity.

## 3.2 B cell development in CD45-deficient mice

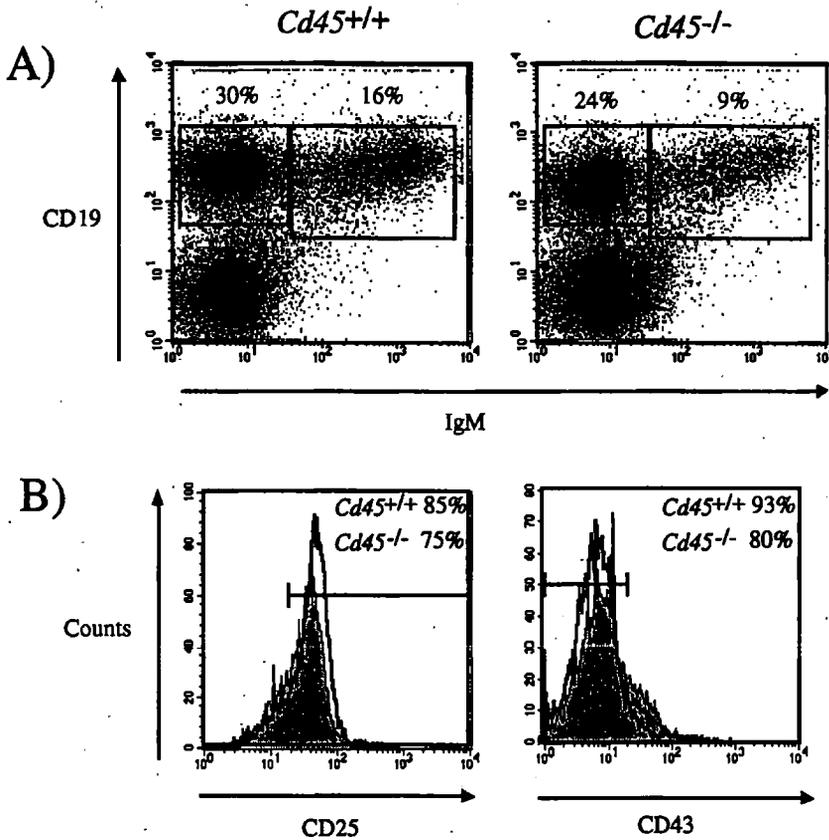
### B cell development in the bone marrow

CD45 has been implicated as both a positive and negative regulator of signal transduction from the BCR in the small number of studies conducted using CD45-deficient B cell lines (Alexander, 1997). I therefore decided to determine the effect of this mutation on the development of B cells within *Cd45<sup>-/-</sup>* mice with particular emphasis on those developmental transitions where antigen receptor mediated signalling has been implicated.

Early B cell development occurs in the bone marrow and is characterised by cell surface expression of distinct markers associated with defined developmental steps. These developmental stages are normally characterised by flow cytometric analysis of the expression of B220 and other cell surface markers (Hardy et al., 1983). As B220 is a B cell specific isoform of CD45, this molecule is not expressed on the surface of *Cd45<sup>-/-</sup>* mice and another marker must be used for this analysis. CD19 is a B cell-specific marker that can be used as a substitute for B220 in this analysis since it is expressed throughout B cell development from the earliest pro-B cell compartment (Rolink et al., 1996). B cell development proceeds from the pro-B (CD19<sup>+</sup>IgM<sup>-</sup>CD25<sup>-</sup>CD43<sup>+</sup>) through the pre-B cell (CD19<sup>+</sup>IgM<sup>-</sup>CD25<sup>+</sup>CD43<sup>-</sup>) and then to the immature B cell (CD19<sup>+</sup>IgM<sup>+</sup>IgD<sup>-</sup>) stage, finally generating mature (CD19<sup>+</sup>IgM<sup>+</sup>IgD<sup>+</sup>) B cells found in splenic and lymph node follicles. As described in the introduction, the developmental transition from pro-B cell to pre-B cells is known to require signalling events through the pre-B cell receptor (pre-BCR). To assay the role of CD45 in this transition I used antibody staining to analyse these populations by flow cytometric analysis. Figure 9A shows a typical flow cytometric analysis of bone marrow from *Cd45<sup>-/-</sup>* mice compared to bone marrow from *Cd45<sup>+/+</sup>* mice stained with anti-CD19 and anti-IgM antibodies. It can be seen that there is a small but consistent reduction in the numbers of B cells that are CD19<sup>+</sup>IgM<sup>-</sup> in *Cd45<sup>-/-</sup>* bone marrow (24%) compared to *Cd45<sup>+/+</sup>* bone marrow (30%). This suggests that there is a partial block at an early stage of B cell

development. To determine if this block is at the pro-B to pre-B cell transition, anti-CD25 and anti-CD43 markers were included in this analysis. By gating on cells that are CD19<sup>+</sup>IgM<sup>-</sup> and displaying the level of CD25 or CD43 expression as histograms (Figure 9B) *Cd45*<sup>-/-</sup> bone marrow was found to have a reduction in both CD43<sup>-</sup> and CD25<sup>+</sup> cells within the CD19<sup>+</sup>IgM<sup>-</sup> compartment (ie. pre-B cells) indicative of a block in B cell development at the pro-B cell to pre-B cell stage. This is a partial developmental block as *Cd45*<sup>-/-</sup> cells develop into CD19<sup>+</sup>IgM<sup>+</sup> immature cells although at reduced levels (9% compared to 16% in *Cd45*<sup>+/+</sup> marrow - Figure 9A). Total cellularity of the bone marrow is no different between mutants and controls.

These results demonstrate that CD45 is required for the efficient development from pro-B cells to pre-B cells, a transition that is associated with signal transduction from the pre-BCR. This suggests that in the absence of CD45, the efficiency of pre-BCR signalling is reduced. There is not an absolute requirement for CD45 during this transition suggesting that CD45 is only partially required for signalling from the pre-BCR or that in its absence other molecules can substitute for its activity.



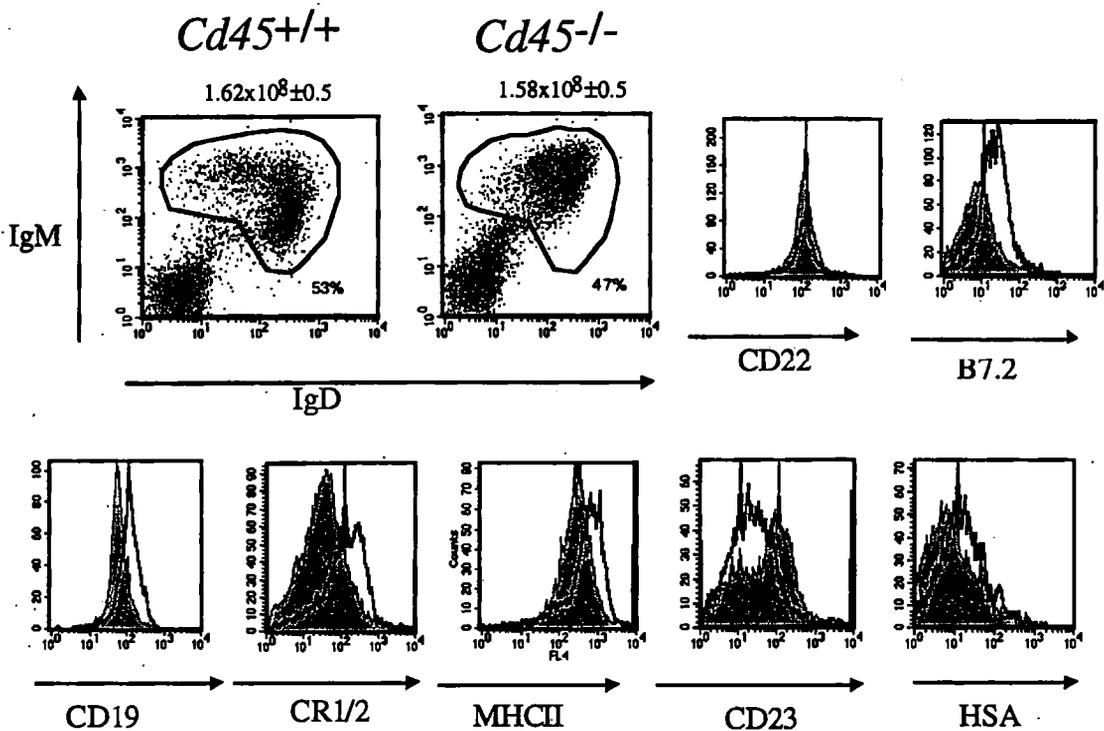
**Figure 9: There is a partial developmental block in the transition from pro- to pre-B cells in *Cd45*<sup>-/-</sup> mice.** Bone marrow from *Cd45*<sup>-/-</sup> and *Cd45*<sup>+/+</sup> outbred mice were stained with CD19, IgM and CD25 or CD43 as three colour stains. (A) Typical flow cytometric analysis displayed as dot plots showing bone marrow staining for IgM and CD19 of 6 wk old outbred female *Cd45*<sup>-/-</sup> mice and *Cd45*<sup>+/+</sup> litter mate control. The two boxed regions represent the CD19<sup>+</sup>IgM<sup>-</sup> cells consisting of the pro- and pre-B cell compartments and CD19<sup>+</sup>IgM<sup>+</sup> cells consisting of the immature and mature B cell compartments and the percentages of cells within these boxes are displayed above them. B) *Cd45*<sup>-/-</sup> (shaded) and *Cd45*<sup>+/+</sup> (broad line) CD19<sup>+</sup>IgM<sup>+</sup> cells were gated on shown in (A) and overlay histograms of CD25 or CD43 expression within these gates are displayed with the percentage of cells that are CD25<sup>+</sup> or CD43<sup>+</sup> pre-B cells calculated and displayed.

### *Cd45*<sup>-/-</sup> B cells have unusual levels of cell surface markers

B cell development from IgM<sup>hi</sup>IgD<sup>lo</sup> B cells to the most mature IgM<sup>lo</sup>IgD<sup>hi</sup> cells is characterised by changes in the expression levels of a number of markers. As CD45 was found to have a role in the pro-B cell to pre-B cell development I decided to assess the role of CD45 in this B cell transition as signal transduction from the BCR has been implicated in this developmental checkpoint (Torres et al., 1996; Turner et al., 1997). The presence and maturity of B cells in the spleen of

*Cd45*<sup>-/-</sup> mice was analysed using antibody staining and flow cytometry. Figure 10 shows dot plot analysis of typical staining from *Cd45*<sup>+/+</sup> and *Cd45*<sup>-/-</sup> splenocytes stained with anti-IgM and anti-IgD antibodies. Splenic B cells from *Cd45*<sup>+/+</sup> mice show the expected IgM/IgD staining patterns with a majority of the B cells having the mature phenotype of a high ratio of IgD to IgM (IgM<sup>lo</sup>IgD<sup>hi</sup>). In *Cd45*<sup>-/-</sup> mice, B cells are present in equivalent numbers to controls although they are unusual in that they have high expression levels of both IgM and IgD (IgM<sup>hi</sup>IgD<sup>hi</sup>) compared to controls (Figure 10). This suggests that the CD45-deficient B cells are less mature than controls as they do not have the most mature population of IgM<sup>lo</sup>IgD<sup>hi</sup> cells. To study this further, I analysed the expression of various markers that alter their expression levels during B cell maturation. I stained splenocytes with multiple antibodies and displayed the level of expression of these markers on B cells, by gating on IgM<sup>+</sup>IgD<sup>+</sup> cells. Figure 10 shows that there are moderately increased levels of expression of the B cell coreceptor molecule CD19, MHC class II, and complement receptor 1 and 2 (CR1/2), all of which are molecules whose expression increases during B cell maturation. This increase in expression was not due to an increase in cell size as measured by forward and side scatter analysis (not shown). There was not a general increase in the levels of all cytoplasmic membrane proteins, as expression of the CD22 was not increased. CD23 is another marker of B cell maturity which increases in expression during B cell maturation. *Cd45*<sup>-/-</sup> B cells express higher levels of this marker than controls suggesting that these cells are immature. HSA expression decreases as B cells mature and as *Cd45*<sup>-/-</sup> B cells express lower levels of this marker this would suggest they are immature. In conclusion CD45-deficient B cells are unusual in that there is a contradiction between the level of expression of HSA, CD23 and IgM/IgD suggesting these cells are immature, however the expression of other developmental markers suggest more advanced maturation. It is difficult to reconcile these observations if these markers are only representing the developmental stage of the B cells in CD45-deficient mice. In addition to looking at the maturity of CD45-deficient B cells I also analysed the levels of expression of the CD28 ligand, B7.2. Increased B7.2 expression is associated with B cell activation (Lenschow et al., 1994). The

increased levels of expression of this marker on CD45-deficient B cells suggests that these cells have an activated phenotype. This could be explained if CD45 is a negative regulator of BCR signalling and thus CD45-deficient B cells are more susceptible to activation on encounter with antigens.

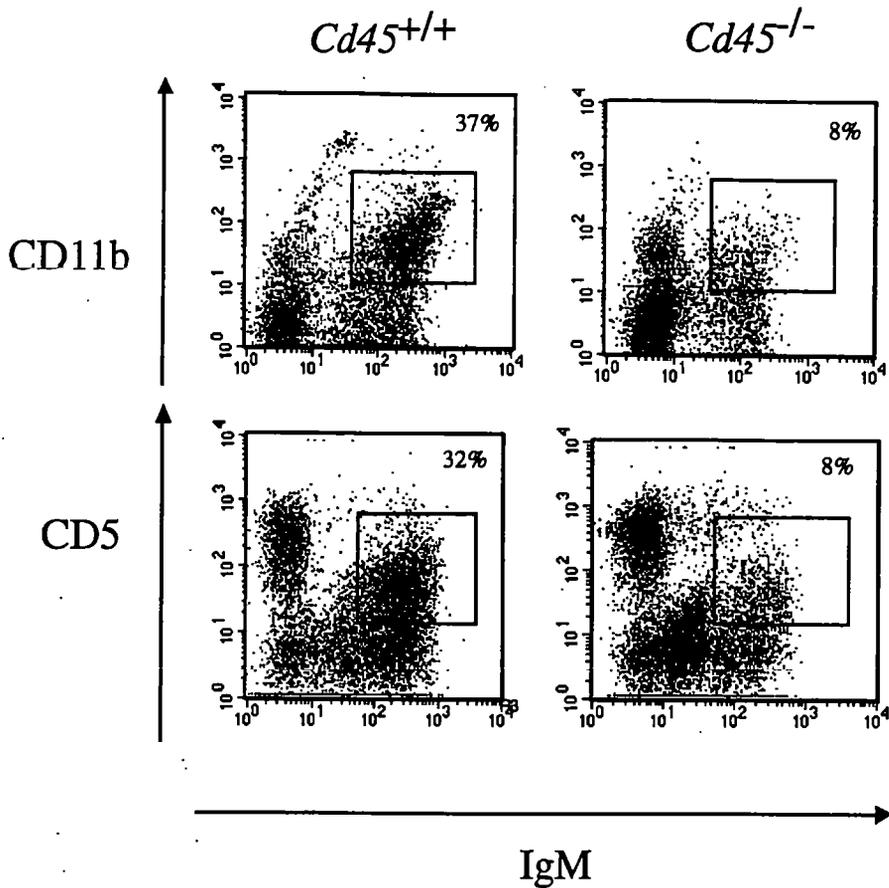


**Figure 10: B cell surface marker expression.** Representative flow cytometric analysis of splenocytes from *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* animals stained with anti-IgM and anti-IgD (dot plots-top left hand side). IgM<sup>+</sup>IgD<sup>+</sup> cells were gated and the percentages of gated lymphocytes displayed. Total B cell numbers are shown at the top of the dot plots. The levels of expression of cellular maturity and activation markers CD19, CR1/2, MHC class II, CD23, HSA, B7.2 and CD22 from gated cells were obtained by three or four colour analysis and are displayed as histograms (*Cd45<sup>+/+</sup>*-Shaded and *Cd45<sup>-/-</sup>*-broad black lines).

### *Cd45<sup>-/-</sup>* B-1 cells are greatly reduced in number

As described in the introduction, the major B cell population consists of so-called B-2 cells, however another population of B cells called B-1 cells exist which reside predominantly in the peritoneal cavity of mice (Tarakhovsky, 1997). Mice deficient in positive regulators of signal transduction, such as CD19, have reduced numbers of B-1 cells, whilst mice defective in negative regulators of signal

transduction, such as CD22, have more B-1 cells. This suggests that the development or maintenance of this population of B cells require signal transduction from the BCR and that the strength of this signal determines the numbers of B-1 cells present in the mouse. To determine whether CD45 maybe involved in this process, flow cytometric analysis was performed using markers of this population on cells flushed from the peritoneal cavity of CD45-deficient mice. B-1 cells can be subdivided into two distinct populations, B-1a and B-1b cells. Both B-1a and B-1b cells can be identified on the basis of their expression of IgM and CD11b, whilst B-1a cells can be specifically identified as IgM<sup>+</sup>CD5<sup>+</sup> cells. The peritoneal cavities of *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* littermates were flushed and the presence of B-1 cells determined by their expression of these markers (Figure 11-boxed areas). Total numbers of B-1 cells (IgM<sup>+</sup>CD11b<sup>+</sup>) were greatly reduced in *Cd45<sup>-/-</sup>* animals compared to *Cd45<sup>+/+</sup>* controls as seen by the reduction in IgM<sup>+</sup>CD11b<sup>+</sup> cells (4.6-fold reduction-Figure 11). Moreover, the B-1a cell population (IgM<sup>+</sup>CD5<sup>+</sup>) is reduced four-fold (Figure 11). Since the development or maintenance of B-1 cells requires signal transduction from the BCR, this suggests that CD45 is a positive regulator of signal transduction from the BCR on B-1 cells.

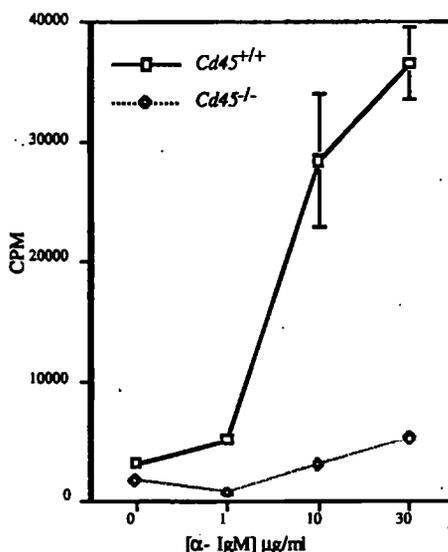


**Figure 11: CD45-deficient mice have a reduced number of B-1 cells.** B-1 cells obtained by flushing the peritoneal cavity of mice were stained with anti-IgM and either anti-CD5 or anti-CD11b. Boxed areas show percentages of IgM<sup>+</sup>CD11b<sup>+</sup> total B-1 cells (upper panels) and the IgM<sup>+</sup>CD5<sup>+</sup> B-1a sub-population (lower panels). All plots are representative of the analysis of multiple mice.

### *Cd45<sup>-/-</sup>* B cells fail to proliferate on antigen stimulation

CD45-deficient B cell lines have been shown to be defective in their proliferative responses to antigen receptor cross linking (Justement, 1997). To determine whether this is also true for mature re-circulating B cells derived from *Cd45<sup>-/-</sup>* mice, I purified splenic B cells using a negative selection protocol and stimulated them to proliferate *in vitro* by incubation with an anti-IgM antibody that crosslinks the BCR. This was conducted in the presence of IL-4 which enhances the proliferative response of the B cells to receptor cross-linking. This stimulation causes the proliferation of *Cd45<sup>+/+</sup>* B cells, as measured by the incorporation of <sup>3</sup>H-thymidine, and the level of proliferation was dependent on the concentration of

the anti-IgM antibody added (Figure 12). In contrast only a very low level of response in *Cd45<sup>-/-</sup>* B cells was detected at the highest doses of anti-IgM used suggesting a severe deficiency in proliferative responses in *Cd45<sup>-/-</sup>* B cells. *Cd45<sup>-/-</sup>* B cells were, however, able to proliferate in the presence of LPS or PMA+Ionomycin and in response to crosslinking of CD40, suggesting that this deficiency was specific to the BCR and not to other receptors (not shown). In conclusion, CD45-deficient mature B cells are defective in receptor-mediated proliferation suggesting that CD45 is a positive regulator of signal transduction from the BCR.

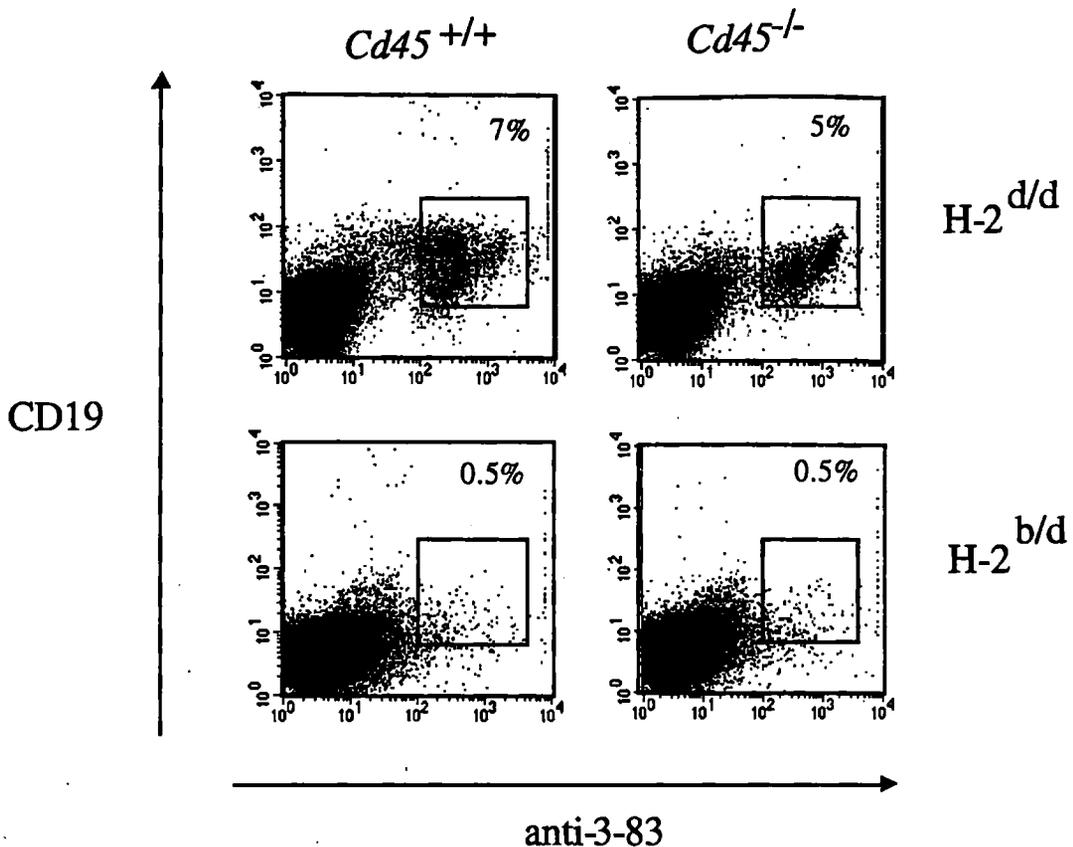


**Figure 12: CD45-deficient B cells fail to proliferate on receptor stimulation.** Purified splenic B cells were stimulated with anti-IgM (b-7-6) at the concentrations shown in the presence of IL4 (10u/ml) for 3 days and proliferation was measured by <sup>3</sup>H-thymidine incorporation in counts per min (CPM). Results shown are the mean of triplicate samples and error bars show standard deviation. Similar results were obtained using a polyclonal goat anti-mouse anti-serum (not shown).

#### ***Cd45<sup>-/-</sup>* B cells expressing a transgenic receptor can be efficiently deleted**

B cells from *Cd45<sup>-/-</sup>* mice fail to proliferate when stimulated by crosslinking of their BCR and this suggests that CD45 is required as a positive regulator of signal transduction in these cells. To examine the effects of this mutation on

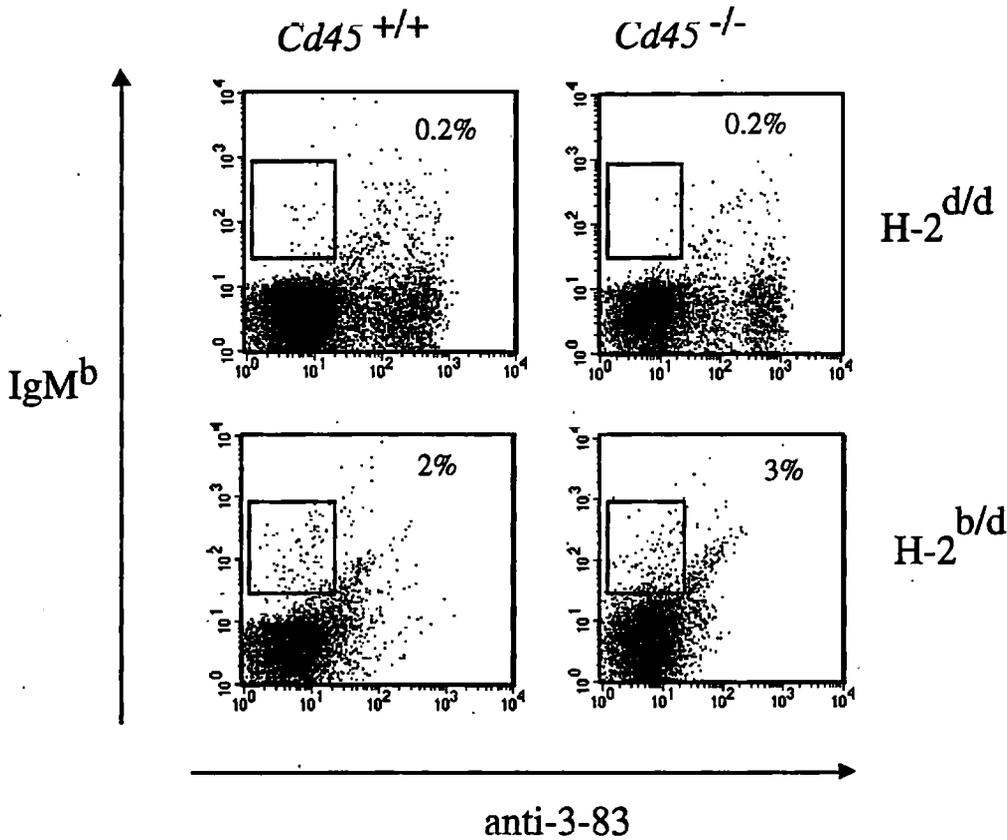
another BCR-mediated phenomenon, that of the negative selection of B cells on encounter with autoantigen, I bred *Cd45<sup>-/-</sup>* mice with mice carrying the 3-83 $\mu\delta$  (3-83) transgene. 3-83 transgenic mice express IgM and IgD specific for H-2K<sup>k</sup> and H-2K<sup>b</sup>, but which do not bind H-2K<sup>d</sup>. In the absence of autoantigen, B cell development can occur with the majority of B cells expressing the 3-83 receptor. In the presence of H-2K<sup>k</sup> or H-2K<sup>b</sup>, B cells in 3-83 transgenic mice are deleted in response to autoantigen. Figure 13 shows flow cytometric analysis of bone marrow from *Cd45<sup>-/-</sup>* and *Cd45<sup>+/+</sup>* mice expressing the 3-83 receptor in the presence or absence of the negatively selecting ligand H-2K<sup>b</sup>. *Cd45<sup>-/-</sup>* B cells expressing the 3-83 transgene were found to be efficiently produced in the marrow of both *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* animals on the non-deleting H-2<sup>d</sup> genetic background (top panels). In the presence of H-2K<sup>b</sup>, the negatively selecting ligand, B cells expressing the 3-83 transgene are efficiently eliminated in the bone marrow of both *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* mice (Figure 13-lower panels) and no 3-83-expressing B cells were found in the lymph node or spleen of these mice (data not shown). Thus although *Cd45<sup>-/-</sup>* B cells are deficient in antigen receptor-mediated proliferative responses to receptor crosslinking *in vitro*, CD45 does not appear to be necessary for BCR mediated negative selection events *in vivo*.



