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The role of Syk-family protein tyrosine kinases in B cell development.

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The role of Syk-family protein tyrosine kinases in B cell development

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Abstract

The related Syk and Zap70 tyrosine kinases play an important role in lymphocyte development and signalling. Gene targeted mutant mice revealed that Syk plays a significant role in B cell development. $Syk^{-/-}$ mice show a partial block in B cell development at the pro-B to pre-B cell transition and a complete block at the immature to mature B cell transition. In contrast, mice deficient for Zap70 were reported to have normal numbers of B cells, but no T cells due to a block in thymic positive selection. Unexpectedly, mice deficient for both Syk and Zap70 show a complete block in B cell development at the pro-B to pre-B cell transition, indicating that Zap70 plays a role in the B cell lineage as well.

I have now further explored the role of Zap70 in B cell development and function. I found that Zap70 is expressed in all developing and mature B cell subsets, although pro-B cells express higher levels of Zap70 than other populations. Analysis of B cell development showed an increase in B1b and marginal zone B cells in $Zap70^{-/-}$ mice, which was due to the lack of Zap70 in the B cell lineage. In contrast, Zap70-deficient B cells were able to mount normal T-dependent and T-independent immune responses and had unaffected BCR-induced calcium release.

Furthermore, I asked whether the distinct effects of Syk and Zap70 mutations on B cell development were due to intrinsic differences in the function of the two kinases, or rather reflected differences in levels of expression. I found that overexpression of Zap70 in $Syk^{-/-}$ mice completely rescued B cell development, arguing that the two kinases are able to perform the same function during B cell development, and that the distinct B cell

phenotypes of *Syk^{-/-}* and *Zap70^{-/-}* mice are caused mainly by differences in levels of expression.

Finally, to be able to address the role of Syk in mature B cells, I set up a system where I made Syk inducible by fusing the protein to the hormone-binding domain of the estrogen receptor (ER-HBD). Three different Syk-ER fusion constructs were expressed in *Syk^{-/-}* DT40 cells and one of these was found to restore BCR-induced calcium fluxes in a hormone-dependent manner.

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Abbreviations

ATA	anti-T cell antibody
B-CLL	B cell chronic lymphocytotic leukaemia
BCR	B cell receptor
BLNK	B linker protein
BM	bone marrow
CLP	common lymphoid progenitor
DAG	diacylglycerol
DN	double-negative
DP	double-positive
ER	estrogen receptor
ES cell	embryonic stem cell
FL	fetal liver
FSC	forward scatter
GEF	guanine-nucleotide exchange factor
GFP	Green fluorescent protein
HBD	hormone binding domain
HC	Heavy chain
HEL	hen egg lysozyme
HSA	heat stable antigen
HSC	haematopoietic stem cell
HSP	heat-shock protein

HuCD2	human CD2
ICOS	inducible T cell costimulator
Ig	Immunoglobulin
IκB	inhibitor of NFκB
IL	Interleukin
IP$_3$	inositol-1,4,5-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motive
ITIM	Immunoreceptor tyrosine-based inhibitory motive
KLH	keyhole limpet haemocyanin
LC	light chain
LN	lymph node
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MRF	mature recirculating follicular
MZ	marginal zone
NF	newly formed
NF-AT	nuclear factor of activated T cells
NFκB	nuclear factor κB
NK	natural killer
4-OHT	4-hydroxytamoxifen
PALS	periarteriolar lymphoid sheath
PEC	peritoneal cavity
PI3K	Phosphoinositide-3-kinase
PKC	protein kinase C

PLCγ	Phospholipase C gamma
PTK	protein tyrosine kinase
rtTA	reverse transactivator
SCF	stem cell factor
SLC	surrogate light chain
SH	Src homology
SHIP	SH2-containing inositol 5'-phosphatase
SHP-1	SH2-containing tyrosine phosphatase-1
SP	single-positive
SSC	side scatter
Syk	Spleen tyrosine kinase
T1, T2	transitional 1, 2
TCR	T cell receptor
TD	thymus-dependent
Tet	tetracycline
TetO	tetracycline operator
TI	thymus-independent
TLR	toll-like receptor
TNP	2,4,6-trinitrophenol
TPO	trombopoietin
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

1. Introduction

1.1 Innate and adaptive immune system

Defence against microbes is mediated by the early reactions of the innate immune system and the later responses of the adaptive immune system. Innate immunity consists of mechanisms that exist before infection, are capable of rapid responses to microbes, and react in essentially the same way to repeated infections. The principal components of innate immunity are physical and chemical barriers, phagocytic cells and natural killer cells, blood proteins and cytokines (Medzhitov and Janeway, 1997).

In contrast to innate immunity, adaptive immunity refers to antigen-specific immune responses. This type of immunity develops as a response to infection and adapts to the infection. One of the most important features of adaptive immunity is that it leads to long-lasting memory of specific antigens (Janeway, 2001).

There are two types of adaptive immunity, called humoral immunity and cellular immunity, which are mediated with different components of the immune system and function to eliminate different types of microbes. Humoral immunity is mediated by antibodies, which are produced by B lymphocytes or B cells and functions in defence against extracellular microbes. Cellular immunity is mediated by T lymphocytes and their products, such as cytokines. T cells destroy intracellular pathogens by killing infected cells and by activating macrophages, but they also have a central role in the destruction of extracellular pathogens by activating B cells (Abbas, 2000).

The macrophages and neutrophils of the innate immune system provide a first line of defence against many common microorganisms and are essential for the control of common bacterial infections. However, they cannot always eliminate infectious organisms, and there are some pathogens that they cannot recognise. The lymphocytes of the adaptive immune system have evolved to provide various means of defence, which in addition provides increased protection against subsequent re-infection with the same pathogen.

The cells of the innate immune system, however, play a crucial part in the initiation and subsequent direction of adaptive immune responses, as well as participating in the removal of pathogens that have been targeted by an adaptive immune response. Furthermore, because there is a delay of 4 to 7 days before the initial adaptive immune response takes effect, the innate immune response has a critical role in controlling infections during this period.

1.1.1   Humoral immunity

As mentioned before, humoral immunity is mediated by antibodies, which are produced by B cells. The simplest way in which antibodies can protect from pathogens such as viruses or bacterial toxins is by binding to them and thereby blocking their access to cells that they might infect or destroy. This process is known as Neutralisation.

Binding by antibodies, however, is not sufficient on its own to arrest the replication of bacteria that multiply outside cells. In this case, antibodies can coat pathogens and this

coating enables a phagocytic cell to ingest and destroy the bacterium. This coating of the bacterium by antibodies is known as Opsonization.

Antibodies also have the function to activate the complement system, which in some cases can form pores that directly destroy bacteria. However the main function of complement is to coat the surface of pathogens, like antibodies do themselves, and enable phagocytes to engulf and destroy the bacteria that they would otherwise not recognise.

1.2   Cellular immunity

Some bacterial pathogens and parasites and all viruses, replicate inside the cell where they cannot be detected by antibodies. The destruction of these invaders is the function of T cells. Cellular immunity or cell-mediated immune responses are dependent on the direct interactions between the T cell and the cell bearing the antigen that the T cell recognises. Cytotoxic T cells (or CD8$^+$ T cells) can recognise e.g. antigens derived from replicating viruses displayed on the surface of infected cells. These cells control the infection by directly killing the infected cell before viral replication is complete