**Figure 13: B cell development in the bone marrow  $Cd45^{-/-}$  and  $Cd45^{+/+}$  mice expressing the 3-83 transgenic B cell receptor.** Flow cytometric dot plots of bone marrow of  $Cd45^{+/+}$  or  $Cd45^{-/-}$  mice carrying the 3-83 $\mu\delta$  transgene stained with anti-CD19 and an anti-idiotypic antibody specific for the 3-83 BCR (anti-3-83) in the presence (lower panels, H-2<sup>b/d</sup>) or absence (upper panels, H-2<sup>d/d</sup>) of the deleting ligand MHC class I H-2K<sup>b</sup>. Cells expressing these markers have been gated (box) and their percentages calculated.

This transgenic mouse model can also be used to assess the role of CD45 in allelic exclusion of both Ig heavy and light chains. As discussed in the introduction, allelic exclusion is a mechanism that requires signalling events from antigen receptors. The transgenic 3-83 receptor is of the IgM<sup>a</sup> allotype so by breeding these mice to have endogenous heavy chains of the IgM<sup>b</sup> allotype it was possible to assess by flow cytometry allelic exclusion of endogenous heavy chain gene rearrangement by the transgenic receptor. Figure 14 shows flow cytometric analysis of splenic B cells stained for the presence of the 3-83 receptor and for the endogenous IgM<sup>b</sup> allotype. In the absence of the deleting ligand the transgene was found to efficiently prevent the expression of endogenous receptors in  $Cd45^{-/-}$  B cells as seen as an absence of IgM<sup>b</sup> expressing B cells (Figure 14-top panels). Only in the presence of the cognate ligand for the 3-83 receptor do we find a small

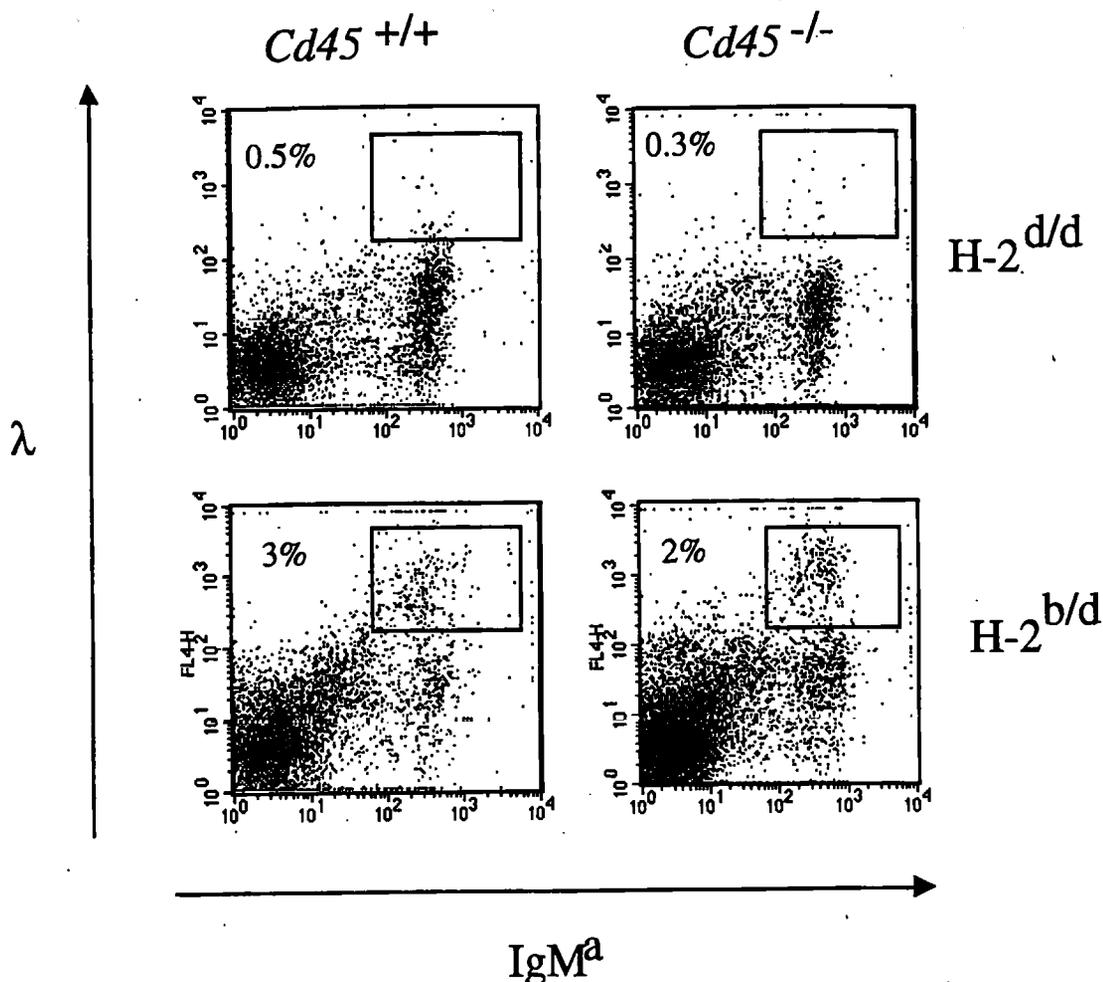
number of B cells in both  $Cd45^{+/+}$  and  $Cd45^{-/-}$  mice that now express  $IgM^b$  (Figure 14-bottom panels). It is likely that these result from a small population of cells in which 3-83 expression has been suppressed by some epigenetic means allowing endogenous heavy chain rearrangement and thus escape from deletion. However in both  $Cd45^{+/+}$  and  $Cd45^{-/-}$  mice there is no evidence of a population of cells expressing both the transgenic  $IgM^a$  and endogenous  $IgM^b$  heavy chains which would be indicative of a failure of heavy chain allelic exclusion. This data suggests that not only is CD45 not required for negative selection but also that heavy chain allelic exclusion does not require CD45.



**Figure 14: Heavy chain allelic exclusion in  $Cd45^{-/-}$  and  $Cd45^{+/+}$  mice expressing the 3-83 transgenic B cell receptor.** Flow cytometric dot plots of bone marrow of  $Cd45^{+/+}$  or  $Cd45^{-/-}$  mice carrying the 3-83 $\mu\delta$  transgene stained with anti- $IgM^b$  and an anti-idiotypic antibody specific for the 3-83 BCR (anti-3-83) in the presence (lower panels,  $H-2^{b/d}$ ) or absence (upper panels,  $H-2^{d/d}$ ) of the deleting ligand MHC class I  $H-2K^b$ . Cells expressing these markers have been gated (box) and their percentages calculated.

The 3-83 transgene uses a  $\kappa$  light chain thus it is also possible to assess light chain allelic exclusion using this mouse model by staining for endogenous  $\lambda$  light chain rearrangement. Figure 15 shows flow cytometric analysis of splenic B cells from *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* mice that express the 3-83 transgene. In the absence of H-2K<sup>b</sup> both *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* mice contain very few cells that express  $\lambda$  light chain suggesting that the 3-83 transgene drives efficient light chain allelic exclusion in the mutant mice (Figure 15-top panels). In the presence of H-2K<sup>b</sup>, both *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* mice have B cells that express endogenous  $\lambda$  light chain (Figure 15-bottom panels). By this rearrangement these cells alter the specificity of their antigen receptor and thus avoid negative selection by altering the specificity of their antigen receptor - a process called receptor editing (Hertz and Nemazee, 1998).

In conclusion, although CD45-deficient B cells fail to undergo proliferation on receptor crosslinking, the processes of negative selection and both heavy and light chain allelic exclusion do not require CD45 and suggest that different signal transduction pathways are used in these receptor mediated events.

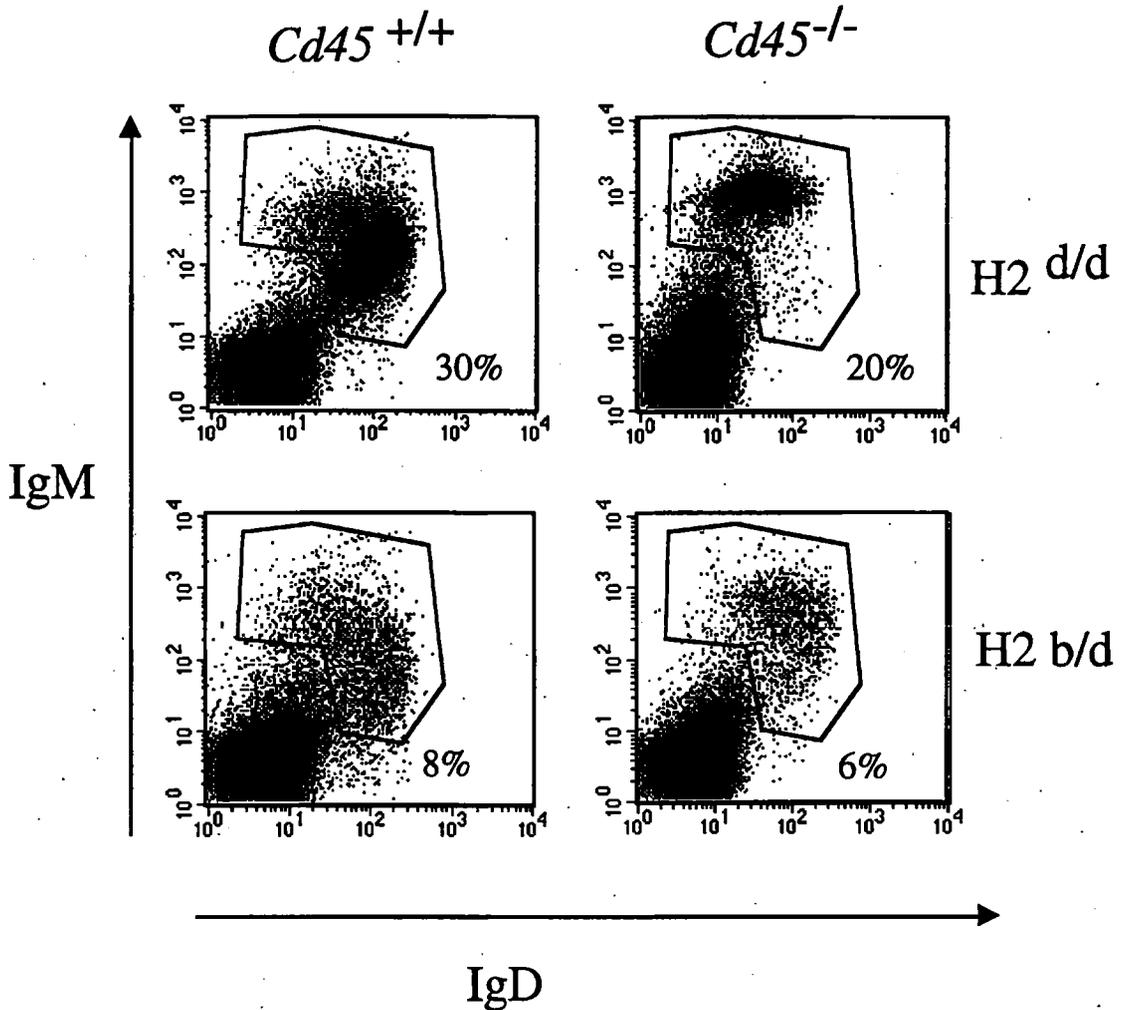


**Figure 15: Light chain allelic exclusion and receptor editing in *Cd45*<sup>-/-</sup> and *Cd45*<sup>+/+</sup> mice expressing the 3-83 transgenic B cell receptor.** Flow cytometric dot plots of bone marrow of *Cd45*<sup>+/+</sup> or *Cd45*<sup>-/-</sup> mice carrying the 3-83 $\mu\delta$  transgene stained with anti-IgM<sup>a</sup> and antibody specific for  $\lambda$  light chain ( $\lambda$ ) in the presence (lower panels, H-2<sup>b/d</sup>) or absence (upper panels, H-2<sup>d/d</sup>) of the deleting ligand MHC class I H-2K<sup>b</sup>. Cells expressing these markers have been gated (box) and their percentages calculated.

***Cd45*<sup>-/-</sup> B cells expressing a transgenic receptor have unusual levels of the receptor.**

B cell development in the bone marrow of *Cd45*<sup>-/-</sup> mice expressing the 3-83 $\mu\delta$  transgene was found to occur normally (Figure 13-top panels), I therefore examined whether these cells were present in the peripheral lymphoid organs. I performed flow cytometric analysis on splenic cells from these mice stained with anti-IgM and anti-IgD antibodies to assess the maturity of these cells and compared them to controls. B cells from both *Cd45*<sup>+/+</sup> and *Cd45*<sup>-/-</sup> transgenic mice expressing the 3-83 BCR accumulate in the spleen in high numbers on the non-deleting H-2<sup>d</sup>

genetic background (Figure 16- top panels). These *Cd45*<sup>-/-</sup> B cells expressing the 3-83 transgene have an altered level of expression of IgM and IgD being IgM<sup>hi</sup>IgD<sup>lo</sup> cells compared to the IgM<sup>hi</sup>IgD<sup>hi</sup> phenotype seen in non-transgenic *Cd45*<sup>-/-</sup> B cells (compare Figure 16-top panels and Figure 10). The failure of these cells to increase expression of IgD could suggest that the B cells are less mature than those cells seen in the absence of the transgenic BCR. In the presence of the deleting MHC ligand, H-2<sup>b</sup>, the majority of B cells are eliminated from the spleen of both *Cd45*<sup>+/+</sup> and *Cd45*<sup>-/-</sup> mice. Some B cells accumulate in the spleen (Figure 16-lower panels) and lymph node (not shown) of both 3-83 transgenic *Cd45*<sup>+/+</sup> and *Cd45*<sup>-/-</sup> mice. These B cells accumulate due to rearrangement of endogenous heavy and light chains which alter the receptor specificity of the BCR allowing these B cells to evade deletion. Due to this alteration of receptor specificity these cells are not recognized by the anti-idotypic antibody (data not shown). CD45-deficient B cells that accumulate in this fashion have a IgM<sup>hi</sup> IgD<sup>hi</sup> phenotype similar to CD45-deficient B cells that develop in the absence of the 3-83 receptor. In conclusion, the expression of the rearranged BCR in transgenic mice appears to alter the level of receptor expression and possibly the maturity of non-transgenic CD45-deficient B cells when compared to CD45-deficient B cells expressing endogenous receptors.

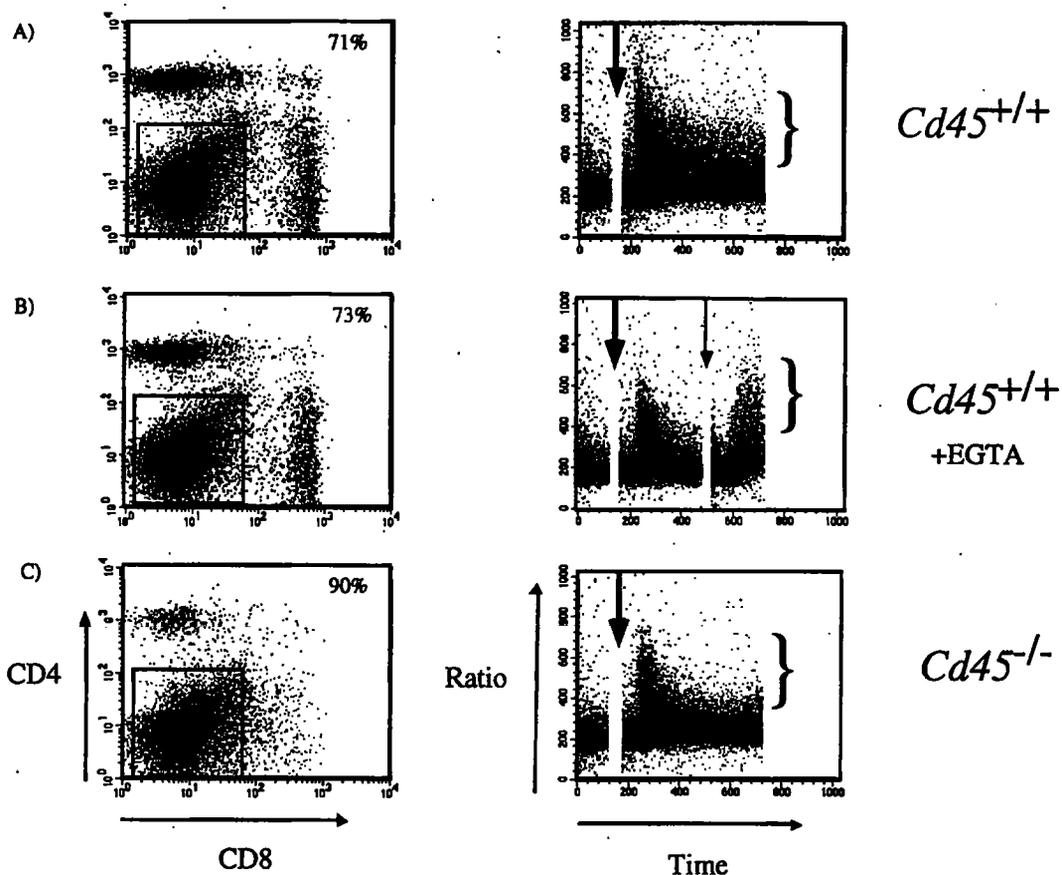


**Figure 16: CD45-deficient B cells expressing the 3-83 transgene have unusual receptor levels.** Flow cytometric analysis of splenic B cells from 8 wk-old female *Cd45*<sup>+/+</sup> and *Cd45*<sup>-/-</sup> mice expressing the 3-83 $\mu\delta$  BCR transgene in the presence (top panels) or the absence (lower panels) of the negatively selecting ligand H-2K<sup>b</sup>, stained with antibodies to IgM and IgD.

***Cd45*<sup>-/-</sup> B cells fail to mobilise calcium correctly following antigen receptor stimulation**

The defective proliferative response of *Cd45*<sup>-/-</sup> B cells *in vitro* is suggestive of a failure of signal transduction events from the BCR. One of the hallmarks of BCR-induced signalling is an increase in intracellular calcium (Ca<sup>2+</sup>) concentration. Initially this occurs by a rapid and transient release of Ca<sup>2+</sup> from intracellular stores and is followed by a slower influx of Ca<sup>2+</sup> from extracellular sources. Figure 17 shows the results of Ca<sup>2+</sup> mobilisation studies performed by flow cytometry. Here

T cells are excluded from the analysis by staining for CD4 and CD8 and gating on those cells negative for these markers (Figure 17-boxed area on dot plot), these are predominately B cells (not shown). Cells were stimulated by the addition of anti-IgM and intracellular  $\text{Ca}^{2+}$  concentrations monitored by the changes in the ratio of violet/blue fluorescence of a  $\text{Ca}^{2+}$  binding dye Indo-1. Both  $\text{Cd45}^{-/-}$  and  $\text{Cd45}^{+/+}$  B cells are able to mobilise  $\text{Ca}^{2+}$  from intracellular stores as seen by the initial peak in dye fluorescence ratio seen shortly after the addition of the antibody (Figure 17 - after broad arrow). The influx of  $\text{Ca}^{2+}$  from extracellular stores occurs shortly after the intracellular release of  $\text{Ca}^{2+}$  and is seen as a prolonged but shallow increase in ratio of the dye fluorescence in the cells (Figure 17A-bracketed area). The addition of the chelating agent EGTA to the medium prevents the influx of extracellular  $\text{Ca}^{2+}$  and cells do not appear in this area (Figure 17B-bracketed area). This can be reversed by the addition of excess  $\text{Ca}^{2+}$  to the cells (Figure 17B-fine arrow).  $\text{Cd45}^{-/-}$  B cells are defective in their ability to mobilise  $\text{Ca}^{2+}$  from extracellular sources as they fail to demonstrate the prolonged increase in  $[\text{Ca}^{2+}]$  (Figure 17C). In conclusion, these results indicate that CD45 is a positive regulator for signal transduction pathways leading from the BCR to extracellular but not intracellular  $\text{Ca}^{2+}$  mobilisation pathways.



**Figure 17: Defective  $\text{Ca}^{2+}$  flux in  $\text{Cd}45^{-/-}$  B cells.** Representative experiment on splenic B cells from 6 wk old female  $\text{Cd}45^{+/+}$  and  $\text{Cd}45^{-/-}$  mice pre-loaded with Indo-1 and stimulated with anti-IgM antibody (b-7-6). (A) Left hand side dot plots show wildtype cells stained with CD4 and CD8. Cells were gated (boxed area) on cells expressing neither CD4 or CD8 and the percentage of cells within this gate displayed. Intracellular calcium concentration in B cells (the predominant cell type gated in the CD4/CD8 plot) is shown as a ratio of Indo-1 violet/blue fluorescence (Ratio: calcium bound dye/ calcium free dye) versus time. Each channel on the time axis corresponds to 500ms. Cells were stimulated with  $50\mu\text{g/ml}$  anti-IgM at the time indicated by the broad arrow. This results in an initial peak representing intracellular  $\text{Ca}^{2+}$  release followed by a shallow broad peak due to extracellular  $\text{Ca}^{2+}$  flux (bracketed area). (B) As above but in the presence of the chelating reagent EGTA which prevents extracellular  $\text{Ca}^{2+}$  flux as indicated by a failure to see cells within the bracketed area. Addition of excess  $\text{Ca}^{2+}$  to the cells (fine arrow) restores the extracellular  $\text{Ca}^{2+}$  flux and cells return to the bracketed area. (C) As for  $\text{Cd}45^{+/+}$  cells. Note the absence of cells in the bracketed area after the initial peak due to intracellular  $\text{Ca}^{2+}$  flux.

## Summary

$\text{Cd}45^{-/-}$  mice produce equivalent numbers of B cells compared to control animals. These B cells are unusual in that they have altered levels of cell surface markers. In apparent contradiction, these cells appear immature on the basis of their

expression levels of IgM and IgD and yet have increased expression of other markers that are associated with increasing B cell maturation. In addition, CD45-deficient B cells have increased expression of B7.2, suggesting that these cells are activated and therefore that the absence of CD45 has made the BCR more susceptible to activation. In contrast, on BCR stimulation *in vitro* these B cells have a diminished BCR-mediated proliferation response. B-1 cell populations are greatly reduced in these mice suggesting that CD45 is also a positive regulator of BCR signalling B-1 cells.

The use of the 3-83 transgenic BCR demonstrates that 3-83/*Cd45*<sup>-/-</sup> B cells can be produced however these cells express unusual levels of IgM and IgD, suggesting that they are less mature than CD45-deficient B cells that develop in the absence of the transgene. *Cd45*<sup>-/-</sup> B cells are deleted normally in the presence of the negatively selecting ligand for the transgenic receptor and undergo both Ig heavy and light chain allelic exclusion normally suggesting that CD45 is not required for these processes. Finally *Cd45*<sup>-/-</sup> B cells have a diminished but not absent Ca<sup>2+</sup> flux on receptor stimulation that could explain the abolition of some BCR-mediated responses.

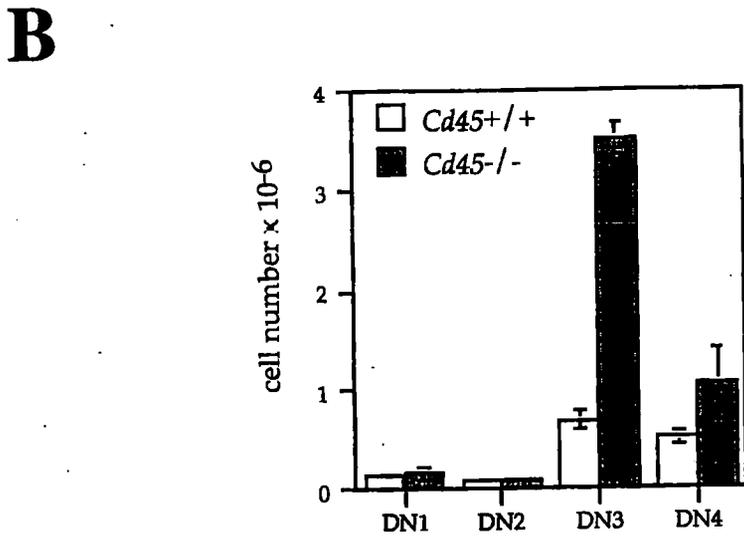
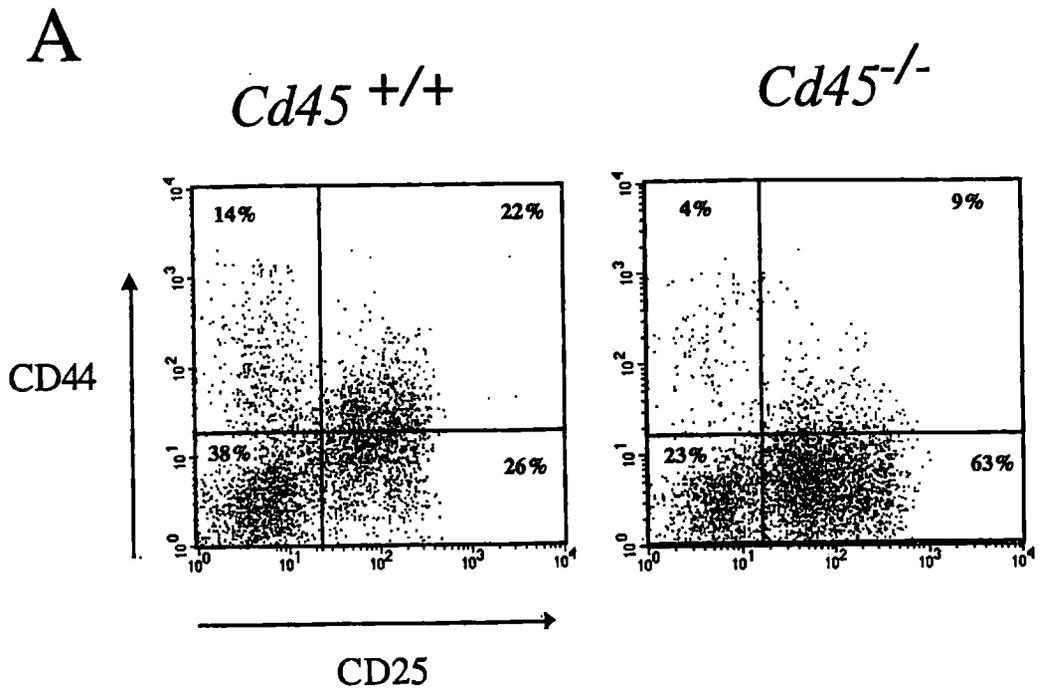
### 3.3 T cell development in *Cd45*<sup>-/-</sup> mice

#### ***Cd45*<sup>-/-</sup> T cells have a partial block in early T cell development**

CD45 has been implicated as a positive regulator of signal transduction from the TCR in the majority of studies conducted on CD45-deficient T cell lines *in vitro*. I therefore decided to assess the effect of CD45 deficiency on the development of T cells in *Cd45*<sup>-/-</sup> mice with particular emphasis of those developmental checkpoints that require signal transduction from the receptor.

To assess the effect of CD45 deficiency on the development of T cells in *Cd45*<sup>-/-</sup> mice, flow cytometric analysis was performed using antibodies specific for markers of early T cell development. T cells develop in the thymus from CD4<sup>+</sup>CD8<sup>-</sup>

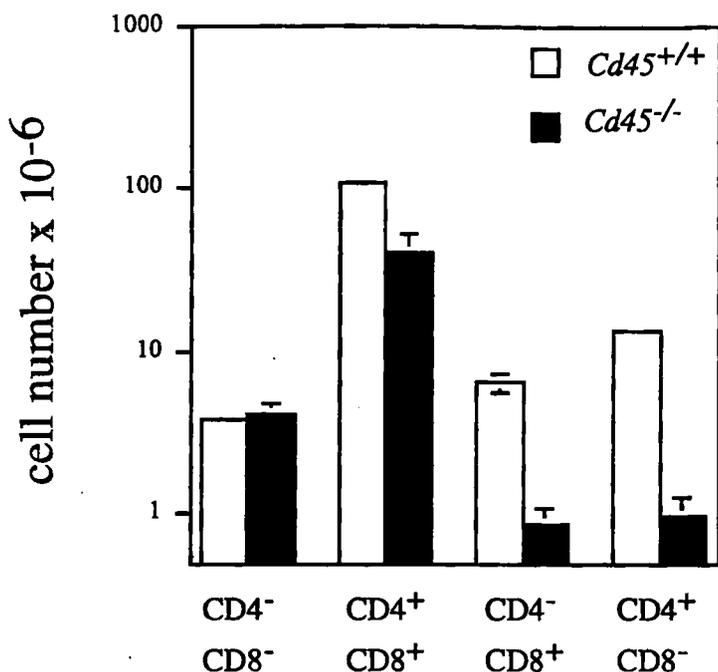
double negative (DN) thymocytes through an intermediate double positive (DP) stage where thymocytes express both the CD4 and CD8 coreceptor molecules, to single positive (SP) mature CD4<sup>+</sup> or CD8<sup>+</sup> T cells. The DN stage of T cell development can be further subdivided into distinct developmental steps based on their cell surface expression of CD25 and CD44 molecules. DN thymocytes develop from CD44<sup>+</sup>CD25<sup>-</sup> (DN1) cells into CD44<sup>+</sup>CD25<sup>+</sup> (DN2) cells, next into CD44<sup>-</sup>CD25<sup>+</sup> (DN3) and finally into CD44<sup>-</sup>CD25<sup>-</sup> (DN4) thymocytes prior to leaving the DN compartment (Godfrey and Zlotnik, 1993; Nikolic-Zugic, 1991). Figure 18A shows a typical flow cytometric analysis using these markers and Figure 18B shows the enumerated results of three mutant and control mice. Cells were stained with antibodies to CD4, CD8, CD44 and CD25 and analysed using four colour flow cytometry. The number of DN cells was found to be similar between mutant and control mice; however, there is a greater than 5-fold accumulation of DN cells that are CD44<sup>-</sup>CD25<sup>+</sup> (DN3) cells in *Cd45*<sup>-/-</sup> mice compared to *Cd45*<sup>+/+</sup> controls. A block at this stage of T cell development has been reported in mice mutant for *Rag-1*, *TCRβ* and *pTα*, suggesting that this block is associated with pre-T cell receptor signalling (Fehling et al., 1995; Levelt et al., 1993; Mombaerts et al., 1992). This deficiency in T cell development in *Cd45*<sup>-/-</sup> mice is a partial block as cells can develop into CD44<sup>-</sup>CD25<sup>-</sup> T cells and leave the DN T cell compartment to become DP thymocytes. This shows that CD45 is required for the efficient progression through the DN3 stage of thymocyte development and suggests that CD45 is a positive regulator of signal transduction from the pre-T cell receptor.



**Figure 18: Early T cell development is impaired in *Cd45<sup>-/-</sup>* mice.** (A) Typical flow cytometric analysis of CD44 and CD25 expression on DN thymocytes from 6 wk old female outbred mice with the percentages shown for the least mature CD44<sup>+</sup>CD25<sup>-</sup> (DN1) fraction, the CD44<sup>+</sup>CD25<sup>+</sup> (DN2) fraction, the CD44<sup>-</sup>CD25<sup>+</sup> (DN3) fraction and the most mature CD44<sup>-</sup>CD25<sup>-</sup> (DN4) fraction of DN thymocytes. (B) Bar graph showing mean numbers of DN thymocytes from 3 control (*Cd45<sup>+/+</sup>*) and 3 mutant (*Cd45<sup>-/-</sup>*) outbred mice (female; 6 weeks old). Error bars represent standard error of mean (s.e.m.). Numbers are shown for the DN1, DN2, DN3 cells and the most mature DN4 thymocytes. Note the five-fold increase in DN3 cells in *Cd45<sup>-/-</sup>* mice.

## The development of single positive thymocytes is blocked in *Cd45*<sup>-/-</sup> mice

T cells develop from the DP stage of thymic development into CD4<sup>+</sup> or CD8<sup>+</sup> SP thymocytes that exit the thymus and enter the periphery. As described in the introduction, the positive and negative selection of thymocytes at the DP stage of T cell development relies on the strength of signal from the TCR. To determine the role of *Cd45* in these developmental steps, flow cytometry was performed on *Cd45*<sup>-/-</sup> thymi using antibodies to CD4 and CD8 and the absolute numbers of cells were calculated. Thymic cellularity in *Cd45*<sup>-/-</sup> mice is typically half that of control mice. This reduction can almost entirely be accounted for by a reduction in the number of DP thymocytes present in *Cd45*<sup>-/-</sup> mice (Figure 19). This reduction is likely to be due to the reduced efficiency of transit into the DP stage of thymocyte development due to the earlier block at the DN3 stage of T cell development. There is also a dramatic 7-13-fold reduction in the number of CD4<sup>+</sup> and CD8<sup>+</sup> SP cells in the thymus of *Cd45*<sup>-/-</sup> mice. A similar decrease was also seen in the numbers of CD4<sup>+</sup> and CD8<sup>+</sup> peripheral T cells in the spleen and lymph nodes of the mutant mice (not shown). This is suggestive of a block in positive selection events. As seen with the earlier block at the DN stage of T cell development, this is not a complete block as some thymocytes develop into CD4<sup>+</sup> SP and CD8<sup>+</sup> SP cells. This suggests that CD45 may be required for the efficient signal transduction from the TCR during positive selection.

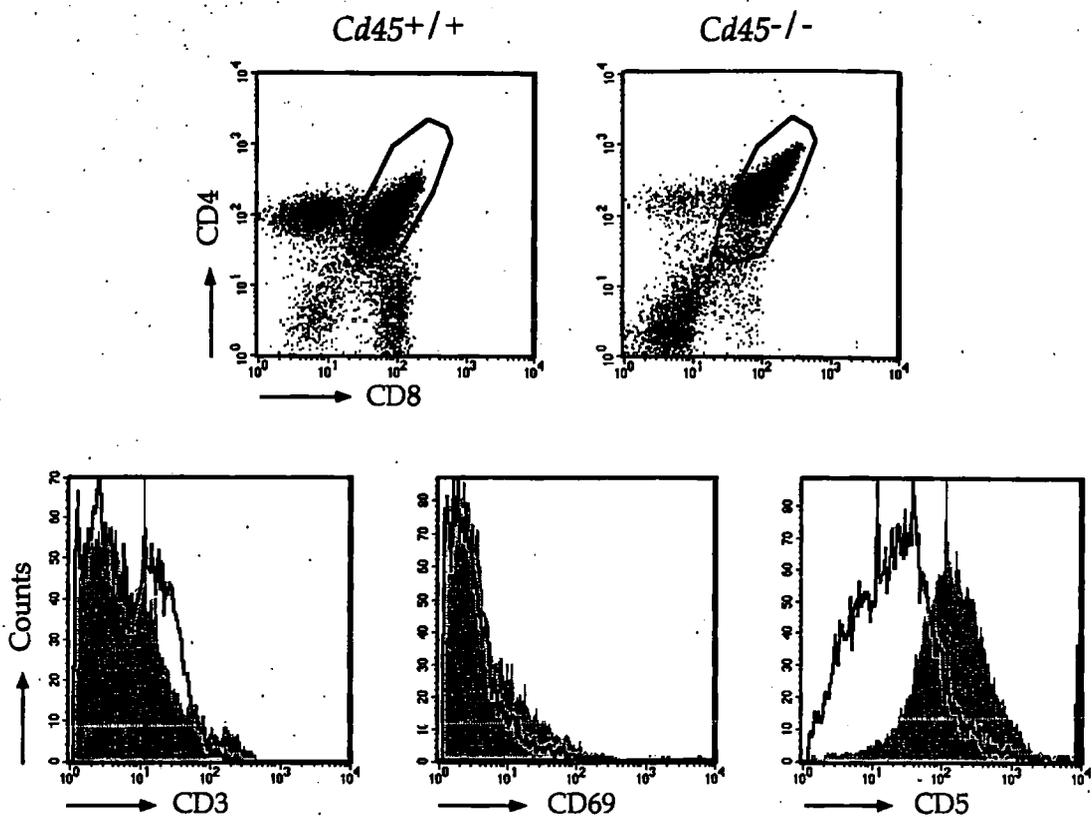


**Figure 19: *Cd45*<sup>-/-</sup> thymic development is impaired.** Bar graph showing mean numbers of thymocytes from 6 control (*Cd45*<sup>+/+</sup>) and 6 mutant (*Cd45*<sup>-/-</sup>) mice (female; 7-8 weeks old) inbred on a BALB/c background. Error bars represent standard error of mean (s.e.m.). Numbers are shown for the least mature double negative (DN) fraction of thymocytes expressing neither CD4 nor CD8 (CD4<sup>-</sup>CD8<sup>-</sup>), the double positive (DP) fraction expressing both (CD4<sup>+</sup>CD8<sup>+</sup>) or the most mature single positive (SP) fractions expressing either CD4 or CD8 alone (CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>+</sup>). CD45-deficient thymocytes show a 2.6-fold reduction in the number of DP and a 7 to 13-fold reduction in the number of SP cells. Note the logarithmic scale.

### Thymocytes from *Cd45*<sup>-/-</sup> mice express altered levels of cell surface markers

A number of cellular markers can be used to assess maturation of T cells during their development. Flow cytometric analysis was performed to assess the expression of these markers during T cell development in *Cd45*<sup>-/-</sup> mice. A hallmark of DP thymocytes undergoing positive selection is an increase in CD3 expression from intermediate to high levels and the induction of CD69 expression. Figure 20 shows flow cytometric analysis of *Cd45*<sup>-/-</sup> thymocytes using these markers in multicolour analysis. Cells were stained with antibodies to CD4 and CD8 and gating was used to display as histograms the levels of expression of these maturity markers in DP cells. Firstly it can be seen that *Cd45*<sup>-/-</sup> DP thymocytes contain cells

that express very high levels of the CD4 and CD8 coreceptor molecules compared to controls. In addition *Cd45*<sup>-/-</sup> DP thymocytes contain very few cells that are CD3<sup>hi</sup>, a characteristic of cells undergoing positive selection. Instead *Cd45*<sup>-/-</sup> mice have many DP cells with intermediate levels of expression of CD3 suggestive of a block at this point in development. *Cd45*<sup>-/-</sup> DP thymocytes also contain fewer CD69<sup>+</sup> cells a further characteristic of a failure of positive selection (Swat et al., 1993; Wang et al., 1995; Yamashita et al., 1993). CD45-deficient DP thymocytes also fail to up-regulate the expression of CD5 (Figure 20) a marker that normally increases in expression during the transition from DN to DP cells (Dutz et al., 1995; Sheard et al., 1996). The few SP thymocytes and peripheral T cells that do develop in *Cd45*<sup>-/-</sup> mice are unusual in that they express higher levels of expression of CD69 and HSA than control cells (not shown) suggesting that a lack of CD45 has had a profound effect on the biology of these cells.

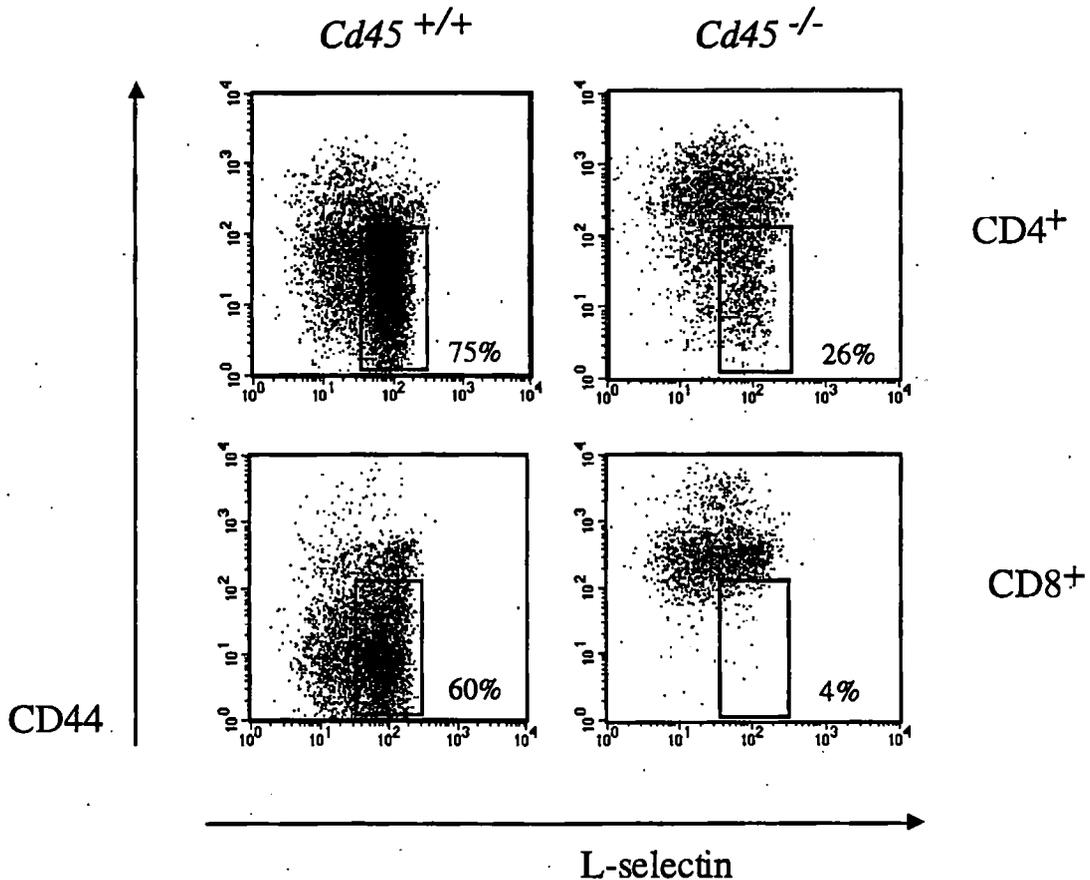


**Figure 20: Thymic development in *Cd45*<sup>-/-</sup> mice.** Typical flow cytometric analysis of *Cd45*<sup>+/+</sup> and *Cd45*<sup>-/-</sup> thymocytes stained with anti-CD4 and anti-CD8. The dot plots at the top show flow cytometric analysis of a *Cd45*<sup>+/+</sup> and *Cd45*<sup>-/-</sup> thymus stained for CD4 and CD8. Levels of expression of cellular maturity and activation markers in the DP population are displayed in the histogram plots (gated as shown) and was achieved by three or four colour flow cytometric analysis (*Cd45*<sup>+/+</sup> -shaded; *Cd45*<sup>-/-</sup> thick black line)

### *Cd45*<sup>-/-</sup> T cells have a “previously activated” phenotype

Lymphocytes on encounter with antigen become activated, and these cells can be distinguished by their expression of the cell surface markers CD44 and L-selectin (Barrat et al., 1995; Gerberick et al., 1997; Khan et al., 1996; Tietz and Hamann, 1997). Those cells which are activated are typically CD44<sup>hi</sup> and L-selectin<sup>lo</sup> whilst naive T cells have CD44<sup>lo</sup> L-selectin<sup>hi</sup> phenotype. As CD45 is thought to be a positive regulator of signal transduction from the TCR I decided to assess *Cd45*<sup>-/-</sup> mice to see if this mutation prevented the activation of CD45-deficient T cells. Figure 21 shows flow cytometric analysis of lymph node T cells from *Cd45*<sup>-/-</sup> mice compared to *Cd45*<sup>+/+</sup> mice, assessed for the expression of these activation markers. It can be seen that in *Cd45*<sup>+/+</sup> mice, both CD4<sup>+</sup> and CD8<sup>+</sup> T

cells are mainly naive ( $CD44^{lo}L\text{-selectin}^{hi}$ ). In contrast, most  $Cd45^{-/-}$   $CD4^{+}$  and  $CD8^{+}$  SP T cells have a “previously activated” phenotype ( $CD44^{hi}L\text{-selectin}^{lo}$ ). This is surprising if CD45 is a positive regulator of TCR signalling, since the mutation would be expected to prevent these cells becoming activated. These results suggest that CD45 may be a negative regulator of TCR signalling and that these cells are more susceptible to activation on encounter with antigen.



**Figure 21: Flow cytometric analysis of lymph node cells for activation markers.** Lymph node cells stained with anti-CD4 and anti-CD8 together anti-CD44 (IM7) and anti-L-selectin (Mel 14).  $CD4^{+}$  or  $CD8^{+}$  T cells were gated and the levels of CD44 and L-selectin expression was then displayed as dot plots by four colour analysis. The gated region represents naive  $L\text{-selectin}^{hi}CD44^{lo}$  cells and the percentages of these cells are displayed.

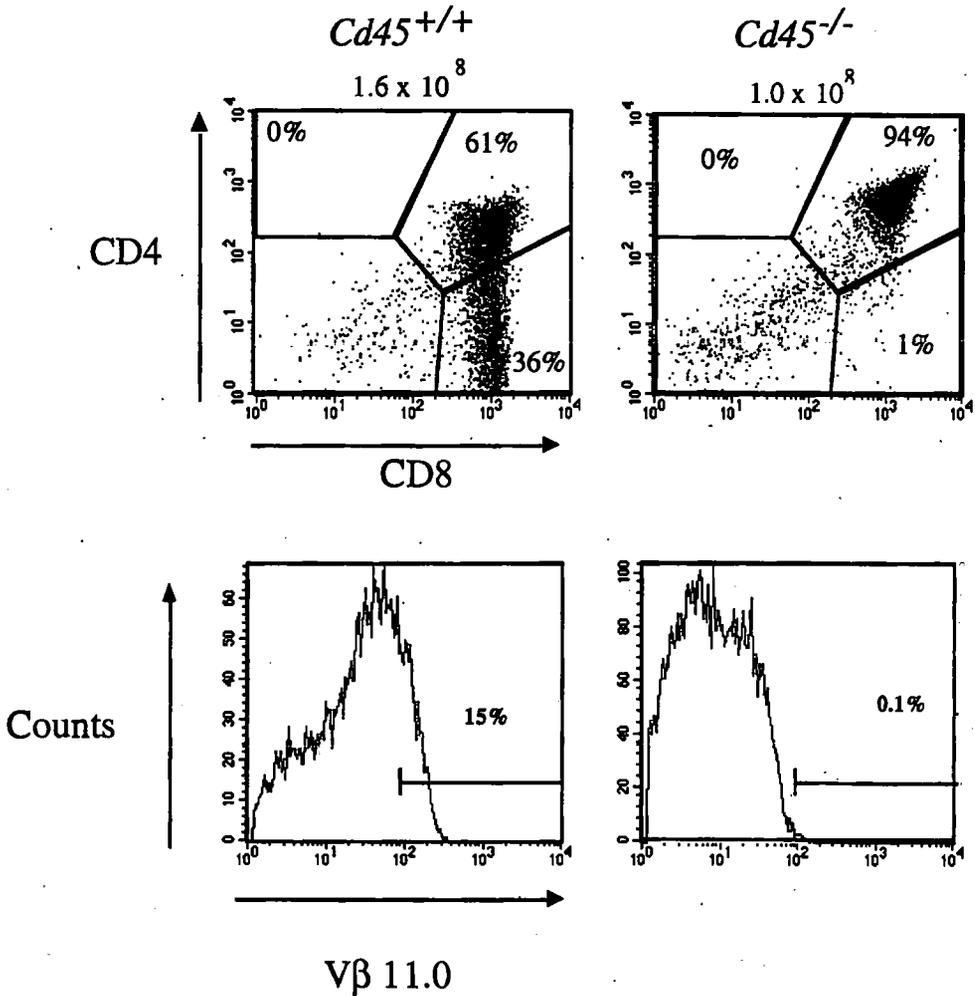
**Positive selection of the F5 Class-I restricted transgene is inhibited in  $Cd45^{-/-}$  mice**

The reduction of the number of  $CD4^{+}$  and  $CD8^{+}$  T cells present in the periphery of  $Cd45^{-/-}$  mice is suggestive of a block in positive selection. DP

thymocytes undergoing positive selection increase their levels of TCR and induce the expression of CD69, whilst there is a concomitant decrease in HSA and CD5 expression (Dutz et al., 1995; Sheard et al., 1996). Alterations in the level of expression of these markers and the failure to accumulate normal numbers of peripheral T cells is consistent with a failure of positive selection of T cells within these mice. However CD45 is expressed on all nucleated haemopoietic cells and it is possible that a defect in cells, other than the thymocytes themselves, is responsible for this failure of selection. To address this, I reconstituted lethally irradiated mice with mixtures of *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* bone marrow. Flow cytometric analysis of these chimeric animals demonstrated that *Cd45<sup>-/-</sup>* marrow gave rise to both B and T cells with identical phenotypic properties to those found in non-chimeric *Cd45<sup>-/-</sup>* mice (data not shown) suggesting that these developmental blocks and phenotypic alterations are cell-autonomous, ie. that CD45 is required within the B or T cells themselves for normal development.

To test the requirement for CD45 in more detail, transgenic TCRs were introduced onto the *Cd45<sup>-/-</sup>* genetic background. The transgenic TCR recognises an influenza nuclear protein peptide in the context of H-2D<sup>b</sup> and is positively selected on the H-2<sup>b</sup> haplotype (Mamalaki et al., 1993). *F5/Cd45<sup>-/-</sup>* mice were further bred to *Rag-1<sup>-/-</sup>* mice to make *F5/Cd45<sup>-/-</sup>/Rag-1<sup>-/-</sup>* mice. The absence of *Rag-1* gene expression prevents endogenous TCR rearrangements and allows the study of the selection of the transgenic receptor in the absence of endogenous TCRs. Flow cytometric analysis was performed on the thymi of these mice and compared to *Cd45<sup>+/+</sup>* controls (Figure 22). The transgenic TCR was identified by an anti-V $\beta$ -11.0 antibody (the V $\beta$  used by the transgenic receptor). Firstly it can be seen that there are similar numbers of CD4<sup>+</sup>CD8<sup>+</sup> DP cells in both *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* mice. However, only *Cd45<sup>+/+</sup>* DP cells are able to express high levels of the transgenic receptor, *Cd45<sup>-/-</sup>* DP cells being either negative or having intermediate levels of the receptor suggesting a deficiency in positive selection in these mice. Consistent with this observation is the profound lack of CD8<sup>+</sup> SP thymocytes in *Cd45<sup>-/-</sup>* mice. The small number of CD4<sup>+</sup>CD8<sup>+</sup> cells found in the mutant thymi are likely to be transitional cells from the DN to DP stage of thymocyte development

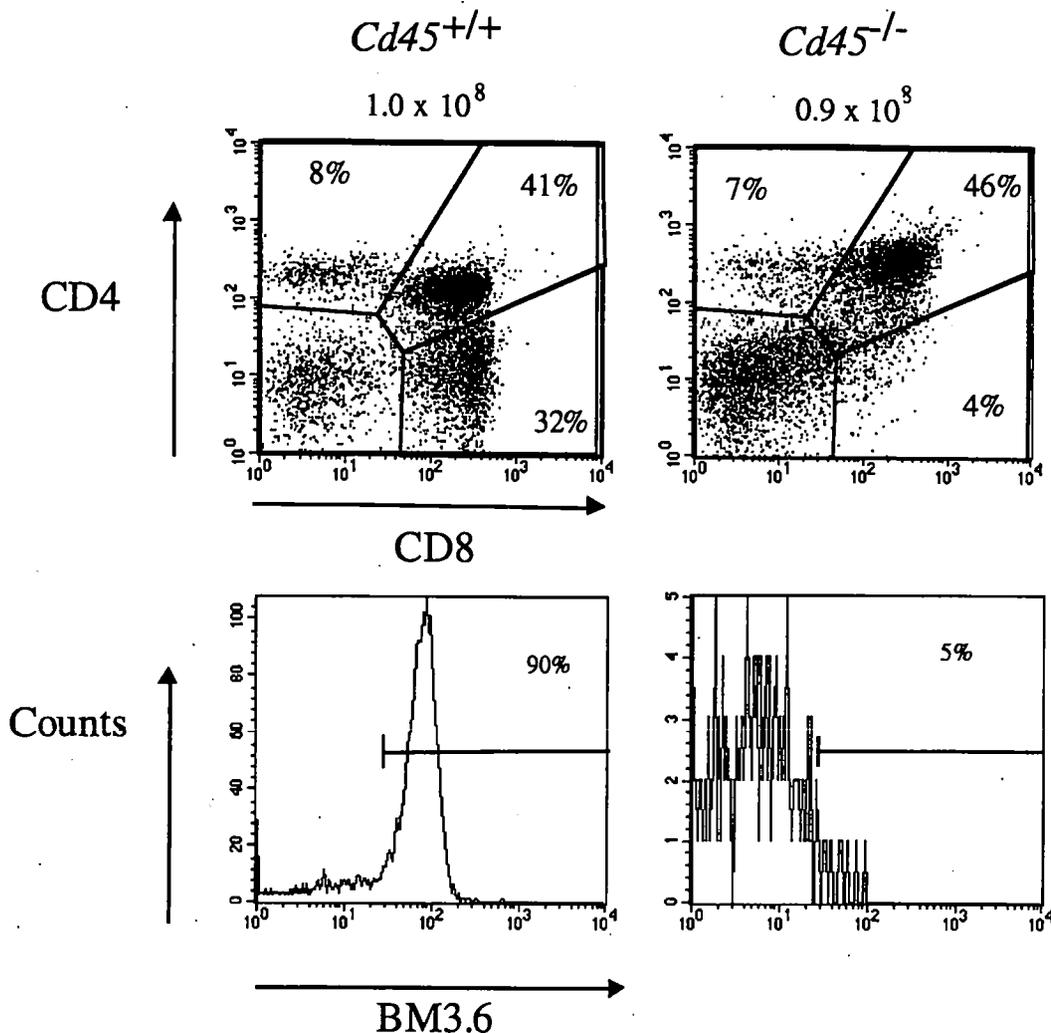
that express low levels of CD8 during this transition. In contrast to control mice, no CD8<sup>+</sup> cells are found in the spleen or lymph node of F5/*Cd45*<sup>+</sup>/*Rag-1*<sup>-</sup> mice (not shown). Thus CD45 is absolutely required for the positive selection of thymocytes bearing the F5 TCR.



**Figure 22: Failure of positive selection in *Cd45*<sup>-</sup> thymocytes expressing the F5 transgenic TCR.** Control (*Cd45*<sup>+/+</sup>) and mutant mice (*Cd45*<sup>-/-</sup>) (6 week old) expressing the transgenic F5 TCR on the positively selecting H-2<sup>b</sup> background in the absence of *Rag-1* gene expression. Expression of the transgenic TCR on DP cells (gated as shown in dot plots) was evaluated using an anti-Vβ-11 antibody and displayed as histograms (bottom). Percentage of Vβ-11<sup>hi</sup> cells is indicated. All plots are representative of multiple experiments.

## Positive selection of the BM3.6 Class-I restricted TCR is inhibited in *Cd45<sup>-/-</sup>* mice

To confirm the deficiency of positive selection of thymocytes found in the F5 expressing transgenic mice the *Cd45<sup>-/-</sup>* mice were bred to express a second class I-restricted transgene, BM3.6. BM3.6 is a class I-restricted anti-H-2K<sup>b</sup> TCR which is positively selected on an H-2K<sup>k</sup> haplotype (Sponaas et al., 1994). These experiments were conducted in the presence of *Rag-1* and *Rag-2* gene expression, so endogenous gene rearrangements can still occur. However the transgenic TCR can be distinguished from endogenously rearranged receptors by the use of an anti-clonotypic antibody which specifically recognises the BM3.6 TCR. Flow cytometric analysis of thymocytes from control animals (*Cd45<sup>+/+</sup>*) expressing the positively selecting ligand H-2K<sup>k</sup> was conducted by staining with anti-CD4 and anti-CD8 antibodies (Figure 23). Many CD8<sup>+</sup> SP cells accumulate in the thymus of *Cd45<sup>+/+</sup>* mice (Figure 23) and these cells express high levels of the transgene (not shown). These develop into peripheral CD8<sup>+</sup> T cells which express high levels of the BM3.6 TCR and are mature as determined by cell surface markers (Figure 23-histograms). *Cd45<sup>-/-</sup>*, BM3.6 mice develop equivalent numbers of DP thymocytes as *Cd45<sup>+/+</sup>* mice (Figure 23). However, very few cells develop from these DP cells to become CD8<sup>+</sup>SP cells, demonstrating a failure of positive selection of this transgene in the absence of CD45 (Figure 23). The small number of cells found in area gating the CD8<sup>+</sup>SP cells in mutant thymi are likely to be transitional cells from the DN to DP, as despite expressing the transgene they do not have the low levels of expression of CD4 characteristic of CD8<sup>+</sup> SP thymocytes and in addition CD8<sup>+</sup> T cells in the spleen or lymph node do not express the transgenic receptor but express TCRs derived from endogenous gene rearrangements (Figure 23-histograms). This demonstrates an absolute requirement for CD45 in the positive selection of a second class-I-restricted TCR.

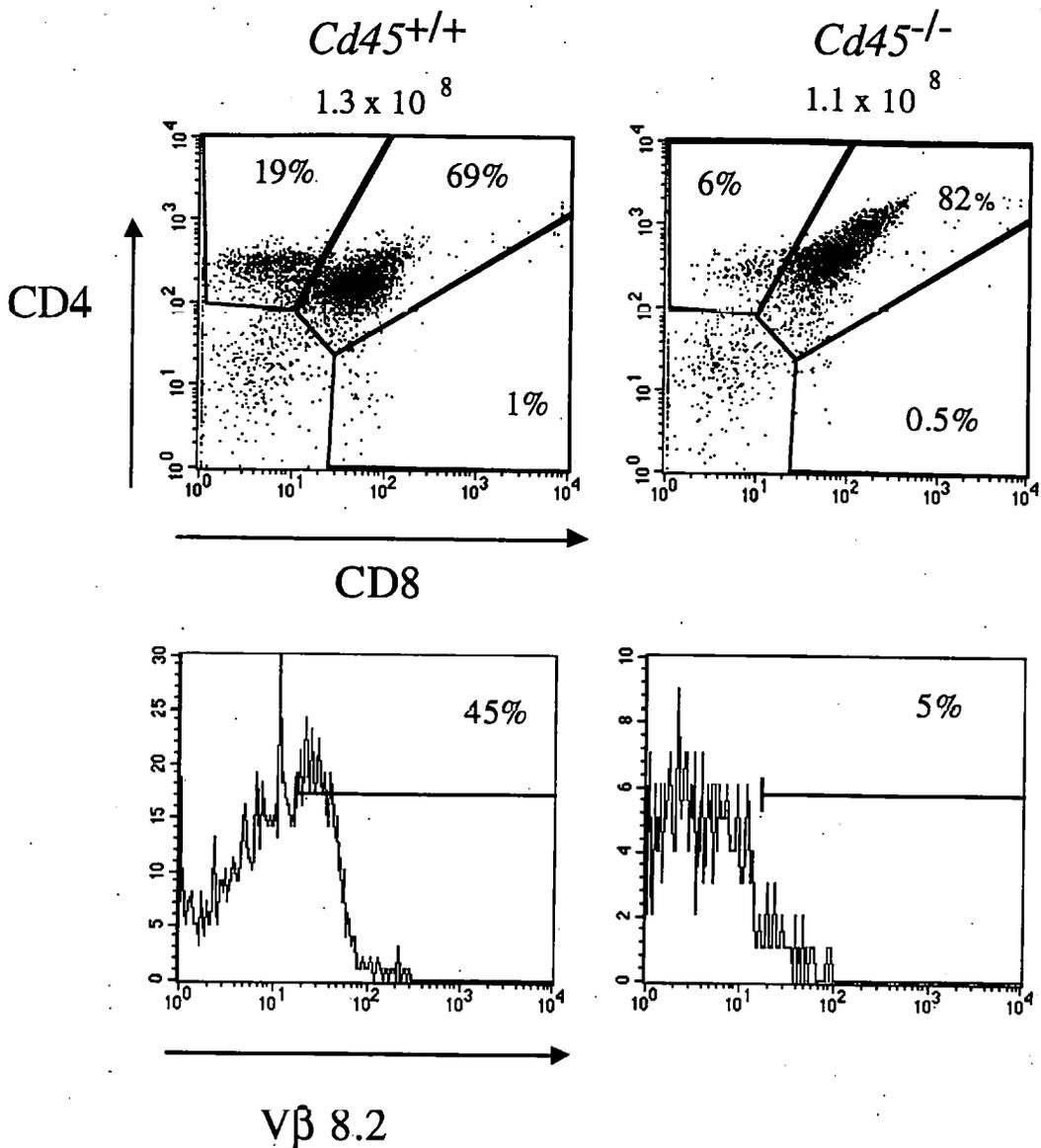


**Figure 23: Failure of positive selection in *Cd45*<sup>-/-</sup> thymocytes expressing the BM3.6 transgenic TCR.** *Cd45*<sup>+/+</sup> and *Cd45*<sup>-/-</sup> mice were bred to contain the BM3.6 transgene and express the positively selecting ligand MHC class I H-2<sup>k</sup>. Flow cytometric analysis was performed on thymocytes stained with anti-CD4 and anti-CD8 together with an anti-clonotypic antibody. Histograms (bottom) show expression of the BM3.6 TCR gated on CD8<sup>+</sup> splenic T cells (top). Percentages of cells within the gated regions are displayed. All plots are representative of multiple experiments.

### Positive selection of the A1 Class-II restricted TCR is inhibited in *Cd45*<sup>-/-</sup> mice

To determine whether the positive selection of class II-restricted thymocytes requires CD45, the *Cd45*<sup>-/-</sup> mice were bred to express the A1 transgenic TCR (to make A1/*Cd45*<sup>-/-</sup> mice). The A1 transgene recognises a peptide from the male specific H-Y antigen presented by MHC class II I-E<sup>k</sup> and is positively selected on the H-2<sup>k</sup> haplotype (Douek et al., 1996). These experiments were conducted in the presence of endogenous *Rag-1* and *Rag-2* gene expression, so the mice are able to

rearrange endogenous TCRs. No-clonotype specific antibody exists for the A1 TCR however the transgenic receptor can be distinguished by its expression of V $\beta$ 8.2. Figure 24 shows a typical flow cytometric analysis of A1/*Cd45*<sup>-/-</sup> and A1/*CD45*<sup>+/+</sup> female mice (where the negative selecting H-Y ligand is absent) on the positively selecting H-2<sup>k</sup> genetic background. Thymocytes were stained with anti-CD4 and anti-CD8 antibodies and the expression of V $\beta$ 8.2 on CD4<sup>+</sup> SP cells displayed as histograms. Firstly it can be seen that there are equivalent numbers of DP cells in both *Cd45*<sup>+/+</sup> and *Cd45*<sup>-/-</sup> mice (Figure 24). In contrast to control mice, very few DP thymocytes develop into CD4<sup>+</sup> SP cells in A1/*Cd45*<sup>-/-</sup> mice and those that do develop express predominately endogenously rearranged receptors. In addition no A1-expressing T cells are found in the periphery of these mice. These observations demonstrate an absolute requirement for CD45 for the efficient positive selection of the A1 class-II restricted TCR.

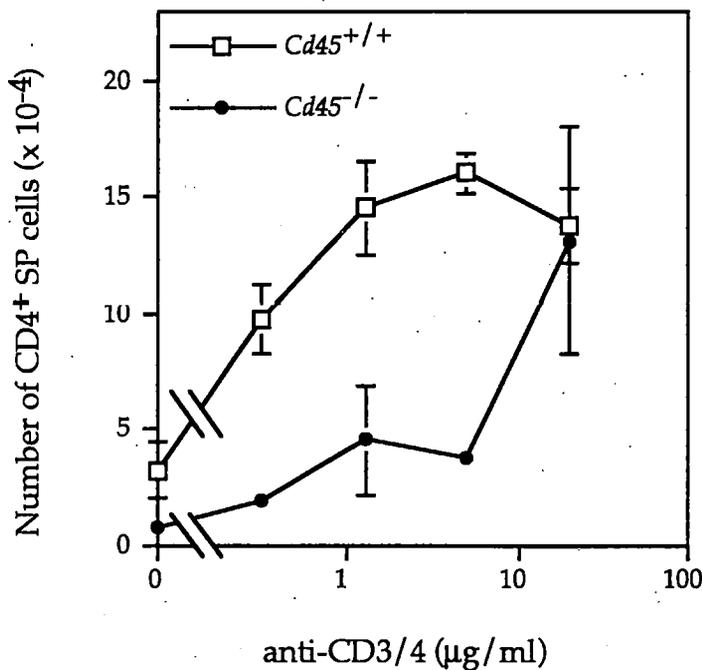


**Figure 24: Thymocyte development in *Cd45<sup>-/-</sup>* and *Cd45<sup>+/-</sup>* mice carrying the A1 transgenic TCR.** *Cd45<sup>+/-</sup>* and *Cd45<sup>-/-</sup>* mice were bred to contain the A1 transgene and express the positively selecting ligand MHC class II I-E<sup>k</sup>. Thymocytes were stained with anti-CD4 and anti-CD8 together with an anti-Vβ8.2 antibody. Histograms (bottom) show expression of the Vβ8.2 expression on CD4<sup>+</sup> SP cells gated as shown in the dot plot (top). Percentages of cells in gated regions are displayed. Percentages on histograms show fraction of cells that are Vβ-8.2<sup>+</sup>. All plots are representative of multiple experiments.

### ***Cd45<sup>-/-</sup>* thymocytes have a reduced sensitivity to a positively selecting ligand**

To further investigate the lack of positive selection of *Cd45<sup>-/-</sup>* thymocytes, a bi-specific antibody that co-crosslinks the CD3 and CD4 molecules was used to induce thymocytes to develop into SP cells in neonatal thymic organ cultures (NTOC) *in vitro* in a dose-dependent manner (Basson et al., 1998; Bommhardt et al., 1997). This reagent allows the determination of whether CD45-deficient

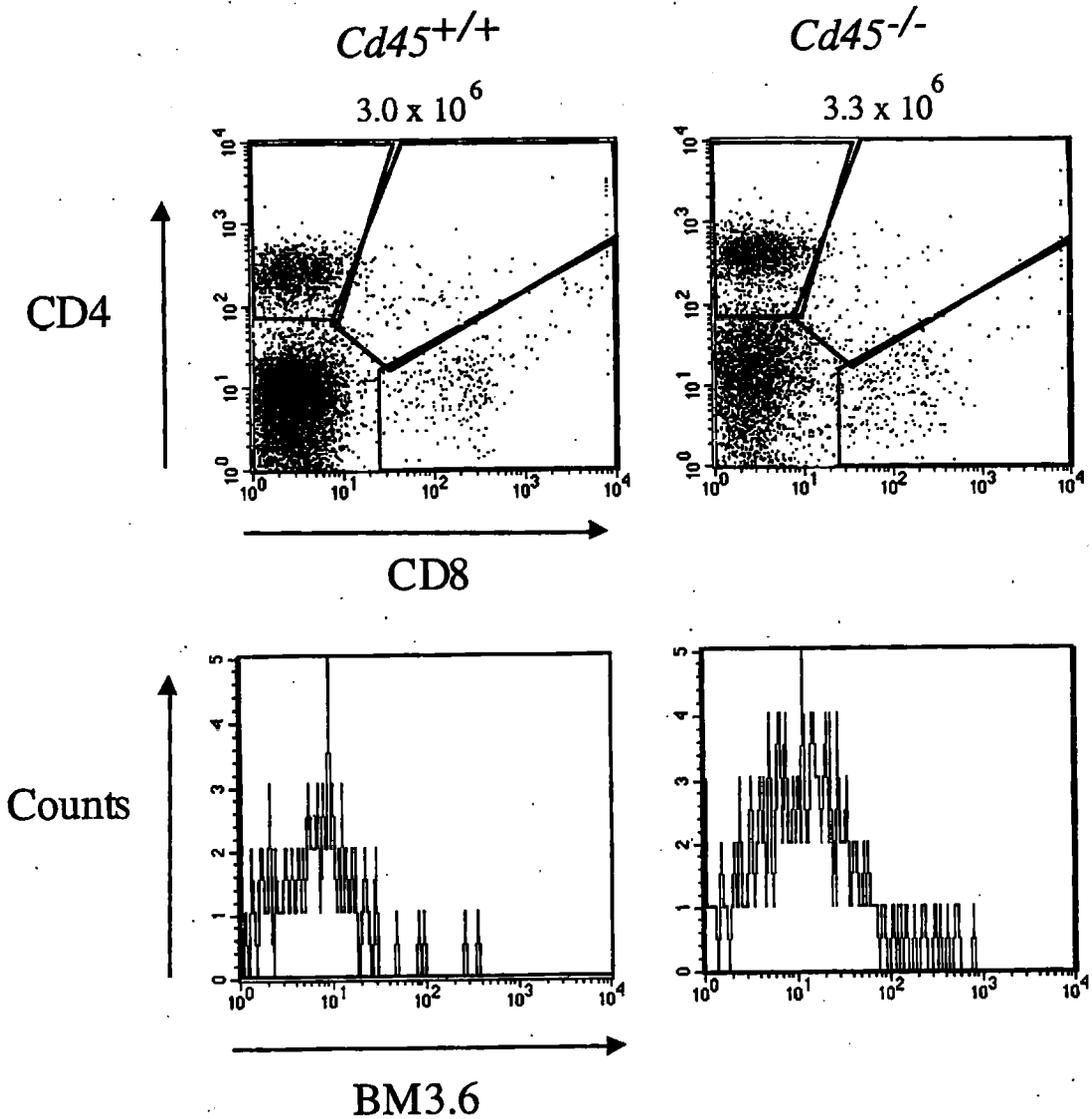
thymocytes can be positively selected by increasing doses of stimulus. Figure 25 shows a graphical representation of total CD4<sup>+</sup> SP cells generated from *Cd45*<sup>+/+</sup> and *Cd45*<sup>-/-</sup> neonatal thymi stimulated *in vitro* with increasing concentrations of the anti-CD3/CD4 bi-specific antibody as assessed by flow cytometric analysis. It can be seen that at very high concentrations of the crosslinking antibody both *Cd45*<sup>+/+</sup> and *Cd45*<sup>-/-</sup> thymocytes are induced to develop into CD4<sup>+</sup> SP cells in equivalent numbers. However, at lower concentrations of this antibody, many fewer CD4<sup>+</sup> SP cells developed in *Cd45*<sup>-/-</sup> thymi. These results are consistent with the idea that *Cd45*<sup>-/-</sup> thymocytes require a greater level of receptor stimulation to induce positive selection than controls and suggests an altered threshold for antigen receptor signalling in *Cd45*<sup>-/-</sup> thymocytes during positive selection.



**Figure 25: *Cd45*<sup>-/-</sup> thymocytes have a reduced sensitivity to a positively selecting ligand.** Graph of total numbers of CD4<sup>+</sup>SP cells generated on stimulation of neonatal thymic cultures from F5/*Cd45*<sup>-/-</sup>/*Rag-1*<sup>-/-</sup> mice stimulated with a bi-specific anti-CD3/CD4 antibody at the indicated concentrations. CD4<sup>+</sup> SP cells were assessed by flow cytometric analysis and total cell numbers calculated. Error bars are standard error of mean.

## ***Cd45<sup>-/-</sup>* thymocytes can undergo negative selection**

To examine the requirement for CD45 in the negative selection of thymocytes *Cd45<sup>-/-</sup>* mice were bred to express the BM3.6 TCR on the negatively selecting H-2K<sup>b</sup> background. Figure 26 shows the result of flow cytometric analysis of thymi from mutant and control mice expressing the transgene on the negatively selecting background. Thymocytes were stained with anti-CD4 and anti-CD8 and the level of expression of the transgene expressed on CD8<sup>+</sup> SP cells displayed as histograms. Firstly it can be seen that there is a dramatic deletion of both *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* DP thymocytes in the presence of the negatively selecting ligand H-2K<sup>b</sup> (Figure 26). This deletion is associated with a decrease in thymic cellularity within these mice (approximately 30 fold - compare cellularity in Figures 23 and 26). A small number of CD4<sup>+</sup>SP and CD8<sup>+</sup> SP thymocytes are present in the thymus of both *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* mice. These cells are predominantly clonotype-negative in both *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* mice and are present due to endogenous TCR gene rearrangements, since these mice were *Rag-1, -2<sup>+</sup>*. Furthermore, no BM3.6-expressing cells are found in the spleen or lymph node of control or mutant mice (not shown). These results demonstrate that negative selection of the BM3.6 TCR occurs normally in *Cd45<sup>-/-</sup>* mice and thus CD45 is not required for the TCR signalling that drives this selection event.

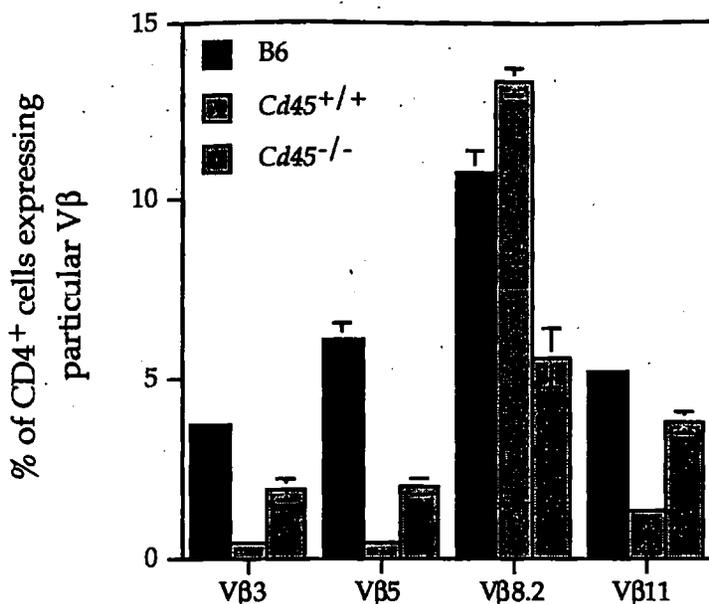


**Figure 26: CD45-deficient thymocytes can undergo negative selection.** Flow cytometric analysis of thymi from control (*Cd45*<sup>+/+</sup>) and mutant (*Cd45*<sup>-/-</sup>) mice (8-10 week old) expressing the BM3.6 transgenic TCR on a deleting H-2<sup>b</sup> background. These mice are *Rag-1*<sup>+/+</sup>, so some endogenous TCR genes are expressed. The total number of thymocytes is shown above each plot. The histograms show expression of the transgene in CD8<sup>+</sup>SP cells.

***Cd45*<sup>-/-</sup> mice have an altered sensitivity to the negative selection of lymphocytes induced by endogenous superantigens**

To further characterise negative selection of thymocytes, deletion in response to endogenous superantigens was studied. As described in the introduction, superantigens are presented by MHC molecules outside the peptide binding groove and are likely therefore to provide a weaker stimulus than conventional antigens. BALB/c mice present proteins encoded by endogenous mouse mammary tumour proviruses (*Mtv* loci) in association with the MHC class II

molecule I-E<sup>d</sup> and this results in the specific deletion of CD4<sup>+</sup> SP cells expressing V $\beta$  3, 5 and 11 (Simpson et al., 1993). In contrast, V $\beta$ 8-expressing SP cells do not undergo *Mtv*-mediated deletion in the BALB/c strain and are used as a control in these experiments. B6 mice which have a deletion in I-E are unable to present these *Mtv* superantigens and cannot delete the relevant V $\beta$ -expressing cells. Figure 27 shows the result of flow cytometric analysis of *Cd45*<sup>-/-</sup> BALB/c mice and *Cd45*<sup>+/+</sup> BALB/c controls compared to B6 mice for their ability to delete T cells expressing particular V $\beta$  genes. *Cd45*<sup>+/+</sup> mice are found to have many fewer V $\beta$  3, 5 and 11 CD4<sup>+</sup> T cells compared to the control B6 mice due to the negative selection of these cells in the presence of the deleting superantigen. In contrast *Cd45*<sup>-/-</sup> mice clearly have more CD4<sup>+</sup> T cells which express V $\beta$  3, 5 and 11 though the numbers of these cells are reduced in comparison to the B6 mice suggesting that there is a partial block in the negative selection of these cells. As expected all three mouse strains were found to contain V $\beta$ 8.2<sup>+</sup> cells, since TCRs expressing this V $\beta$  do not bind to the *Mtv* superantigen. This demonstrates at least a partial requirement for CD45 in the deletion of superantigen-specific T cells. In an additional experiment *Cd45*<sup>-/-</sup> mice were found to delete V $\beta$  3, 5 and 11-expressing T cells in a similar manner to *Cd45*<sup>+/+</sup> mice (not shown).

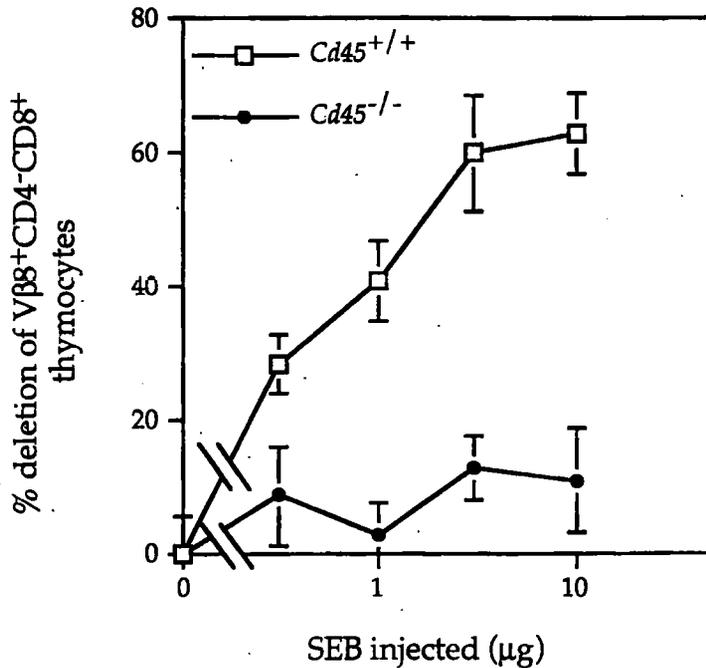


**Figure 27: *Mtv* superantigen deletion of CD4<sup>+</sup> lymph node T cells expressing particular Vβ genes.** Bar graph representing the mean percentage of CD4<sup>+</sup> T cells expressing Vβ3, 5, 8.2 or 11 in lymph nodes taken from three *Cd45*<sup>-/-</sup> mice backcrossed onto BALB/c (*Cd45*<sup>-/-</sup>) and from three *Cd45*<sup>+/+</sup> littermates (*Cd45*<sup>+/+</sup>). Errors bars show standard error of mean. Percentages of CD4<sup>+</sup> T cells expressing the same Vβ genes in an age-matched C57Bl/6 (B6) mouse are shown for comparison. B6 mice do not express MHC class II I-E molecules and are thus unresponsive to *Mtv* superantigens. Error bars represent s.e.m.

### *Cd45*<sup>-/-</sup> T cells respond poorly to exogenous superantigen deletion

To further examine the altered sensitivity of *Cd45*<sup>-/-</sup> thymocytes to negative selection, I analysed the deletion of thymocytes by *Staphylococcus* enterotoxin B (SEB) in these mice. This system has the advantage that the deleting stimulus can be titrated by using increasing concentrations of SEB. SEB binds to class II molecules and to Vβ3, 7, 8 and 17 (Marrack and Kappler, 1990), leading to the selective elimination of T cells expressing these Vβs. SEB deletion of thymocytes occurs specifically at the SP stage of thymic development (Marrack and Kappler, 1990). Figure 28 shows the result of flow cytometric analysis of Vβ8-expressing CD4<sup>+</sup> SP thymocytes in *Cd45*<sup>-/-</sup> BALB/c mice and *Cd45*<sup>+/+</sup> BALB/c mice injected with increasing concentrations of SEB. Control animals show a gradual increase in the percentage deletion of Vβ8<sup>+</sup>CD4<sup>+</sup> SP cells within the thymus with increasing concentrations of SEB. In contrast, there is clearly a substantial deficiency in SEB-induced deletion in *Cd45*<sup>-/-</sup> mice. At the highest SEB concentration used,

*Cd45<sup>+/+</sup>* mice delete around 60% of all V $\beta$ 8-expressing CD4<sup>+</sup> SP thymocytes whilst this concentration has only a minor effect on V $\beta$ 8<sup>+</sup> CD4<sup>+</sup> thymocytes in *Cd45<sup>-/-</sup>* mice. These results therefore support a positive role for CD45 in the negative selection of T cells in superantigen deletion and suggest that in the absence of CD45 the threshold of TCR signalling for negative selection is increased.

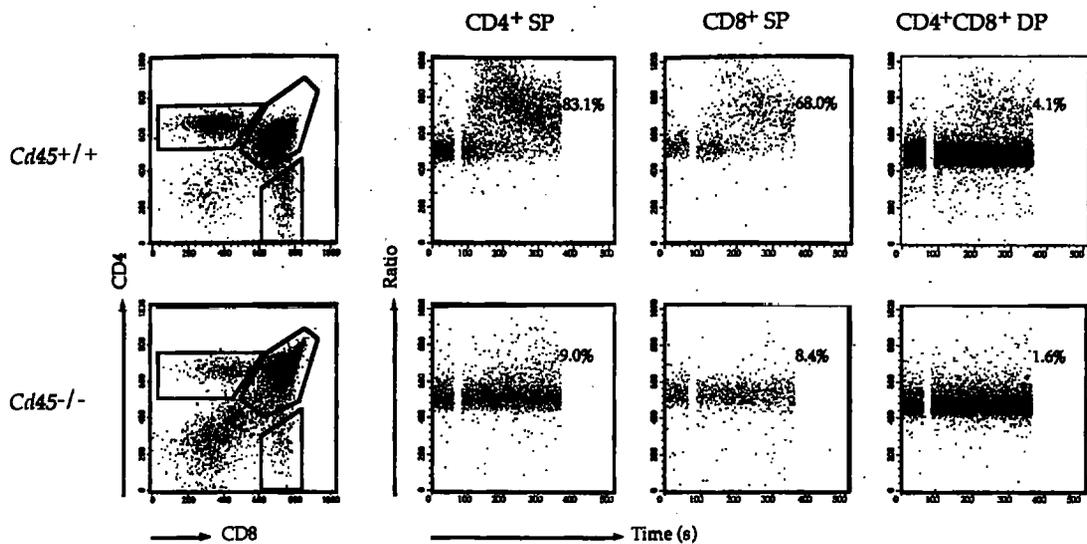


**Figure 28: Defective negative selection of thymocytes to exogenous superantigen.** *Staphylococcus* enterotoxin B (SEB)-induced deletion of V $\beta$ 8<sup>+</sup>CD4<sup>+</sup>SP thymocytes. CD45-deficient (*Cd45<sup>-/-</sup>*) or control (*Cd45<sup>+/+</sup>*) mice which have been backcrossed to the BALB/c background were injected three times every other day with the dose of SEB shown. Mice were analysed the day after the third injection. Graph shows the percentage deletion of V $\beta$ 8<sup>+</sup>CD4<sup>+</sup>SP within these mice as determined by flow cytometric analysis.

### ***Cd45<sup>-/-</sup>* T cells show defective Ca<sup>2+</sup> mobilisation following TCR stimulation**

The deficiency of *Cd45<sup>-/-</sup>* thymocytes in response to both positive and negative selection stimuli could be due to a failure of signal transduction events from the TCR. One of the hallmarks of this signal transduction is the mobilisation of Ca<sup>2+</sup> on antigen stimulation which results in a transient increase in intracellular Ca<sup>2+</sup> concentrations. Thymocytes from *Cd45<sup>+/+</sup>* and *Cd45<sup>-/-</sup>* mice were assessed for their ability to flux calcium in response to receptor crosslinking using four colour

flow cytometry. Thymocytes loaded with the  $\text{Ca}^{2+}$  sensitive dye Indo-1 were stained with anti-CD4 and anti-CD8, to allow gating, and an anti-CD3 hamster antibody. TCR stimulation was achieved by the crosslinking of CD3 using an anti-hamster antibody and changes in fluorescence of the Indo-1 dye was measured over a six minute time course. In initial experiments increasing doses of crosslinker were used to stimulate the cells and  $\text{Cd45}^{+/+}$  cells were found to be sensitive to receptor-induced  $\text{Ca}^{2+}$  flux with as little as 10  $\mu\text{g/ml}$  cross-linker (not shown). Figure 29 shows the results of stimulation of thymocytes with 100  $\mu\text{g/ml}$  cross-linker. As expected  $\text{Cd45}^{+/+}$  thymocytes respond strongly to this high level of receptor crosslinking. In contrast  $\text{Cd45}^{-/-}$  thymocytes only respond very poorly to this level of receptor stimulation in any of their thymocyte sub-populations. Although  $\text{Cd45}^{-/-}$  thymocytes have lower levels of TCR on their  $\text{CD4}^+$  SP and  $\text{CD8}^+$  SP thymocytes, since these cells fail to respond even to very high levels of crosslinking antibody this suggests that CD45 is required for the coupling of the TCR to  $\text{Ca}^{2+}$  signalling pathways.



**Figure 29: Greatly reduced TCR-driven intracellular calcium flux in CD45-deficient thymocytes.** Flow cytometric analysis showing CD4 and CD8 staining of *Cd45*<sup>+/+</sup> or *Cd45*<sup>-/-</sup> thymocytes preloaded with Indo-1 and stained with anti-CD3. Intracellular [Ca<sup>2+</sup>] in either the CD4<sup>+</sup>CD8<sup>-</sup> SP, CD4<sup>+</sup>CD8<sup>+</sup> SP or CD4<sup>+</sup>CD8<sup>+</sup> DP thymocytes (gated as shown in the CD4/CD8 plots) is shown as a ratio of Indo-1 violet/blue fluorescence (Ratio; calcium bound dye/calcium free dye) versus time. Cells were stimulated with 100 $\mu$ g/ml goat anti-hamster antibody to crosslink the anti-CD3 at the time indicated by the break in the calcium trace (about 60s after start of data collection). The percentage of cells responding is indicated on each plot, defined as the percentage of cells exceeding a ratio of 600 between 140s and 360s after the start of the experiment. All plots are representative of multiple experiments.

## Summary

*Cd45*<sup>-/-</sup> mice have been shown to have a severe deficiency in T cell development. There are two distinct developmental blocks. Firstly there is a block at the CD44<sup>+</sup>CD25<sup>+</sup> DN3 stage of CD4<sup>+</sup>CD8<sup>-</sup> DN thymocyte development, suggesting that CD45 plays a role in signalling from the pre-TCR. The second developmental block is at the DP stage in thymocyte development which is associated with the positive and negative selection events of T cell selection. Some *Cd45*<sup>-/-</sup> T cells are able to reach the periphery, albeit in greatly reduced numbers. These cells are unusual in that they have both an immature (CD3<sup>lo</sup>/HSA<sup>hi</sup>) and yet an activated phenotype (CD44<sup>hi</sup>/L-selectin<sup>lo</sup>) as assessed by expression of cell surface markers. To determine the role of CD45 during positive selection of thymocytes both class I and class II transgenic TCR receptors were used. *Cd45*<sup>-/-</sup> mice were found to display a profound block in the development of mature T cells expressing the

transgenic TCRs suggesting an important role for CD45 in the positive selection of these receptors. Moreover, this deficiency appears to be due to altered thresholds of TCR signalling as assessed using bi-specific antibodies to induce positive selection events in a dose dependent manner. The use of a transgenic TCR to investigate T cell negative selection events demonstrates that *Cd45<sup>-/-</sup>* thymocytes can undergo negative selection events induced by the cognate ligand. However, negative selection induced by endogenous (*Mtv*) and exogenous (SEB) superantigens are deficient in *Cd45<sup>-/-</sup>* thymocytes. In addition *Cd45<sup>-/-</sup>* T cells are found to have a profound block in their ability to mobilise  $Ca^{2+}$  in response to antigen receptor stimulation.

# Chapter Four

## DISCUSSION

### 4.1 Creation of the *Cd45*<sup>-/-</sup> mice

The aim of this project was to create a mouse strain deficient in CD45 by genetic targeting using homologous recombination in ES cells and to characterise lymphocyte development in these mice with particular emphasis on the positive and negative selection events that occur during development. In this discussion I will examine the results of these studies in the context of what is known about the role of CD45 in signal transduction and compare and contrast these results with published data using other CD45-deficient mouse strains.

The first published CD45-deficient mice (*Cd45*<sup>ex6/ex6</sup>) were created by genetic targeting of exon 6 of the *Cd45* gene, which is one of the differentially expressed exons. These mice were found to have some *Cd45* expression on around 20% of their T cells although no expression on B cells (Kishihara et al., 1993). More recently *Cd45*<sup>ex9/ex9</sup> mice have been published which have a targeted disruption of an exon used in all isoforms of CD45 (Byth et al., 1996). These mice do not express any isoforms of CD45 on any lymphocytes studied and do not produce any truncated form of the protein. Here I describe the construction and characterisation of mice with a targeted disruption of exon 12 of the *Cd45* gene (*Cd45*<sup>-/-</sup>), an exon also in all isoforms of CD45. Like the *Cd45*<sup>ex9/ex9</sup> mice, no isoforms of CD45 can be detected on the surface of the lymphocytes of these mice. In these mice a small amount of a truncated protein appears to be produced from the targeted allele. Further analysis of this truncated protein has been performed by immunoprecipitation studies and has confirmed it has phosphatase activity consistent with it being derived from CD45. Despite the presence of this truncated

protein these *Cd45*<sup>-/-</sup> mice demonstrate the same deficiencies in T and B cell development as described for mice where no truncated form of CD45 is present (see later and Byth et al., 1996). This is best explained by the fact that the mutation of exon 12 was engineered in such a way that if any truncated form of the protein containing the phosphatase domain was to be produced, it would lack the necessary signal sequences that allow it to be located in the membrane. Studies using truncated forms of CD45 in transfection studies have shown that membrane localisation is a prerequisite for the contribution of CD45 to signal transduction (Alexander, 1997).

The *Cd45*<sup>-/-</sup> mice were found to have strain-specific phenotypes. The mutation was found to be lethal on the 129/Sv mouse strain whilst *Cd45*<sup>-/-</sup> BALB/c mice display splenomegaly. Strain-specific differences in phenotype, as well as in the severity of the phenotype, have been noted previously in other gene targeted mice (Sibilia and Wagner, 1995; Threadgill et al., 1995). *Cd45*<sup>-/-</sup> mice inbred on a 129/Sv genetic background suffer from chronic diarrhoea which leads to a progressive wasting disease and death. This particular phenotype is reminiscent of the symptoms seen in other immunodeficient mice. Mice deficient in the cytokines IL-2 and IL-10 both suffer from chronic enterocolitis and develop similar symptoms to 129/Sv *Cd45*<sup>-/-</sup> mice (Kuhn et al., 1993; Sadlack et al., 1993). Moreover spontaneous inflammatory bowel disease is associated with TCR $\alpha$ , TCR $\beta$  and TCR $\delta$  double knockout mice (Mombaerts et al., 1993). The reason these mice develop these symptoms is not clear however it has been suggested that this may be due to autoimmune disease caused by an inappropriate immune response to the normally non-pathogenic microbial flora of the gut (Sadlack et al., 1995). Furthermore, splenomegaly similar to that seen in *Cd45*<sup>-/-</sup> BALB/c mice is also associated with autoimmune disease in mice deficient in the Lyn Src-family kinase (Hibbs et al., 1995; Nishizumi et al., 1995). Thus it appears that *Cd45*<sup>-/-</sup> mice have autoimmune-like diseases, although much further work would have to be done to fully characterise these phenotypes. If these symptoms are those of autoimmunity then this would suggest that genetic background of the mouse is important in the presentation of the disease. Currently I am attempting to analyse anti-DNA

antibodies in *Cd45<sup>-/-</sup>* BALB/c mice. The production of this type of antibody and its deposition in the kidneys of Lyn-deficient mice is characteristic of their autoimmune disease. Preliminary data suggest that older *Cd45<sup>-/-</sup>* BALB/c mice develop kidney pathology however initial attempts to analyse anti-DNA antibodies have been unsuccessful.

## 4.2 B lymphocyte development in *Cd45<sup>-/-</sup>* mice

To examine the effects of the *Cd45* mutation on B cell development, flow cytometric analysis was performed using antibodies specific for markers of B cell maturation and activation. Analysis of bone marrow from *Cd45<sup>-/-</sup>* mice has revealed a developmental block that has not been reported previously in the other CD45-deficient mouse strains. *Cd45<sup>-/-</sup>* B cell development in the bone marrow is seen to be partially defective at the transition from pro- to pre-B cell. This transition is associated with signal transduction from the pre-BCR. Failure to transit from the pro- to pre-B cell compartment is seen in mice that cannot form a functional pre-BCR, such as in RAG 1/2-deficient mice (Mombaerts et al., 1992; Shinkai et al., 1992; Weaver and Alt, 1997),  $\lambda$ 5-deficient mice (Kitamura et al., 1992; Rolink et al., 1993), or in mice that do not have Ig $\beta$  molecules (Gong and Nussenzweig, 1996) which are necessary to couple the receptor to the signal transduction pathways in the cell. This partial block in pro- to pre-B cell transition seen in CD45-deficient mice would suggest a need for CD45 in the signalling events from the pre-BCR. This requirement is not absolute as many B cells can still make this transition.

Despite the partial block in early B cell development, *Cd45<sup>-/-</sup>* B cells accumulate in the spleen in similar numbers to controls. Normally B cell development progresses from the most immature peripheral IgM<sup>hi</sup>IgD<sup>lo</sup> cells to IgM<sup>lo</sup>IgD<sup>hi</sup> cells (Rajewsky, 1992). *Cd45<sup>-/-</sup>* B cells are IgM<sup>hi</sup>IgD<sup>hi</sup> and this failure to down-regulate IgM could mean that these B cells are blocked in their development.

HSA is another maturity marker that is normally down-regulated during B cell development. *Cd45<sup>-/-</sup>* B cells are seen to have higher levels of this marker, again suggesting an immature B cell phenotype. In contrast, *Cd45<sup>-/-</sup>* B cells also have altered levels of expression of other markers that are associated with increasing B cell maturation. The levels of expression of both MHC class II and CR1/2 normally increase during the transition from IgM<sup>hi</sup>IgD<sup>lo</sup> cells to the most mature IgM<sup>lo</sup>IgD<sup>hi</sup> cells and yet *Cd45<sup>-/-</sup>* mice have levels of these markers higher than those seen in *Cd45<sup>+/+</sup>* mice. This B cell phenotype has been reported for both the other published CD45-deficient mouse strains (Byth et al., 1996; Kishihara et al., 1993). Given the conflicting results on the expression of these development markers of B maturity it is therefore difficult to conclude if the B cells in these mice are more or less mature than controls. Interestingly, *Cd45<sup>ca/lox</sup>* mice crossed to mice deficient in SHP-1 show an apparent maturation of the B cell phenotype to IgD<sup>hi</sup>IgM<sup>lo</sup> cells suggesting that mutations in these two phosphatase cancel each other, consistent with CD45 and SHP-1 being positive and negative regulators of BCR signalling respectively (Pani et al., 1997).

*Cd45<sup>-/-</sup>* B cells also express the co-regulatory molecule CD19 at higher levels than controls. As CD19 is thought to be a positive regulator of receptor signalling, this increase in expression would suggest that *Cd45<sup>-/-</sup>* B cells may have a lower threshold for activation than control cells. This could however be counteracted if there was a general increase in all cell surface molecules including negative regulators. CD22 is a major negative regulator of BCR signalling and this is not elevated in *Cd45<sup>-/-</sup>* B cells. Other negative regulators of signalling exist and it is possible that they compensate for CD19 over-expression. If *Cd45<sup>-/-</sup>* B cells are more sensitive to BCR stimulation due to CD19 over-expression, then the B cells within these mice would be more likely to become activated by autoantigens and this could explain the splenomegaly seen in *Cd45<sup>-/-</sup>* mice. However, although B cells from transgenic mice overexpressing CD19 are hyper-responsive to receptor stimulation, no autoimmunity has been reported in these animals.

The elevation of the surface level of expression of B7.2 on *Cd45*<sup>-/-</sup> B cells is interesting as this molecule is generally considered as a marker of B cell activation (Lenschow et al., 1994). Studies from all the CD45-deficient mice strains analysed, including those presented in this thesis, show that *in vitro* CD45-deficient B cells fail to proliferate following BCR crosslinking, suggesting a decrease in signal transduction. Moreover, signal transduction through the BCR in *Cd45*<sup>-/-</sup> B cells also appears to be defective as judged by the greatly reduced intracellular Ca<sup>2+</sup> flux in response to crosslinking of the receptor in agreement with studies performed on the *Cd45*<sup>ext6/ext6</sup> mice (Benatar et al., 1996). This means there is a contradiction in that CD45-deficient mice appear to have an activated B cell phenotype and yet these B cells fail to signal properly from the receptor. Could this be explained if *Cd45*<sup>-/-</sup> B cells were anergic? Anergic cells are those that develop in the presence of autoantigen but fail to respond to it. If CD45 is a negative regulator of BCR signalling then this would mean that the BCR of CD45-deficient B cells would be more likely to result in B cell activation during receptor mediated selection events. To avoid autoimmune disease these activated *Cd45*<sup>-/-</sup> B cells must subsequently become unresponsive to antigen receptor stimulation i.e. they become anergic. As discussed in the introduction, B cell anergy has been studied extensively in the anti-HEL transgenic mice bred to express sHEL. Anergic B cells within these mice share some characteristic with *Cd45*<sup>-/-</sup> B cells in that they fail to proliferate in response to antigen crosslinking and have reduced Ca<sup>2+</sup> flux. However anergic B cells have a characteristic very low level of expression of IgM such that these B cells are IgM<sup>lo</sup>/IgD<sup>hi</sup> which is not seen with CD45-deficient B cells (Cyster and Goodnow, 1995; Goodnow et al., 1991).

When the *CD45*<sup>-/-</sup> mice were bred to transgenic mice expressing the 3-83 $\mu$ 8 BCR, in the absence of the cognate ligand for this receptor (H-2 K<sup>b</sup>), 3-83/*Cd45*<sup>-/-</sup> mice develop large numbers of B cells that display a IgM<sup>hi</sup>IgD<sup>lo</sup> phenotype, i.e. they resemble immature rather than anergic cells. This suggests that CD45 is required for the developmental up-regulation of IgD on B cells expressing the 3-83 BCR. A similar experiment has been conducted using the *Cd45*<sup>ext6/ext6</sup> mice bred to the anti-HEL transgenic mice (Cyster et al., 1996). These mice have the advantage

that cognate ligand for this receptor can be expressed in mice in soluble form (sHEL) which acts as a weak ligand, or a membrane bound form (mHEL) that can act as a strong highly crosslinking ligand. When this group made radiation chimeras from the bone marrow of *Cd45<sup>ex6/ex6</sup>* anti-HEL mice in the absence of the cognate ligand they found that the *Cd45<sup>ex6/ex6</sup>* B cells also display this  $\text{IgM}^{\text{hi}}\text{IgD}^{\text{lo}}$  phenotype, again suggesting a reduction in the maturity of CD45-deficient B cells. When bone marrow from anti-HEL/sHEL mice is mixed with wildtype bone marrow and used to reconstitute lethally irradiated mice to form chimaeras, then the anergic cells die by negative selection due to competition by the wildtype cells for entry into the follicles (Cyster et al., 1994). When similar radiation chimaeras were made with bone marrow from anti-HEL transgenic *Cd45<sup>ex6/ex6</sup>* mice in the absence of sHEL, these were found to behave in a similar manner to wildtype anergic B cells in that they had a reduced capacity to compete with wildtype cells to populate the spleen and lymph nodes of reconstituted mice (Cyster et al., 1996). However when anti-HEL transgenic *Cd45<sup>ex6/ex6</sup>* mice were bred to express sHEL then a surprising result was obtained. B cells from these mice now appear to behave like naive wildtype cells having an increased capacity to compete with wildtype bone marrow than those CD45-deficient B cells that developed without exposure to sHEL (Cyster et al., 1996). In addition they appear to have increased in their maturity to become  $\text{IgM}^{\text{int}}\text{IgD}^{\text{hi}}$  cells. This result is more consistent with CD45-deficient B cells having a reduced signalling capability. In the absence of CD45 there is insufficient signal for this transition to occur. When sHEL is present this autoantigen acts to stimulate the receptor further to allow for efficient B cell development. If the CD45-deficient B cells were truly anergic then they would be expected to be unresponsive to further stimulus through their antigen receptor. In summary CD45-deficient B cells are less responsive to BCR stimulation, and when expressing a transgene BCR they are arrested in their development at an immature  $\text{IgM}^{\text{hi}}\text{IgD}^{\text{lo}}$  stage. However it is difficult to reconcile this with increased expression of certain activation markers.

When 3-83/*Cd45<sup>-/-</sup>* mice were bred to express the negatively selecting cognate ligand, H-2K<sup>b</sup>, the B cells expressing the 3-83 receptor were efficiently

deleted. A similar result was obtained with *Cd45<sup>ex6/ex6</sup>* mHEL mice. However some B cells were found in the 3-83/*Cd45<sup>-/-</sup>*, H-2<sup>b</sup> mice but these developed by rearranging endogenous receptor chains and using these to alter the specificity of the receptor such that they no longer recognise H-2K<sup>b</sup>. The B cells that developed in this manner had the IgM<sup>hi</sup>IgD<sup>hi</sup> phenotype found in non-transgenic *Cd45<sup>-/-</sup>* mice. B cells expressing rearranged endogenous receptors or B cells expressing a rearranged transgenic receptor in the presence of a weak cognate ligand (sHEL), also up-regulate IgD and become IgM<sup>int</sup>IgD<sup>hi</sup>. One model to explain this is that B cells developing from IgM<sup>hi</sup>IgD<sup>lo</sup> cells require a weak signal from an as yet unknown ligand. CD45-deficient B cells expressing rearranged endogenous receptors will have a variety of different receptor affinities and this could mean that those cells which have a high enough affinity to this putative ligand can overcome the deficiency in CD45 and develop into IgM<sup>hi</sup>IgD<sup>hi</sup> cells. This is presumably mimicked by the addition of soluble HEL in the *Cd45<sup>ex6/ex6</sup>* anti-HEL transgenic mice. This process could be regarded as the B cell equivalent of the positive selection of T cells (Thompson and Allison, 1997). Indeed both Syk-deficient (Turner et al., 1997) and Ig $\alpha$ -deficient mice (Torres et al., 1996) have a block in B cell development at this stage suggesting that BCR signalling is required for this transition.

The numbers of B-1 cells within *Cd45<sup>-/-</sup>* mice are greatly reduced. Mice deficient in each of the BCR coreceptor components CD19, CD21 and CD81 also have a reduction in the numbers of B-1 cells (Maecker and Levy, 1997; Rickert et al., 1995; Tsitsikov et al., 1997). This is thought to be due to the reduced levels of BCR signalling in the absence of these molecules being insufficient to allow the development of this population. Similarly, Btk- and PKC $\beta$ -deficient mice have very much reduced levels of B-1 cells and it has been suggested that these molecules are required for signalling from the receptor during B-1 cell development (Khan et al., 1995; Leitges et al., 1996). In contrast CD22-deficient mice and mice deficient in SHP-1 have increased numbers of B-1 cells (O'Keefe et al., 1996). These molecules play an important role in the negative regulation of the BCR signalling and it is thought that the absence of these molecules increases the sensitivity of the

receptor leading to increased B-1 cell development. Thus B-1 cell development or maintenance is sensitive to the levels of signals being transduced from the receptor. Those mutations which reduce BCR signalling have lower numbers of B-1 cells whilst those with increased BCR signalling have more B-1 cells. As *Cd45*<sup>-/-</sup> mice have reduced numbers of B-1 cells this would suggest that CD45 is required for the efficient transmission of signals from the B-1 receptor.

In conclusion, most of the data from all three CD45-deficient mouse lines suggests that CD45 is a positive regulator of BCR signalling, and that in its absence there are several blocks in B cell development at checkpoints requiring pre-BCR or BCR signals.

### 4.3 T lymphocyte development in *Cd45*<sup>-/-</sup> mice

To investigate the role of CD45 in T cell development I used flow cytometric analysis of T cell markers of maturity and activation. T cell development is severely affected in *Cd45*<sup>-/-</sup> mice. Firstly, there is a partial block at the DN stage of thymocyte development at the transition from CD44<sup>+</sup>CD25<sup>+</sup> to CD44<sup>+</sup>CD25<sup>-</sup> cell populations. A similar partial block at this transition has also been reported for the *Cd45*<sup>ex9/ex9</sup> mice (Byth et al., 1996). Thymocyte development is also blocked at this developmental stage in mice unable to make a functional pre-TCR, such as pT $\alpha$ - and RAG-deficient mice (Fehling et al., 1995), or TCR $\beta$ -deficient mice (Jacobs et al., 1996; Levelt et al., 1993; Mombaerts et al., 1992) and mice that do not have CD3 $\epsilon$  molecules which are necessary to couple the pre-TCR to signal transduction pathways (Malissen et al., 1995). The block in this transition in the *Cd45*<sup>-/-</sup> mice would suggest that CD45 is a positive regulator of signal transduction from the pre-TCR in a similar manner to its role in signal transduction from the pre-BCR. Again there is not an absolute requirement for CD45 in this transition as many cells develop into DP thymocytes.

The most dramatic block in thymocyte development in *Cd45*<sup>-/-</sup> mice is at the DP to SP transition resulting in greatly reduced numbers of CD4<sup>+</sup> and CD8<sup>+</sup> SP cells and a concurrent decrease in CD4<sup>+</sup> and CD8<sup>+</sup> peripheral T cells. This stage of development is associated with positive and negative selection events that are governed by interactions between the TCR and MHC/peptide complexes on thymic stromal cells. *Cd45*<sup>-/-</sup> mice have a deficiency in DP thymocytes that are CD3<sup>hi</sup> CD69<sup>+</sup> which are thought to be cells that are being positively selected (Swat et al., 1993; Wang et al., 1995; Yamashita et al., 1993). The reduced levels of cells undergoing positive selection suggests that CD45 may be important in this process. To address whether this deficiency in positive selection was T cell-autonomous or due to deficiencies in other cell types such as thymic stromal cells, I made radiation chimaeras with mixtures of increasing proportions of bone marrow from *Cd45*<sup>-/-</sup> mice mixed with bone marrow from wildtype mice (not shown). The results of these studies suggested that the T cell phenotype in *Cd45*<sup>-/-</sup> mice was intrinsic to the T cells and could not be overcome in the presence of wildtype cells.

*Cd45*<sup>-/-</sup> DP thymocytes have lower levels of expression of CD5 than controls. In wildtype mice, CD5 expression is induced as thymocytes transit from the DN to DP compartments. CD5 is thought to be a negative regulator of TCR signalling (Tarakhovsky et al., 1995; Tarakhovsky et al., 1994) and as such, its down regulation in *Cd45*<sup>-/-</sup> mice during DP development may be a mechanism to compensate for the lower levels of signals passing through the pre-TCR in the absence of CD45. In contrast the levels of CD5 expressed on SP T cells and peripheral T cells in *Cd45*<sup>-/-</sup> mice are equivalent to wildtype levels.

To investigate the role of CD45 in T cell selection events, *Cd45*<sup>-/-</sup> mice were bred to transgenic mice expressing class I- and class II-restricted rearranged receptors. With all three different receptors studied, in the absence of CD45 no T cells developed that expressed the transgenic receptor, confirming that CD45 is required for their positive selection. A similar defect in the positive selection of transgenic mice carrying a rearranged receptor has also been described in the *Cd45*<sup>ex6/ex6</sup> mice (Wallace et al., 1997). To study whether efficient *Cd45*<sup>-/-</sup> T cell

development could be induced by increasing the signal to the TCR, a protocol using neonatal thymic organ cultures (NTOC) was used. Bi-functional antibodies that aggregate CD4 with CD3 can be used to efficiently induce CD4<sup>+</sup> SP thymocyte development in control thymi *in vitro* (Basson et al., 1998; Bommhardt et al., 1997). This system can therefore be used for the titration of this stimulus by altering the concentration of the bi-specific anti-CD4/anti-CD3 reagent. In this way we have shown that *Cd45*<sup>-/-</sup> CD4<sup>+</sup> SP T cell development can be induced to levels equivalent to those in controls when high concentrations of bi-specific reagent were used. At lower concentrations of the bi-specific reagent, the development of *Cd45*<sup>-/-</sup> CD4<sup>+</sup> SP cells was much less efficient than in control NTOCs. When a greater dose of bi-specific antibody is used a stronger signal is provided which overcomes the deficiency in the CD45-deficient T cells allowing for efficient positive selection. Together this suggests that TCR signalling in *Cd45*<sup>-/-</sup> T cells is lower than in control cells and that CD45 is a positive regulator of TCR signalling in thymocytes.

In the case of *Cd45*<sup>-/-</sup> mice expressing the BM3 and A1 transgenes, some *Cd45*<sup>-/-</sup> SP thymocytes develop but these express endogenously rearranged TCRs rather than the transgenic TCRs. Thus, positive selection can occur with randomly generated endogenous TCRs but there is no positive selection of *Cd45*<sup>-/-</sup> thymocytes expressing transgenic TCRs. One explanation for this is based on the idea there is a particular level of TCR signalling required during the DP to SP transition. During positive selection, thymocytes interact with the thymic stromal cells which present S/M and drive their development. This involves a signal from the newly rearranged TCR and the strength of that signal determines the fate of the developing cells. Those cells that fail to receive a high enough signal die at the DP stage of development by neglect, those with too high a signal die by negative selection with only those cells given with the correct levels of signalling developing in SP thymocytes. As discussed above, in *Cd45*<sup>-/-</sup> thymocytes the TCR signalling is reduced. Because of this, in thymocytes expressing a defined transgenic TCR those signals that would be normally elicited through the TCR on interaction with S/M are now not sufficient to drive positive selection and the cells die of neglect. Rearrangement of endogenous receptors allows the development of cells with a

wide variety of specificity's. There is likely to be a spectrum of affinities of S/M and TCR interactions. In this way, those cells which receive levels of signalling during this interaction that are of sufficient strength to overcome the signalling deficiency in the absence of CD45 are positively selected.

#### 4.4 T cell negative selection in *Cd45*<sup>-/-</sup> mice

As there is a reduced sensitivity of *Cd45*<sup>-/-</sup> thymocytes for positive selection it is possible that these cells would also have an altered sensitivity to negative selection. Analysis of negative selection in *Cd45*<sup>-/-</sup> mice expressing the BM3.6 transgene shows that deletion of autoreactive thymocytes occurred efficiently in the absence of CD45 in a similar manner to wildtype mice. The deleting ligand for the receptor, H-2K<sup>b</sup> is expressed at high levels in the thymus in these transgenic mice and therefore is likely to provide a strong negative selection stimulus. To see if there is any difference in the efficiency of CD45-deficient mice to negatively select thymocytes, I studied the effects of a weaker stimulus.

*Cd45*<sup>-/-</sup> BALB/c mice were used to investigate superantigen induced deletion of V $\beta$  expressing T cell subsets. Superantigen presentation differs from the presentation of conventional antigens in that superantigens bind, without processing, directly to the lateral surface of the MHC class II molecules rather than in the peptide binding groove. It is likely that this provides a weaker TCR signal than conventional peptide antigens (Simpson et al., 1993). *Cd45*<sup>-/-</sup> thymocytes cells expressing V $\beta$  3, 5 and 11 were not deleted as efficiently as wildtype thymocytes in the presence of endogenous MMTV-encoded superantigens. This result is in direct contrast to observations on the *Cd45*<sup>ext/ext</sup> mice where V $\beta$  deletion was reported to occur normally (Kishihara et al., 1993). *Cd45*<sup>ext/ext</sup> mice are known to have CD45 expression on a proportion of their thymocytes and T cells which may explain the contradictory results.

To further analyse this deficiency in negative selection, I injected varying doses of the bacterial endotoxin superantigen SEB into mutant and control mice and measured specific V $\beta$  deletion. These studies demonstrate that *Cd45*<sup>-/-</sup> mice have a reduced level of negative selection compared to controls. Failure of superantigen deletion of thymocytes from CD45<sup>ex9/ex9</sup> mice treated *in vitro* with SEB has recently been reported (Conroy et al., 1996). Thus in conclusion, *Cd45*<sup>-/-</sup> thymocytes can undergo negative selection when exposed to a negatively selecting ligand expressed at high concentrations. The use of weaker negatively selecting ligands has revealed a deficiency in negative selection of *Cd45*<sup>-/-</sup> thymocytes at least in response to superantigens. Together this suggests that CD45-deficiency leads to reduced TCR signalling during negative selection whereby strong signals still work but weaker signals do not.

## 4.5 Peripheral T cells in *Cd45*<sup>-/-</sup> mice

The expression levels of the adhesion molecules CD44 and L-selectin can be used to distinguish cells that have a previously activated/memory phenotype (Barrat et al., 1995; Gerberick et al., 1997; Khan et al., 1996; Tietz and Hamann, 1997). CD45-deficient mature CD4<sup>+</sup> and CD8<sup>+</sup> peripheral T cells have a predominantly CD44<sup>hi</sup>L-selectin<sup>lo</sup> phenotype characteristic of activated T cells. Despite this activated phenotype, *Cd45*<sup>-/-</sup> T cells have major deficiencies in Ca<sup>2+</sup> mobilisation following crosslinking of their antigen receptors (not shown). The accumulation of T cells with a previously activated phenotype that are unresponsive to receptor stimulation is akin to the observations described earlier for B cells. Could *Cd45*<sup>-/-</sup> peripheral T cells be anergic? Studies of anergic T cells show that these cells have low TCR levels, fail to proliferate, flux Ca<sup>2+</sup> or to produce IL-2 following TCR stimulation (Fields et al., 1996; Gallichio et al., 1994; Quill, 1996; Schwartz, 1996; Sloan-Lancaster et al., 1994). All of these features have been reported in CD45-deficient T cells suggesting they too may be anergic (Alexander, 1997). These observations seem paradoxical in view of the reduced TCR

signalling seen in *Cd45<sup>-/-</sup>* thymocytes. It may be that whilst CD45 is a positive regulator of TCR signalling in thymocytes, it is less important or unnecessary for TCR signalling in peripheral T cells. Since, as I have argued earlier, these cells are likely to have been selected for a higher affinity for autoantigens, it may be that since they have exited into the periphery they become autoreactive and as a consequence assume an anergic phenotype. This may also explain the autoimmune-like enteritis seen in *Cd45<sup>-/-</sup>* 129/Sv mice.

## 4.6 Signalling

Thymocytes from *Cd45<sup>-/-</sup>* mice have a deficiency in both positive and negative selection events. Many other signals have been implicated in the regulation of positive and negative selection during thymocyte development. Inactivation of *Lck*, *ZAP-70* and *Vav* profoundly affects both positive and negative selection of T cells (Molina et al., 1992; Negishi et al., 1995; Turner et al., 1997). On the other hand the *Ras/Raf/MEK/ERK* pathway has been shown to be required for positive but not negative selection (Aberola-Ila et al., 1996), whilst *CD40* ligand and *Jak3* have been shown to play a role in negative but not positive selection. I have demonstrated that *CD45*-deficient thymocytes have a defect in  $Ca^{2+}$  mobilisation following receptor stimulation. This could explain the failure of positive selection, as a role for calcium fluxes in thymocyte selection events has been suggested by a number of studies. A combination of calcium ionophore and phorbol esters can replace the TCR signals that lead to positive selection (Takahama and Nakauchi, 1996), whilst the use of intracellular calcium chelators to block calcium fluxes, interferes with negative selection (Kane and Hedrick, 1996). Moreover the use of inhibitors to abrogate the function of the calcium-activated phosphatase calcineurin inhibits positive selection and negative selection by weakly deleting ligands (Wang et al., 1995). Together these results clearly demonstrate an important role for calcium mobilisation in regulating thymocyte selection events and thus its inhibition in *CD45*-deficient thymocytes could explain the failure of these processes.

How does CD45 regulate calcium mobilisation in thymocytes? Studies have been conducted using lymphocytes derived from the *Cd45<sup>ex9/ex9</sup>* mice into the phosphorylation status and activity of many of the proteins associated with proximal signalling from the TCR (Stone et al., 1997; Stone et al., 1997). This study found that *Cd45<sup>ex9/ex9</sup>* thymocytes demonstrated hyperphosphorylation of Lck and Fyn with Lck in an inactive conformation. This was associated with reduced phosphorylation of TCR- $\zeta$  and CD3- $\epsilon$  and a concurrent failure to recruit ZAP-70 to the TCR. The signalling in these cells, although decreased, was not totally ablated supporting the idea that CD45 controls the levels of TCR signal transduction. These observations support the hypothesis that a major function of CD45 is to activate Lck and Fyn following TCR engagement which leads to the subsequent phosphorylation of ITAMs in the TCR-associated chains by these kinases. This in turn allows for the recruitment of ZAP-70 and further downstream signalling (Chan et al., 1994). The absence of CD45 in cell lines has been associated with the disengagement of the TCR from the ability to generate second messengers such as inositol-1,4,5-trisphosphate ( $IP_3$ ) which is responsible for the mobilisation of calcium from intracellular stores (Koretzky et al., 1990). TCR engagement leads to the activation of phospholipase C  $\gamma 1$  (PLC $\gamma 1$ ) which metabolises phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ) to generate  $IP_3$ . How receptor activation leads to PLC $\gamma 1$  activation is not clear. It appears unlikely that CD45 directly regulates PLC $\gamma 1$  activity as cell lines deficient in CD45 that fail to generate  $IP_3$  in response to TCR stimulation can still do so when stimulated through other classes of receptor (Alexander, 1997). ZAP-70 has been shown to be associated with PLC $\gamma 1$  following TCR engagement (Nel et al., 1995) suggesting that CD45 regulation of ZAP-70 activation (via the Src-family kinases) may in turn regulate PLC $\gamma 1$  activity by direct phosphorylation. An even more complex pathway could be via ZAP-70 interactions with Vav. Vav is an exchange factor for Rho family members which have been implicated in the activation of phosphatidylinositol 4-phosphate 5-kinase (PIP5K). PIP5K activation leads to the production of  $PIP_2$  (Chong et al., 1994; Ren et al., 1996; Toliás et al., 1995) which in turn is a

substrate for PLC $\gamma$ , whose activation, on TCR stimulation, leads to production of IP $_3$  (O'Rourke et al., 1998; Turner et al., 1997).

*Cd45*<sup>-/-</sup> B cells have a partial deficiency in calcium mobilisation such that on receptor stimulation the initial peak of intracellular calcium concentration due to mobilisation of intracellular Ca<sup>2+</sup> stores occurs normally whilst influx from extracellular stores was abrogated. *Cd45*<sup>ex9/ex9</sup> mice also display this defect (Benatar et al., 1996). How does CD45 regulate Ca<sup>2+</sup> mobilisation in B cells? The current model for the role of CD45 in BCR signalling is thought to be through the regulation of the activation status of Src-family kinases such as Lyn or Fyn by dephosphorylation of a negative regulatory tyrosine at the carboxy-terminal end of these proteins. BCR stimulation results in the activation of these Src-family kinases and the subsequent phosphorylation of ITAMs in the Ig $\alpha$  and Ig $\beta$  molecules of the receptor. This leads to the recruitment of Syk, its activation and further downstream signal transduction events. If this model was correct then *Cd45*<sup>-/-</sup> mice would have a B cell phenotype similar to mice deficient in these Src-family kinases. Studies of Lyn-deficient mice suggest that B cells from these mice are hyper-responsive to BCR stimulation rather than hyporesponsive as seen in *Cd45*<sup>-/-</sup> mice (Chan et al., 1998; Wang et al., 1996). Furthermore B cell development and signalling in Fyn-deficient mice is seen to be unperturbed, including having normal receptor-mediated Ca<sup>2+</sup> flux (Sillman and Monroe, 1994). Syk-deficient (*Syk*<sup>-/-</sup>) mice fail to develop mature B cells (Cheng et al., 1995; Turner et al., 1995) and Syk-deficient DT40 cells fail to flux calcium (Takata et al., 1994) demonstrating the importance of Syk in signalling events from the BCR. Defects in Syk activity are however unlikely to explain the defects in Ca<sup>2+</sup> seen in CD45-deficient B cells. Proximal signal transduction pathways in *Cd45*<sup>ex9/ex9</sup> B cells, which demonstrated that the BCR-induced phosphorylation of Ig $\alpha$  and Ig $\beta$  is apparently normal as is Syk activation suggesting that this is not the means by which CD45 signals into the calcium mobilisation pathway.

In summary, it is not clear how CD45 regulates antigen receptor-mediated Ca<sup>2+</sup> fluxes. The most likely route is by regulation of the activation state of Src-

family kinases. The observation that no single Src-family kinase knockout mimics the defective  $\text{Ca}^{2+}$  flux in *Cd45*<sup>-/-</sup> B or T cells may reflect redundancy among multiple Src-family kinases expressed in lymphocytes.

## 4.7 Concluding remarks

There is a striking similarity between B and T cells from *Cd45*<sup>-/-</sup> mice in that they both display profound developmental blocks at stages of development that require signal transduction from the receptors of their cells. In addition those B and T cells within the periphery look immature and yet have increased expression of markers that are associated with having an activated phenotype. Much interest has focused recently on the topology of TCR and S/M interactions and the physical exclusion of proteins from the site of interaction, and recently it has been proposed that CD45 exclusion may play an important role in signal transduction events. The deficiency in both positive and negative selection of thymocytes in *Cd45*<sup>-/-</sup> mice clearly points to a positive role for CD45 in these phenomena. However, the activated phenotype of both B and T cells within the periphery of these mice could suggest a negative role for CD45 in signal transduction from the receptors of these cells. This could be explained if CD45 plays different roles at different stages of development. Clearly much work needs to be done to fully understand the role of CD45 in signal transduction events.

# References

Aberola-Ila, J., Hogquist, K. A., Swan, K. A., Bevan, M. J., and Perlmutter, R. M. (1996). Positive and negative selection invoke distinct signalling pathways. *J. Exp. Med.* *184*, 9-18.

Aifantis, I., Buer, J., von Boehmer, H., and Azogui, O. (1997). Essential role of the pre-T cell receptor in allelic exclusion of the T cell receptor beta locus. *Immunity* *7*, 601-7.

Alexander, D. (1997). The Role of the CD45 Phosphotyrosine Phosphatase in Lymphocyte Signalling. In *Lymphocyte Signalling: Mechanisms, Subversion and Manipulation.*, M. M. Harnett and K. P. Rigley, eds.: John Wiley and Sons Ltd.), pp. 107-139.

Alt, F. W., Oltz, E. M., Young, F., Gorman, J., Taccioli, G., and Chen, J. (1992). VDJ recombination. *Immunol. Today* *13*, 306-14.

Altin, J. G., Pagler, E. B., Kinnear, B. F., and Warren, H. S. (1994). Molecular associations involving CD16, CD45 and zeta and gamma chains on human natural killer cells. *Immunol. and Cell Biol.* *72*, 87-96.

Aoki, Y., Isselbacher, K. J., Cherayil, B. J., and Pillai, S. (1994). Tyrosine phosphorylation of Blk and Fyn Src homology 2 domain-binding proteins occurs in response to antigen-receptor ligation in B cells and constitutively in pre-B cells. *Proc. Natl. Acad. Sci. USA* *91*, 4204-8.

Appleby, M. W., Gross, J. A., Cooke, M. P., Levin, S. D., Qian, X., and Perlmutter, R. M. (1992). Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. *Cell* 70, 751-763.

Arnold, B., Goodnow, C., Hengartner, H., and Hammerling, G. (1990). The coming of transgenic mice: tolerance and immune reactivity. *Immunol. Today* 11, 69-72.

Ashton-Rickardt, P. G., and Tonegawa, S. (1994). A differential-avidity model for T-cell selection. *Immunol. Today* 15, 362-6.

Bachmann, M. F., Kundig, T. M., Speiser, D. E., McKall-Faienza, K., Kishara, K., Mak, T. W., and Ohashi, P. S. (1997). T-cell-independent antiviral B cell responses in CD45-deficient mice. *Cell. Immunol.* 175, 12-5.

Barber, E. K., Dasgupta, J. D., Schlossman, S. F., Trevillyan, J. M., and Rudd, C. E. (1989). The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex. *Proc. Natl. Acad. Sci. USA* 86, 3277-81.

Barrat, F., Haegel, H., Louise, A., Vincent-Naulleau, S., Boulouis, H. J., Neway, T., Ceredig, R., and Pilet, C. (1995). Quantitative and qualitative changes in CD44 and MEL-14 expression by T cells in C57BL/6 mice during aging. *Res. Immunol.* 146, 23-34.

Basson, M. A., Bommhardt, U., Cole, M. S., Tso, J. Y., and Zamoyska, R. (1998). CD3 ligation on immature thymocytes generates antagonist-like signals appropriate for CD8 lineage commitment, independently of T cell receptor specificity. *J. Exp. Med.* 187, 1249-60.

Bazzi, M. D., and Nelsestuen, G. L. (1989). Differences in the effects of phorbol esters and diacylglycerols on protein kinase C. *Biochem.* 28, 9317-23.

Benatar, T., Carsetti, R., Furlonger, C., Kamalia, N., Mak, T., and Paige, C. J. (1996). Immunoglobulin-mediated signal transduction in B cells from CD45-deficient mice. *J. Exp. Med.* 183, 329-34.

Biffen, M., McMichael-Phillips, D., Larson, T., Venkitaraman, A., and Alexander, D. (1994). The CD45 tyrosine phosphatase regulates specific pools of antigen receptor-associated p59fyn and CD4-associated p56lck tyrosine in human T-cells. *EMBO J.* 13, 1920-9.

Bilwes, A. M., den Hertog, J., Hunter, T., and Noel, J. P. (1996). Structural basis for inhibition of receptor protein-tyrosine phosphatase-alpha by dimerization. *Nature* 382, 555-9.

Bommhardt, U., Cole, M. S., Tso, J. Y., and Zamoyska, R. (1997). Signals through CD8 or CD4 can induce commitment to the CD4 lineage in the thymus. *Eur. J. Immunol.* 27, 1152-63.

Borrello, M. A., and Phipps, R. P. (1996). The B/macrophage cell: an elusive link between CD5+ B lymphocytes and macrophages. *Immunol. Today* 17, 471-5.

Borst, J., Jacobs, H., and Brouns, G. (1996). Composition and function of T-cell receptor and B-cell receptor complexes on precursor lymphocytes. *Curr. Opin. Immunol.* 8, 181-90.

Brown, V. K., Ogle, E. W., Burkhardt, A. L., Rowley, R. B., Bolen, J. B., and Justement, L. B. (1994). Multiple components of the B cell antigen receptor complex associate with the protein tyrosine phosphatase, CD45. *J. Biol.Chem.* 269, 17238-44.

Buhl, A. M., and Cambier, J. C. (1997). Co-receptor and accessory regulation of B-cell antigen receptor signal transduction. *Immunol. Revs.* 127-38 160, 127-38.

Burkhardt, A. L., Brunswick, M., Bolen, J. B., and Mond, J. J. (1991). Anti-immunoglobulin stimulation of B lymphocytes activates src-related protein-tyrosine kinases. *Proc. Natl. Acad. Sci. USA* 88, 7410-4.

Byth, K. F., Conroy, L. A., Howlett, S., Smith, A. J. H., May, J., Alexander, D. R., and Holmes, N. (1996). CD45-null transgenic mice reveal a positive regulatory role for CD45 in early thymocyte development, in the selection of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, and in B cell maturation. *J. Exp. Med.* 183, 1707-1718.

Cambier, J. C., Pleiman, C. M., and Clark, M. R. (1994). Signal transduction by the B cell antigen receptor and its coreceptors. *Annu. Rev. Immunol.* 12, 457-86.

Campbell, M. A., and Sefton, B. M. (1990). Protein tyrosine phosphorylation is induced in murine B lymphocytes in response to stimulation with anti-immunoglobulin. *EMBO J.* 9, 2125-31.

Cantrell, D. (1996). T cell antigen receptor signal transduction pathways. *Ann. Rev. Immunol.* 14, 259-274.

Carlow, D. A., Teh, S. J., van Oers, N. S., Miller, R. G., and Teh, H. S. (1992). Peripheral tolerance through clonal deletion of mature CD4-CD8+ T cells. *Int. Immunol.* 4, 599-610.

Chambers, C. A., Cado, D., Truong, T., and Allison, J. P. (1997). Thymocyte development is normal in CTLA-4-deficient mice. *Proc. Natl. Acad. Sci. USA* 94, 9296-301.

Chan, A. C., van, O. N., Tran, A., Turka, L., Law, C. L., Ryan, J. C., Clark, E. A., and Weiss, A. (1994). Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TCR signaling. *J. Immunol.* 152, 4758-66.

Chan, S. H., Cosgrove, D., Waltzinger, C., Benoist, C., and Mathis, D. (1993). Another view of the selective model of thymocyte selection. *Cell* 73, 225-36.

Chan, V. W., Lowell, C. A., and DeFranco, A. L. (1998). Defective negative regulation of antigen receptor signaling in Lyn-deficient B lymphocytes. *Curr. Biol.* 8, 545-53.

Chan, V. W. F., Meng, F., Soriano, P., DeFranco, A. L., and Lowell, C. A. (1997). Characterization of the B lymphocyte populations in Lyn-deficient mice and the role of Lyn in signal initiation and down-regulation. *Immunity* 7, 69-81.

Chang, H. L., Lefrancois, L., Zaroukian, M. H., and Esselman, W. J. (1991). Developmental expression of CD45 alternate exons in murine T cells. Evidence of additional alternate exon use. *J. Immunol.* 147, 1687-93.

Chen, J., and Alt, F. W. (1993). Gene rearrangement and B-cell development. *Curr. Opin. Immunol.* 5, 194-200.

Cheng, A. M., Negishi, I., Anderson, S. J., Chan, A. C., Bolen, J., Loh, D. Y., and Pawson, T. (1997). The Syk and ZAP-70 SH2-containing tyrosine kinases are implicated in pre-T cell receptor signalling. *Proc. Natl. Acad. Sci. USA* 94, 9797-9801.

Cheng, A. M., Rowley, B., Pao, W., Hayday, A., Bolen, J. B., and Pawson, T. (1995). Syk tyrosine kinase required for mouse viability and B-cell development. *Nature* 378, 303-306.

Cheng, G., Ye, Z. S., and Baltimore, D. (1994). Binding of Bruton's tyrosine kinase to Fyn, Lyn, or Hck through a Src homology 3 domain-mediated interaction. *Proc. Natl. Acad. Sci. USA* 91, 8152-5.

Chong, L. D., Traynor Kaplan, A., Bokoch, G. M., and Schwartz, M. A. (1994). The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell* 79, 507-513.

Chu, D. H., Spits, H., Peyron, J. F., Rowley, R. B., Bolen, J. B., and Weiss, A. (1996). The Syk protein tyrosine kinase can function independently of CD45 or Lck in T cell antigen receptor signaling. *EMBO J.* 15, 6251-61.

Conroy, L. A., Byth, K. F., Howlett, S., Holmes, N., and Alexander, D. R. (1996). Defective depletion of CD45-null thymocytes by the *Staphylococcus aureus* enterotoxin B superantigen. *Immunol. Letts.* 54, 119-22.

Crompton, T., Outram, S. V., Buckland, J., and Owen, M. J. (1997). A transgenic T cell receptor restores thymocyte differentiation in interleukin-7 receptor alpha chain-deficient mice. *Eur. J. Immunol.* 27, 100-4.

Cronin, F. E., Jiang, M., Abbas, A. K., and Grupp, S. A. (1998). Role of mu heavy chain in B cell development. I. Blocked B cell maturation but complete allelic exclusion in the absence of Ig alpha/beta. *J. Immunol.* 161, 252-9.

Cyster, J. G., and Goodnow, C. C. (1995). Antigen-induced Exclusion from Follicles and Anergy Are Separate and Complimentary Processes that Influence Peripheral B cell Fate. *Immunity* 3, 691-701.

Cyster, J. G., Hartley, S. B., and Goodnow, C. C. (1994). Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. *Nature* 371, 389-395.

Cyster, J. G., Healy, J. I., Kishihara, K., Mak, T. W., Thomas, M. L., and Goodnow, C. C. (1996). Regulation of B-lymphocyte negative and positive selection by tyrosine phosphatase CD45. *Nature* 381, 325-328.

D'Ambrosio, D., Hippen, K. L., Minskoff, S. A., Mellman, I., Pani, G., Siminovitch, K. A., and Cambier, J. C. (1995). Recruitment and activation of PTP1C in negative regulation of antigen receptor signaling by Fc $\gamma$ RIIB1. *Science* 268, 293-7.

DeFranco, A. L. (1996). The two-headed antigen. B-cell co-receptors. *Curr. Biol.* 6, 548-50.

DeFranco, A. L., Richards, J. D., Blum, J. H., Stevens, T. L., Law, D. A., Chan, V. W., Datta, S. K., Foy, S. P., Hourihane, S. L., Gold, M. R., and et al. (1995). Signal transduction by the B-cell antigen receptor. *Annal. NY Acad. Sci.* 766, 195-201.

Desai, D. M., Sap, J., Schlessinger, J., and Weiss, A. (1993). Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. *Cell* 73, 541-54.

Donovan, J., Goldman, F., and Koretsky, G. (1994). Restoration of CD2-mediated signalling by a chimeric membrane-protein including the cytoplasmic sequences of CD45. *Hum. Immunol.* 40, 123-130.

Doody, G. M., Dempsey, P. W., and Fearon, D. T. (1996). Activation of B lymphocytes: integrating signals from CD19, CD22 and Fc gamma RIIb1. *Curr. Opin. Immunol.* 8, 378-82.

Douek, D. C., Corley, K. T. T., Zal, T., Mellor, A., Dyson, P. J., and Altmann, D. M. (1996). Negative selection by endogenous antigen and superantigen occurs at multiple thymic sites. *Int. Immunol.* 8, 1413-1420.

Dudley, E. C., Petrie, H. T., Shah, L. M., Owen, M. J., and Hayday, A. C. (1994). T cell receptor beta chain gene rearrangement and selection during thymocyte development in adult mice. *Immunity* 1, 83-93.

Durie, F. H., Foy, T. M., Masters, S. R., Laman, J. D., and Noelle, R. J. (1994). The role of CD40 in the regulation of humoral and cell-mediated immunity. *Immunol. Today* 15, 406-11.

Dutz, J. P., Ong, C. J., Marth, J., and Teh, H. S. (1995). Distinct differentiative stages of CD4+CD8+ thymocyte development defined by the lack of coreceptor binding in positive selection. *J. Immunol.* 154, 2588-99.

Engel, P., Zhou, L. J., Ord, D. C., Sato, S., Koller, B., and Tedder, T. F. (1995). Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity* 3, 39-50.

Engelhard, V. H. (1994). How cells process antigens. *Scientific American* 271, 54-61.

Engelhard, V. H. (1994). Structure of peptides associated with class I and class II MHC molecules. *Ann. Rev. Immunol.* 12, 181-207.

Falasca, M., Logan, S. K., Lehto, V. P., Baccante, G., Lemmon, M. A., and Schlessinger, J. (1998). Activation of phospholipase C $\gamma$  by PI 3-kinase-induced PH domain-mediated membrane targeting. *EMBO J.* 17, 414-422.

Fearon, D. T. (1993). The CD19-CR2-TAPA-1 complex, CD45 and signaling by the antigen receptor of B lymphocytes. *Curr. Opin. Immunol.* 5, 341-8.

Fearon, D. T. (1997). Seeking wisdom in innate immunity. *Nature* 388, 323-4.

Fehling, H. J., Krotkova, A., Saint Ruf, C., and von Boehmer, H. (1995). Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. *Nature* 375, 795-8.

Ferber, I., Schonrich, G., Schenkel, J., Mellor, A. L., Hammerling, G. J., and Arnold, B. (1994). Levels of peripheral T cell tolerance induced by different doses of tolerogen. *Science* 263, 674-6.

Fields, P., Fitch, F., and Gajewski, T. (1996). Control of T lymphocyte signal transduction through clonal anergy. *J. Mol. Med.* 74, 673-83.

Fluckiger, A.-C., Li, Z., Kato, R. M., Wahl, M. I., Ochs, H. D., Longnecker, R., Kinet, J.-P., Witte, O. N., Scharenberg, A. M., and Rawlings, D. J. (1998).

Btk/Tec kinases regulate sustained increases in intracellular  $\text{Ca}^{2+}$  following B-cell receptor activation. *EMBO J.* 17, 1973-1985.

Forster, I., Gu, H., Muller, W., Schmitt, M., Tarlinton, D., and Rajewsky, K. (1991). CD5 B cells in the mouse. *Curr. Top. Microbiol. Immunol.* 173, 247-51.

Fung-Leung, W. P., Schilham, M. W., Rahemtulla, A., Kundig, T. M., Vollenweider, M., Potter, J., van Ewijk, W., and Mak, T. W. (1991). CD8 is needed for development of cytotoxic T cells but not helper T cells. *Cell* 65, 443-9.

Gallichio, M., Conti, D., Lempert, N., Gruber, S., Geiselhart, L., and Freed, B. (1994). Calcium-mediated induction of anergy in the Jurkat T-cell line. *Transplant. Proc.* 26, 3458-9.

Gay, D., Saunders, T., Camper, S., and Weigert, M. (1993). Receptor Editing: An Approach by Autoreactive B cells to Escape Tolerance. *J. Exp. Med.* 177, 999-1008.

Gerberick, G. F., Cruse, L. W., Miller, C. M., Sikorski, E. E., and Ridder, G. M. (1997). Selective modulation of T cell memory markers CD62L and CD44 on murine draining lymph node cells following allergen and irritant treatment. *Tox. and App. Pharmacol.* 146, 1-10.

Giordanengo, V., Limouse, M., Peyron, J. F., and Lefebvre, J. C. (1995). Lymphocytic CD43 and CD45 bear sulfate residues potentially implicated in cell to cell interactions. *European Journal of Immunology* 25, 274-8.

Godfrey, D. I., and Zlotnik, A. (1993). Control points in early T-cell development. *Immunol. Today* 14, 547-53.

Gold, M. R., Law, D. A., and DeFranco, A. L. (1990). Stimulation of protein tyrosine phosphorylation by the B-lymphocyte antigen receptor. *Nature* 345, 810-3.

Goldman, S. J., Uniyal, S., Ferguson, L. M., Golan, D. E., Burakoff, S. J., and Kiener, P. A. (1992). Differential activation of phosphotyrosine protein phosphatase activity in a murine T cell hybridoma by monoclonal antibodies to CD45. *J. Biol. Chem.* 267, 6197-204.

Gong, Q., White, L., Johnson, R., White, M., Negishi, I., Thomas, M., and Chan, A. C. (1997). Restoration of thymocyte development and function in *zap-70*<sup>-/-</sup> mice by the Syk protein tyrosine kinase. *Immunity* 7, 369-377.

Gong, S., and Nussenzweig, M. C. (1996). Regulation of an early developmental checkpoint in the B cell pathway by Ig beta. *Science* 272, 411-4.

Goodnow, C. C. (1992). B-cell tolerance. *Curr. Opin. Immunol.* 4, 703-10.

Goodnow, C. C. (1996). Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. *Proc. Natl. Acad. Sci. USA* 93, 2264-71.

Goodnow, C. C. (1992). Transgenic mice and analysis of B-cell tolerance. *Ann. Rev. Immunol.* 10, 489-518.

Goodnow, C. C., Brink, R., and Adams, E. (1991). Breakdown of self-tolerance in anergic B lymphocytes. *Nature* 352, 532-6.

Goodnow, C. C., Crosbie, J., Adelstein, S., Lavoie, T. B., Smith-Gill, S. J., Brink, R. A., Pritchard-Briscoe, H., Wotherspoon, J. S., Loblay, R. H., and Raphael, K. (1988). Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* 334, 676-82.

Goodnow, C. C., Crosbie, J., Jorgensen, H., Brink, R. A., and Basten, A. (1989). Induction of self-tolerance in mature peripheral B lymphocytes. *Nature* 342, 385-91.

Groves, T., Smiley, P., Cooke, M. P., Forbush, K., Perlmutter, R. M., and Guidos, C. J. (1996). Fyn Can Partially Substitute for Lck in T Lymphocyte Development. *Immunity* 5, 417-428.

Grusby, M. J., Auchincloss, H., Jr., Lee, R., Johnson, R. S., Spencer, J. P., Zijlstra, M., Jaenisch, R., Papaioannou, V. E., and Glimcher, L. H. (1993). Mice lacking major histocompatibility complex class I and class II molecules. *Proc. Natl. Acad. Sci. USA* 90, 3913-7.

Hamilton, A. M., Lehuen, A., and Kearney, J. F. (1994). Immunofluorescence analysis of B-1 cell ontogeny in the mouse. *Int. Immunol.* 6, 355-61.

Hardy, R. R., Carmack, C. E., Shinton, S. A., Kemp, J. D., and Hayakawa, K. (1991). Resolution and characterization of pro-B and pre-pre-B cell stages in normal mouse bone marrow. *J. Exp. Med.* 173, 1213-1225.

Hardy, R. R., Hayakawa, D. R., Parks, D. R., and Herzenberg, L. A. (1983). Demonstration of B-cell maturation in X-linked immunodeficient mice by simultaneous three-colour immunofluorescence. *Nature* 306, 270-272.

Hentges, F. (1994). B lymphocyte ontogeny and immunoglobulin production. *Clin. and Exp. Immunol.* 97, 3-9.

Hertz, M., and Nemazee, D. (1998). Receptor editing and commitment in B lymphocytes. *Curr. Opin. Immunol.* 10, 208-13.

Hibbs, M. L., Tarlinton, D. M., Armes, J., Grail, D., Hodgson, G., Maglitto, R., Stacker, S. A., and Dunn, A. R. (1995). Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. *Cell* 83, 301-11.

Hippen, K. L., Buhl, A. M., D'Ambrosio, D., Nakamura, K., Persin, C., and Cambier, J. C. (1997). FcγRIIB1 inhibition of BCR-mediated phosphoinositide hydrolysis and Ca<sup>2+</sup> mobilization is integrated by CD19 dephosphorylation. *Immunity* 7, 49-58.

Hovis, R., Donovan, J., and Musci, M. (1993). Rescue of signalling by a chimeric protein containing the cytoplasmic domain of CD45. *Science* 260, 544-546.

Hurley, T. R., Hyman, R., and Sefton, B. M. (1993). Differential effects of expression of the CD45 tyrosine protein phosphatase on the tyrosine phosphorylation of the lck, fyn, and c-src tyrosine protein kinases. *Mol. and Cell. Biol.* *13*, 1651-6.

Isakov, N. (1997). ITIMs and ITAMs. The Yin and Yang of antigen and Fc receptor-linked signaling machinery. *Immunol. Res.* *16*, 85-100.

Jacobs, H., Iacomini, J., van de Ven, M., Tonegawa, S., and Berns, A. (1996). Domains of the TCR beta-chain required for early thymocyte development. *J. Exp. Med.* *184*, 1833-43.

Jameson, S. C., Hogquist, K. A., and Bevan, M. J. (1995). Positive selection of thymocytes. *Annu. Rev. Immunol.* *13*, 93-126.

Janeway, C. A., Jr., and Bottomly, K. (1994). Signals and signs for lymphocyte responses. *Cell* *76*, 275-85.

Janeway, C. A., Jr., Goodnow, C. C., and Medzhitov, R. (1996). Danger - pathogen on the premises: Immunological tolerance. *Curr. Biol.* *6*, 519-22.

Johnson, P., Ostergaard, H. L., Wasden, C., and Trowbridge, I. S. (1992). Mutational analysis of CD45. A leukocyte-specific protein tyrosine phosphatase. *J. Biol. Chem.* *267*, 8035-41.

Justement, L. B. (1997). The role of CD45 in signal transduction. *Adv. Immunol.* 66, 1-65.

Justement, L. B., Campbell, K. S., Chien, N. C., and Cambier, J. C. (1991). Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. *Science* 252, 1839-42.

Kane, L. P., and Hedrick, S. M. (1996). A role for calcium influx in setting the threshold for CD4<sup>+</sup>CD8<sup>+</sup> thymocyte negative selection. *J. Immunol.* 156, 4594-601.

Katagiri, T., Ogimoto, M., Hasegawa, K., Mizuno, K., and Yakura, H. (1995). Selective regulation of Lyn tyrosine kinase by CD45 in immature B cells. *J. Biol. Chem.* 270, 27987-90.

Kawakami, Y., Miura, T., Bissonnette, R., Hata, D., Khan, W. N., Kitamura, T., Maeda-Yamamoto, M., Hartman, S. E., Yao, L., Alt, F. W., and Kawakami, T. (1997). Bruton's tyrosine kinase regulates apoptosis and JNK/SAPK kinase activity. *Proc. Natl. Acad. Sci. USA* 94, 3938-42.

Kawauchi, K., Lazarus, A. H., Rapoport, M. J., Harwood, A., Cambier, J. C., and Delovitch, T. L. (1994). Tyrosine kinase and CD45 tyrosine phosphatase activity mediate p21ras activation in B cells stimulated through the antigen receptor. *J. Immunol.* 152, 3306-3316.

Khan, I. A., Matsuura, T., and Kasper, L. H. (1996). Activation-mediated CD4+ T cell unresponsiveness during acute *Toxoplasma gondii* infection in mice. *Int. Immunol.* 8, 887-96.

Khan, W. N., Alt, F. W., Gerstein, R. M., Malynn, B. A., Larsson, I., Rathbun, G., Davidson, L., Muller, S., Kantor, A. B., Herzenberg, L. A., and et al. (1995). Defective B cell development and function in Btk-deficient mice. *Immunity* 3, 283-99.

Kiener, P. A., Rankin, B. M., Burkhardt, A. L., Schieven, G. L., Gilliland, L. K., Rowley, R. B., Bolen, J. B., and Ledbetter, J. A. (1993). Cross-linking of Fc gamma receptor I (Fc gamma RI) and receptor II (Fc gamma RII) on monocytic cells activates a signal transduction pathway common to both Fc receptors that involves the stimulation of p72 Syk protein tyrosine kinase. *J. Biol. Chem.* 268, 24442-8.

Killeen, N., and Littman, D. R. (1993). Helper T-cell development in the absence of CD4-p56lck association. *Nature* 364, 729-32.

Kishihara, K., Penninger, J., Wallace, V. A., Kundig, T. M., Kawai, K., Wakeham, A., Timms, E., Pfeffer, K., Ohashi, P. S., Thomas, M. L., Furlonger, C., Paige, C. J., and Mak, T. W. (1993). Normal B lymphocyte development but impaired T cell maturation in CD45-exon6 protein tyrosine phosphatase-deficient mice. *Cell* 74, 143-156.

Kisielow, P., and Miazek, A. (1995). Positive selection of T cells: rescue from programmed cell death and differentiation require continual engagement of the T cell receptor. *J. Exp. Med.* 181, 1975-84.

Kitamura, D., Kudo, A., Schaal, S., Muller, W., Melchers, F., and Rajewsky, K. (1992). A critical role of lambda 5 protein in B cell development. *Cell* 69, 823-31.

Kitamura, D., and Rajewsky, K. (1992). Targeted disruption of  $\mu$  chain membrane exon causes loss of heavy-chain allelic exclusion. *Nature* 356, 154-6.

Kitamura, D., Roes, J., Kühn, R., and Rajewsky, K. (1991). A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* 350, 423-6.

Kong, Y. Y., Kishihara, K., Sumichika, H., Nakamura, T., Kaneko, M., and Nomoto, K. (1995). Differential requirements of CD45 for lymphocyte development and function. *Eur. J. Immunol.* 25, 3431-6.

Koretzky, G., Kohmetscher, M., Kadleck, T., and A., W. (1992). Restoration of T-cell receptor-mediated signal transduction by transfection of CD45 cDNA into a CD45-deficient variant of the Jurkat T-cell line. *J. Immunol* 149, 1138-1142.

Koretzky, G. A., Picus, J., Thomas, M. L., and Weiss, A. (1990). Tyrosine phosphatase CD45 is essential for coupling T-cell antigen receptor to the phosphatidyl inositol pathway. *Nature* 346, 66-8.

Kostelny, S. A., Cole, M. S., and Tso, J. Y. (1992). Formation of a bispecific antibody by the use of leucine zippers. *J. Immunol.* 148, 1547-53.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., and Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75, 263-74.

Leitges, M., Schmedt, C., Guinamard, R., Davoust, J., Schaal, S., Stabel, S., and Tarakhovsky, A. (1996). Immunodeficiency in protein kinase C $\beta$ -deficient mice. *Science* 273, 788-791.

Lenschow, D. J., Sperling, A. I., Cooke, M. P., Freeman, G., Rhee, L., Decker, D. C., Gray, G., Nadler, L. M., Goodnow, C. C., and Bluestone, J. A. (1994). Differential up-regulation of the B7-1 and B7-2 costimulatory molecules after Ig receptor engagement by antigen. *J. Immunol.* 153, 1990-7.

Levelt, C. N., Mombaerts, P., Iglesias, A., Tonegawa, S., and Eichmann, K. (1993). Restoration of early thymocyte differentiation in T-cell receptor beta-chain-deficient mutant mice by transmembrane signaling through CD3 epsilon. *Proc. Natl. Acad. Sci. USA* 90, 11401-5.

Linsley, P. S., Greene, J. L., Brady, W., Bajorath, J., Ledbetter, J. A., and Peach, R. (1994). Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity* 1, 793-801.

Loffert, D., Ehlich, A., Muller, W., and Rajewsky, K. (1996). Surrogate light chain expression is required to establish immunoglobulin heavy chain allelic exclusion during early B cell development. *Immunity* 4, 133-44.

Lorenz, M., Jung, S., and Radbruch, A. (1995). Switch transcripts in immunoglobulin class switching. *Science* 267, 1825-8.

Lorenz, M., and Radbruch, A. (1996). Developmental and molecular regulation of immunoglobulin class switch recombination. *Curr. Topics Microbiol. Immunol.* 217, 151-69.

Maecker, H. T., and Levy, S. (1997). Normal lymphocyte development but delayed humoral immune response in CD81-null mice. *J. Exp. Med.* 185, 1505-1510.

Majeti, R., Bilwes, A. M., Noel, J. P., Hunter, T., and Weiss, A. (1998). Dimerization-induced inhibition of receptor protein tyrosine phosphatase function through an inhibitory wedge. *Science* 279, 88-91.

Malissen, M., Gillet, A., Ardouin, L., Bouvier, G., Trucy, J., Ferrier, P., Vivier, E., and Malissen, B. (1995). Altered T cell development in mice with a targeted mutation of the CD3-epsilon gene. *EMBO J.* 14, 4641-53.

Malissen, M., Trucy, J., Jouvin-Marche, E., Cazenave, P. A., Scollay, R., and Malissen, B. (1992). Regulation of TCR alpha and beta gene allelic exclusion during T-cell development. *Immunol. Today* 13, 315-22.

Mamalaki, C., Elliot, J., Norton, T., Yannoutsos, N., Townsend, A. R., Chandler, P., Simpson, E., and Kioussis, D. (1993). Positive and negative

selection in transgenic mice expressing a T-cell receptor specific for influenza nucleoprotein and endogenous superantigen. *Dev. Immunol.* 3, 159-174.

Maroun, C., and Julius, M. (1994). Distinct involvement of CD45 in antigen receptor signalling in CD4+ and CD8+ primary T cells. *Eur. J. Immunol.* 24, 967-973.

Marrack, P., and Kappler, J. (1990). The staphylococcal enterotoxins and their relatives. *Science* 248, 705-11.

Mason, D. Y., Jones, M., and Goodnow, C. C. (1992). Development and follicular localization of tolerant B lymphocytes in lysozyme/anti-lysozyme IgM/IgD transgenic mice. *Int. Immunol.* 4, 163-75.

Matsuda, A., Motoya, S., Kimura, S., McInnis, R., Maizel, A. L., and Takeda, A. (1998). Disruption of lymphocyte function and signaling in CD45-associated protein-null mice. *J. Exp. Med.* 187, 1863-1870.

May, M. J., and Ghosh, S. (1998). Signal transduction through NF- $\kappa$ B. *Immunol. Today* 19, 80-88.

Melchers, F., Karasuyama, H., Haasner, D., Bauer, S., Kudo, A., Sakaguchi, N., Jameson, B., and Rolink, A. (1993). The surrogate light chain in B-cell development. *Immunol. Today* 14, 60-8.

Mittler RS, R. B., Kiener PA (1991). Physical association between CD45 and CD4 or CD8 occur as late activation events in antigen receptor-stimulated human T-cells. *J. Immunol.* 1991 *147*, 3434-3440.

Molina, T. J., Kishihara, K., Siderovski, D. P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C. J., Hartmann, K. U., Veillette, A., Davidson, D., and Mak, T. W. (1992). Profound block in thymocyte development in mice lacking p56lck. *Nature* *357*, 161-164.

Mombaerts, P., Clarke, A. R., Rudnicki, M. A., Iacomini, J., Itohara, S., Lafaille, J. J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M. L., and et al. (1992). Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature* *360*, 225-31.

Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S., and Papaioannou, V. F. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* *68*, 869-77.

Mombaerts, P., Mizoguchi, E., Grusby, M. J., Glimcher, L. H., Bhan, A. K., and Tonegawa, S. (1993). Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. *Cell* *75*, 274-82.

Motyka, B., and Teh, H. S. (1998). Naturally occurring low affinity peptide/MHC class I ligands can mediate negative selection and T cell activation. *J. Immunol.* *160*, 77-86.

Murray, J. S. (1998). How the MHC selects Th1/Th2 immunity. *Immunol. Today* 19, 157-63.

Nakayama, T., Ueda, Y., Yamada, H., Shores, E. W., Singer, A., and June, C. H. (1992). In vivo calcium elevations in thymocytes with T cell receptors that are specific for self ligands. *Science* 257, 96-9.

Negishi, I., Motoyama, N., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A. C., and Loh, D. Y. (1995). Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature* 376, 435-438.

Nel, A. E., Gupta, S., Lee, L., Ledbetter, J. A., and Kanner, S. B. (1995). Ligation of the T-cell antigen receptor (TCR) induces association of hSos1, ZAP-70, phospholipase C-gamma 1, and other phosphoproteins with Grb2 and the zeta-chain of the TCR. *J. Biol. Chem.* 270, 18428-18436.

Nemazee, D., Russell, D., Arnold, B., Haemmerling, G., Allison, J., Miller, J. F., Morahan, G., and Buerki, K. (1991). Clonal deletion of autospecific B lymphocytes. *Immunol. Rev.* 122, 117-32.

Ng, D. H., Maiti, A., and Johnson, P. (1995). Point mutation in the second phosphatase domain of CD45 abrogates tyrosine phosphatase activity. *Biochem. Biophys. Res. Comm.* 206, 302-9.

Nikolic-Zugic, J. (1991). Phenotypic and functional stages in the intrathymic development of alpha beta T cells. *Immunol. Today* 12, 65-70.

Nishinakamura, R., Miyajima, A., Mee, P. J., Tybulewicz, V. L., and Murray, R. (1996). Hematopoiesis in mice lacking the entire granulocyte-macrophage colony-stimulating factor/interleukin-3/interleukin-5 functions. *Blood* 88, 2458-64.

Nishizumi, H., Horikawa, K., Mlinaric-Rascan, I., and Yamamoto, T. (1998). A double-edged kinase Lyn: a positive and negative regulator for antigen receptor-mediated signals. *J. Exp. Med.* 187, 1343-8.

Nishizumi, H., Taniuchi, I., Yamanashi, Y., Kitamura, D., Ilic, D., Mori, S., Watanabe, T., and Yamamoto, T. (1995). Impaired proliferation of peripheral B cells and indication of autoimmune disease in lyn-deficient mice. *Immunity* 3, 549-60.

Nitschke, L., Carsetti, R., Ocker, B., Kohler, G., and Lamers, M. C. (1997). CD22 is a negative regulator of B-cell receptor signalling. *Curr. Biol.* 7, 133-143.

Novak, T. J., Farber, D., Leitenberg, D., Hong, S. C., Johnson, P., and Bottomly, K. (1994). Isoforms of the transmembrane tyrosine phosphatase CD45 differentially affect T cell recognition. *Immunity* 1, 109-19.

O'Keefe, T. L., Williams, G. T., Davies, S. L., and Neuberger, M. S. (1996). Hyperresponsive B cells in CD22-deficient mice. *Science* 274, 798-801.

O'Rourke, L., Tooze, R., Turner, M., Sandoval, D. M., Carter, R. H., Tybulewicz, V. L. J., and Fearon, D. T. (1998). CD19 as a membrane-anchored

adaptor protein of B lymphocytes: costimulation of lipid and protein kinases by recruitment of Vav. *Immunity* 8, 635-645.

Ogimoto, M., Katagiri, T., Mashima, K., Hasegawa, K., Mizuno, K., and Yakura, H. (1994). Negative regulation of apoptotic death in immature B cells by CD45. *Int. Immunol.* 6, 647-654.

Ohbo, K., Suda, T., Hashiyama, M., Mantani, A., Ikebe, M., Miyakawa, K., Moriyama, M., Nakamura, M., Katsuki, M., Takahashi, K., Yamamura, K., and Sugamura, K. (1996). Modulation of hematopoiesis in mice with a truncated mutant of the interleukin-2 receptor gamma chain. *Blood* 87, 956-67.

Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T., and Nakagawa, H. (1991). CSK: a protein-tyrosine kinase involved in regulation of src family kinases. *J. Biol. Chem.* 266, 24249-52.

Ostergaard, H. L., Shackelford, D. A., Hurley, T. R., Johnson, P., Hyman, R., Sefton, B. M., and Trowbridge, I. S. (1989). Expression of CD45 alters phosphorylation of the lck-encoded tyrosine protein kinase in murine lymphoma T-cell lines. *Proc. Natl. Acad. Sci. USA* 86, 8959-63.

Otipoby, K. L., Andersson, K. B., Draves, K. E., Klaus, S. J., Farr, A. G., Kerner, J. D., Perlmutter, R. M., Law, C. L., and Clark, E. A. (1996). CD22 regulates thymus-independent responses and the lifespan of B cells. *Nature* 384, 634-637.

Owen, M. J., and Venkitaraman, A. R. (1996). Signalling in lymphocyte development. *Curr. Opin. Immunol.* 8, 191-8.

Page, D. M., Kane, L. P., Allison, J. P., and Hedrick, S. M. (1993). Two signals are required for negative selection of CD4+CD8+ thymocytes. *J. Immunol.* 151, 1868-80.

Pani, G., Fischer, K. D., Mlinaric Rascan, I., and Siminovitch, K. A. (1996). Signaling capacity of the T cell antigen receptor is negatively regulated by the PTP1C tyrosine phosphatase. *J. Exp. Med.* 184, 839-852.

Pani, G., Siminovitch, K. A., and Paige, C. J. (1997). The motheaten mutation rescues B cell signaling and development in CD45-deficient mice. *J. Exp. Med.* 186, 581-588.

Pelanda, R., Schwers, S., Sonoda, E., Torres, R. M., Nemazee, D., and Rajewsky, K. (1997). Receptor editing in a transgenic mouse model: site, efficiency, and role in B cell tolerance and antibody diversification. *Immunity* 7, 765-75.

Peschon, J. J., Morrissey, P. J., Grabstein, K. H., Ramsdell, F. J., Maraskovsky, E., Gliniak, B. C., Park, L. S., Ziegler, S. F., Williams, D. E., Ware, C. B., and et al. (1994). Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J. Exp. Med.* 180, 1955-60.

Pingel, J. T., and Thomas, M. L. (1989). Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell* 58, 1055-65.

Powell, L. D., Sgroi, D., Sjoberg, E. R., Stamenkovic, I., and Varki, A. (1993). Natural ligands of the B cell adhesion molecule CD22 beta carry N-linked oligosaccharides with alpha-2,6-linked sialic acids that are required for recognition. *J. Biol. Chem.* 268, 7019-27.

Qian, D., and Weiss, A. (1997). T cell antigen receptor signal transduction. *Curr. Opin. Cell Biol.* 9, 205-12.

Quill, H. (1996). Anergy as a mechanism of peripheral T cell tolerance. *J. Immunol.* 156, 1325-7.

Rahemtulla, A., Shahinian, A., Kundig, T., Zinkernagel, R., and Mak, T. W. (1993). CD4 negative mice as a model for immunodeficiency. *Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences* 342, 57-8.

Rajewsky, K. (1992). Early and late B-cell development in the mouse. *Curr. Opin. Immunol.* 4, 171-6.

Rawlings, D. J., Scharenberg, A. M., Park, H., Wahl, M. I., Lin, S., Kato, R. M., Fluckiger, A., Witte, O. N., and Kinet, J. P. (1996). Activation of BTK by a phosphorylation mechanism initiated by src family kinases. *Science* 271, 822-825.

Ren, X. D., Bokoch, G. M., Traynor Kaplan, A., Jenkins, G. H., Anderson, R. A., and Schwartz, M. A. (1996). Physical association of the small GTPase Rho with a 68-kDa phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells. *Mol. Biol. Cell.* 7, 435-42.

Rickert, R. C., Rajewsky, K., and Roes, J. (1995). Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature* 376, 352-355.

Rodgers, W., and Rose, J. K. (1996). Exclusion of CD45 inhibits activity of p56lck associated with glycolipid-enriched membrane domains. *J. Cell Biol.* 135, 1515-23.

Rolink, A., Grawunder, U., Winkler, T. H., Karasuyama, H., and Melchers, F. (1994). IL-2 receptor alpha chain (CD25, TAC) expression defines a crucial stage in pre-B cell development. *Int. Immunol.* 6, 1257-64.

Rolink, A., Karasuyama, H., Grawunder, U., Haasner, D., Kudo, A., and Melchers, F. (1993). B cell development in mice with a defective lambda 5 gene. *Eur. J. Immunol.* 23, 1284-8.

Rolink, A., and Melchers, F. (1996). B-cell development in the mouse. *Immunol. Lett.* 54, 157-61.

Rolink, A., ten Boekel, E., Melchers, F., Fearon, D. T., Krop, I., and Andersson, J. (1996). A subpopulation of B220+ cells in murine bone marrow does not

express CD19 and contains natural killer cell progenitors. *J. Exp. Med.* 183, 187-94.

Roth, P. E., and DeFranco, A. L. (1995). Lymphocyte development. Intrinsic checkpoints for lineage progression. *Curr. Biol.* 5, 349-52.

Roth, P. E., and DeFranco, A. L. (1996). Receptor tails unlock developmental checkpoints for B lymphocytes. *Science* 272, 1752-4.

Russell, D. M., Dembic, Z., Morahan, G., Miller, J. F., Burki, K., and Nemazee, D. (1991). Peripheral deletion of self-reactive B cells. *Nature* 354, 308-11.

Sadlack, B., Lohler, J., Schorle, H., Klebb, G., Haber, H., Sickel, E., Noelle, R. J., and Horak, I. (1995). Generalized autoimmune disease in interleukin-2-deficient mice is triggered by an uncontrolled activation and proliferation of CD4+ T cells. *Eur. J. Immunol.* 25, 3053-9.

Sadlack, B., Merz, H., Schorle, H., Schimpl, A., Feller, A. C., and Horak, I. (1993). Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* 75, 253-61.

Saga, Y., Furukawa, K., Rogers, P., Tung, J. S., Parker, D., and Boyse, E. A. (1990). Further data on the selective expression of Ly-5 isoforms. *Immunogenetics* 31, 296-306.

Saga, Y., Tung, J. S., Shen, F. W., Pancoast, T. C., and Boyse, E. A. (1988). Organization of the Ly-5 gene. *Mol. Cell. Biol.* 8, 4889-95.

Saint-Ruf, C., Ungewiss, K., Groettrup, M., Bruno, L., Fehling, H. J., and von Boehmer, H. (1994). Analysis and expression of a cloned pre-T cell receptor gene. *Science* 266, 1208-12.

Sato, S., Miller, A. S., Howard, M. C., and Tedder, T. F. (1997). Regulation of B lymphocyte development and activation by the CD19/CD21/CD81/Leu 13 complex requires the cytoplasmic domain of CD19. *J. Immunol.* 159, 3278-3287.

Sato, S., Miller, A. S., Inaoki, M., Bock, C. B., Jansen, P. J., Tang, M. L., and Tedder, T. F. (1996). CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. *Immunity* 5, 551-562.

Sato, S., Steeber, D. A., Jansen, P. J., and Tedder, T. F. (1997). CD19 expression levels regulate B lymphocyte development: human CD19 restores normal function in mice lacking endogenous CD19. *J. Immunol.* 158, 4662-4669.

Sato, T., Furukawa, K., Autero, M., Gahmberg, C. G., and Kobata, A. (1993). Structural study of the sugar chains of human leukocyte common antigen CD45. *Biochemistry* 32, 12694-704.

Satterthwaite, A. B., Lowell, C. A., Khan, W. N., Sideras, P., Alt, F. W., and Witte, O. N. (1998). Independent and opposing roles for Btk and lyn in B and myeloid signaling pathways. *J. Exp. Med.* 188, 833-44.

Scharenberg, A. M., El-Hillal, O., Fruman, D. A., Beitz, L. O., Li, Z., Lin, S., Gout, I., Cantley, L. C., Rawlings, D. J., and Kinet, J.-P. (1998). Phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P<sub>3</sub>)/Tec kinase-dependent calcium signalling pathway: a target for SHIP-mediated inhibitory signals. *EMBO J.* *17*, 1961-1972.

Scharenberg, A. M., and Kinet, J. P. (1998). PtdIns-3,4,5-P<sub>3</sub>: a regulatory nexus between tyrosine kinases and sustained calcium signals. *Cell* *94*, 5-8.

Schwartz, R. (1996). Models of T cell anergy: is there a common molecular mechanism? *J. Exp. Med.* *184*, 1-8.

Sefton, B. M., and Taddie, J. A. (1994). Role of tyrosine kinases in lymphocyte activation. *Curr. Opin. Immunol.* *6*, 372-9.

Sgroi, D., Varki, A., Braesch-Andersen, S., and Stamenkovic, I. (1993). CD22, a B cell-specific immunoglobulin superfamily member, is a sialic acid-binding lectin. *J. Biol. Chem.* *268*, 7011-8.

Shaw, A. S., and Dustin, M. L. (1997). Making the T cell receptor go the distance: a topological view of T cell activation. *Immunity* *6*, 361-9.

Sheard, M., Sugaya, K., Furuta, M., Tsuda, S., and Takahama, Y. (1996). Heterogeneous expression of recombination activating genes and surface CD5 in CD3<sup>low</sup> CD4<sup>+</sup> CD8<sup>+</sup> thymocytes. *Scan. J. Immunol.* *43*, 619-25.

Shinkai, Y., Koyasu, S., Nakayama, K., Murphy, K. M., Loh, D. Y., Reinherz, E. L., and Alt, F. W. (1993). Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. *Science* 259, 822-5.

Shinkai, Y., Rathbun, G., Lam, K.-P., Oltz, E. M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A. M., and Alt, F. W. (1992). RAG-2 deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68, 855-867.

Shiroo, M., L., G., Biffen, E., Shivnan, E., and Alexander, D. (1992). CD45-tyrosine phosphatase-activated p59fyn couples the T cell antigen receptor to pathways of diacylglycerol production, protein kinase C activation and calcium influx. *EMBO J.* 11, 4887.

Sibilia, M., and Wagner, E. F. (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* 269, 234-8.

Sieh, M., Bolen, J. B., and Weiss, A. (1993). CD45 specifically modulates binding of Lck to a phosphopeptide encompassing the negative regulatory tyrosine of Lck. *EMBO J.* 12, 315-21.

Sillman, A. L., and Monroe, J. G. (1994). Surface IgM-stimulated proliferation, inositol phospholipid hydrolysis, Ca<sup>2+</sup> flux, and tyrosine phosphorylation are not altered in B cells from p59fyn<sup>-/-</sup> mice. *J. Leukoc. Biol.* 56, 812-816.

Simpson, E., Dyson, P. J., Knight, A. M., Robinson, P. J., Elliott, J. I., and Altmann, D. M. (1993). T-cell receptor repertoire selection by mouse mammary tumor viruses and MHC molecules. *Immunol. Rev.* 131, 93-115.

Sloan-Lancaster, J., Shaw, A., Rothbard, J., and PM., A. (1994). Partial T cell signaling: altered phospho-zeta and lack of zap70 recruitment in APL-induced T cell anergy. *Cell.* 79, 913-22.

Sponaas, A. M., Tomlinson, P. D., Antoniou, J., Auphan, N., Langlet, C., Malissen, B., Schmitt Verhulst, A. M., and Mellor, A. L. (1994). Induction of tolerance to self MHC class I molecules expressed under the control of milk protein or beta-globin gene promoters. *Int. Immunol.* 6, 277-87.

Stamenkovic, I., SgROI, D., Aruffo, A., Sy, M. S., and Anderson, T. (1991). The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45RO on T cells and  $\alpha$ 2-6 sialyltransferase, CD75, on B cells. *Cell* 66, 1133-1144.

Stein, P. L., Lee, H.-M., Rich, S., and Soriano, P. (1992). p59<sup>fyn</sup> mutant mice display differential signalling in thymocytes and peripheral T cells. *Cell* 70, 741-750.

Stone, J. D., Conroy, L. A., Byth, K. F., Hederer, R. A., Howlett, S., Takemoto, Y., Holmes, N., and Alexander, D. R. (1997). Aberrant TCR-mediated signalling in CD45-null thymocytes involves dysfunctional regulation of Lck, Fyn, TCR- $\zeta$ , and ZAP-70. *J. Immunol.* 158, 5773-5782.

Stone, J. D., Howlett, S., Byth, K. F., Holmes, N., and Alexander, D. R. (1997).

T-cell antigen receptor signal transduction in CD45<sup>-/-</sup> thymocytes. *Biochem. Soc. Trans.* 25, 302S.

Streuli, M., Krueger, N. X., Thai, T., Tang, M., and Saito, H. (1990). Distinct functional roles of the two intracellular phosphatase like domains of the receptor-linked protein tyrosine phosphatases LCA and LAR. *EMBO J.* 9, 2399-407.

Swat, W., Dessing, M., von Boehmer, H., and Kisielow, P. (1993). CD69 expression during selection and maturation of CD4<sup>+</sup>8<sup>+</sup> thymocytes. *Eur. J. Immunol.* 23, 739-46.

Takahama, Y., and Nakauchi, H. (1996). Phorbol ester and calcium ionophore can replace TCR signals that induce positive selection of CD4 T cells. *J. Immunol.* 157, 1508-13.

Takai, T. (1996). Multiple loss of effector cell functions in FcR gamma-deficient mice. *Int. Rev. Immunol.* 13, 369-81.

Takai, T., Ono, M., Hikida, M., Ohmori, H., and Ravetch, J. V. (1996). Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. *Nature* 379, 346-9.

Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H., and Kurosaki, T. (1994). Tyrosine kinases Lyn and Syk regulate B cell

receptor-coupled  $\text{Ca}^{2+}$  mobilization through distinct pathways. *EMBO J.* 13, 1341-9.

Takeda, A., Wu, J., and Maizell, A. (1992). Evidence for monomeric and dimeric forms of CD45 associated with a 30-kDa phosphorylated protein. *J. Biol. Chem.* 267, 16651-16659.

Tarakhovsky, A. (1997). Bar Mitzvah for B-1 cells: how will they grow up? *J. Exp. Med.* 185, 981-4.

Tarakhovsky, A., Kanner, S. B., Hombach, J., Ledbetter, J. A., Muller, W., Killeen, N., and Rajewsky, K. (1995). A role for CD5 in TCR-mediated signal transduction and thymocyte selection. *Science* 269, 535-7.

Tarakhovsky, A., Muller, W., and Rajewsky, K. (1994). Lymphocyte populations and immune responses in CD5-deficient mice. *Eur. J. Immunol.* 24, 1678-84.

Tedder, T. F., Inaoki, M., and Sato, S. (1997). The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. *Immunity* 6, 107-118.

Teh, Y. M., and Neuberger, M. S. (1997). The immunoglobulin (Ig)alpha and Igbeta cytoplasmic domains are independently sufficient to signal B cell maturation and activation in transgenic mice. *J. Exp. Med.* 185, 1753-8.

Thomas, M. L. (1995). Of ITAMs and ITIMs: turning on and off the B cell antigen receptor. *J. Exp. Med.* 181, 1953-6.

Thomas, M. L., and Lefrancois, L. (1988). Differential expression of the leucocyte-common antigen family. *Immunol. Today* 9, 320-6.

Thompson, C. B. (1995). Distinct roles for the costimulatory ligands B7-1 and B7-2 in T helper cell differentiation? *Cell* 81, 979-82.

Thompson, C. B., and Allison, J. P. (1997). The emerging role of CTLA-4 as an immune attenuator. *Immunity* 7, 445-50.

Threadgill, D. W., Dlugosz, A. A., Hansen, L. A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., and Harris, R. C. (1995). Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* 269, 230-4.

Tiegs, S. L., Russell, D. M., and Nemazee, D. (1993). Receptor editing in self-reactive bone marrow B cells. *J. Exp. Med.* 177, 1009-1020.

Tietz, W., and Hamann, A. (1997). The migratory behavior of murine CD4+ cells of memory phenotype. *Eur. J. Immunol.* 27, 2225-32.

Tolias, K. F., Cantley, L. C., and Carpenter, C. L. (1995). Rho family GTPases bind to phosphoinositide kinases. *J. Biol. Chem.* 270, 17656-9.

Torimoto, Y., Dang, N. H., Streuli, M., Rothstein, D. M., Saito, H., Schlossman, S. F., and Morimoto, C. (1992). Activation of T cells through a T cell-specific epitope of CD45. *Cell. Immunol.* *145*, 111-29.

Torimoto, Y., Dang, N. H., Vivier, E., Tanaka, T., Schlossman, S. F., and Morimoto, C. (1991). Coassociation of CD26 (dipeptidyl peptidase IV) with CD45 on the surface of human T lymphocytes. *J. Immunol.* *147*, 2514-7.

Torres, R. M., Flaswinkel, H., Reth, M., and Rajewsky, K. (1996). Aberrant B cell development and immune response in mice with a compromised BCR complex. *Science* *272*, 1804-1808.

Tsitsikov, E. N., Gutierrez-Ramos, J. C., and Geha, R. S. (1997). Impaired CD19 expression and signalling, enhanced antibody response to type II T independent antigen and reduction of B-1 cells in CD81-deficient mice. *Proc. Natl. Acad. Sci. USA* *94*, 10844-10849.

Tsui, H. W., Siminovitch, K. A., de Souza, L., and Tsui, F. W. (1993). Motheaten and viable motheaten mice have mutations in the haematopoietic cell phosphatase gene. *Nat. Genet.* *4*, 124-9.

Turner, M., Gulbranson-Judge, A., Quinn, M. E., Walters, A. E., MacLennan, I. C. M., and Tybulewicz, V. L. J. (1997). Syk tyrosine kinase is required for the positive selection of immature B cells into the recirculating B cell pool. *J. Exp. Med.* *186*, 2013-2021.

Turner, M., Mee, P. J., Costello, P. S., Williams, O., Price, A. A., Duddy, L. P., Furlong, M. T., Geahlen, R. L., and Tybulewicz, V. L. J. (1995). Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature* 378, 298-302.

Turner, M., Mee, P. J., Walters, A., Quinn, M. E., Mellor, A. L., Zamoyska, R., and Tybulewicz, V. L. J. (1997). A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. *Immunity* 7, 451-460.

Tybulewicz, V. L., Crawford, C. E., Jackson, P. K., Bronson, R. T., and Mulligan, R. C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. *Cell* 65, 1153-63.

Unkeless, J. C., and Jin, J. (1997). Inhibitory receptors, ITIM sequences and phosphatases. *Curr. Opin. Immunol.* 9, 338-43.

van Oers, N. S., Killeen, N., and Weiss, A. (1996). Lck regulates the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 in murine thymocytes. *J. Exp. Med.* 183, 1053-62.

van Oers, N. S. C., Lowin-Kropf, B., Finlay, D., Connolly, K., and Weiss, A. (1996).  $\alpha\beta$  T Cell Development Is Abolished in Mice Lacking Both Lck and Fyn Protein Tyrosine Kinases. *Immunity* 5, 429-436.

Volarevic, S., Burns, C. M., Sussman, J. J., and Ashwell, J. D. (1990). Intimate association of Thy-1 and the T-cell antigen receptor with the CD45 tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA* 87, 7085-9.

Volarevic, S., Niklinska, B. B., Burns, C. M., June, C. H., Weissman, A. M., and Ashwell, J. D. (1993). Regulation of TCR signaling by CD45 lacking transmembrane and extracellular domains. *Science* 260, 541-4.

von Boehmer, H. (1996). CD4/CD8 lineage commitment: back to instruction? *J. Exp. Med.* 183, 713-5.

von Boehmer, H. (1991). Positive and negative selection of the alpha beta T-cell repertoire in vivo. *Curr. Opin. Immunol.* 3, 210-5.

von Boehmer, H., and Fehling, H. J. (1997). Structure and function of the pre-T cell receptor. *Ann. Rev. Immunol.* 15, 433-52.

Wagner, S. D., and Neuberger, M. S. (1996). Somatic hypermutation of immunoglobulin genes. *Ann. Rev. Immunol.* 14, 441-57.

Wallace, V. A., Penninger, J. M., Kishihara, K., Timms, E., Shahinian, A., Pircher, H., Kundig, T. M., Ohashi, P. S., and Mak, T. W. (1997). Alterations in the level of CD45 surface expression affect the outcome of thymic selection. *J. Immunol.* 158, 3205-3214.

Wallace, V. A., Rahemtulla, A., Timms, E., Penninger, J., and Mak, T. W. (1992). CD4 expression is differentially required for deletion of MLS-1a-reactive T cells. *J. Exp. Med.* *176*, 1459-63.

Wang, C.-R., Hashimoto, K., Kubo, S., Yokochi, T., Kubo, M., Suzuki, M., Suzuki, K., Tada, T., and Nakayama, T. (1995). T cell receptor-mediated signaling events in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes undergoing thymic selection: requirement of calcineurin activation for thymic positive selection but not negative selection. *J. Exp. Med.* *181*, 927-41.

Wang, J., Koizumi, T., and Watanabe, T. (1996). Altered antigen receptor signaling and impaired Fas-mediated apoptosis of B cells in Lyn-deficient mice. *J. Exp. Med.* *184*, 831-838.

Weaver, C., Pingel, J., Nelson, J., and Thomas, M. (1991). CD8<sup>+</sup> T-cell clones deficient in the expression of the CD45 protein tyrosine phosphatase have impaired responses to T-cell receptor stimuli. *Mol. Cell. Biol.* *11*, 4415-4422.

Weaver, D. T., and Alt, F. W. (1997). V(D)J recombination. From RAGs to stitches. *Nature* *388*, 428-9.

Webb, S. R., and Gascoigne, N. R. (1994). T-cell activation by superantigens. *Curr. Opin. Immunol.* *6*, 467-75.

Weiss, A., and Littman, D. R. (1994). Signal transduction by lymphocyte antigen receptors. *Cell* *76*, 263-274.

Welge, T., Wolf, M., Jaggle, C., and Luckenbach, G. A. (1993). Human naive T cells are preferentially stimulated by crosslinking of CD3 and CD45RA with monoclonal antibodies. *Cell. Immunol.* *148*, 218-25.

Williams, O., Tanaka, Y., Tarazona, R., and Kioussis, D. (1997). The agonist-antagonist balance in positive selection. *Immunol. Today* *13*, 121-126.

Yamashita, I., Nagata, T., Tada, T., and Nakayama, T. (1993). CD69 cell surface expression identifies developing thymocytes which audition for T cell antigen receptor-mediated positive selection. *Int. Immunol.* *5*, 1139-50.

Yanagi, S., Sugawara, H., Kurosaki, M., Sabe, H., Yamamura, H., and Kurosaki, T. (1996). CD45 modulates phosphorylation of both autophosphorylation and negative regulatory tyrosines of Lyn in B cells. *J. Biol. Chem.* *271*, 30487-30492.

Yao, L., Kawakami, Y., and Kawakami, T. (1994). The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C. *Proc. Natl. Acad. Sci. USA* *91*, 9175-9179.

Zamoyska, R. (1998). CD4 and CD8: modulators of T-cell receptor recognition of antigen and of immune responses? *Curr. Opin. Immunol.* *10*, 82-7.

Zenner, G., Dirk zur Hausen, J., Burn, P., and Mustelin, T. (1995). Towards unraveling the complexity of T cell signal transduction. *Bioessays* *17*, 967-75.

Zhang, W., Sloan-Lancaster, J., Kitchen, J., Tribble, R. P., and Samelson, L. E. (1998). LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* 92, 83-92.

Zijlstra, M., Bix, M., Simister, N. E., Loring, J. M., Raulet, D. H., and Jaenisch, R. (1990). Beta 2-microglobulin deficient mice lack CD4-8+ cytolytic T cells. *Nature* 344, 742-6.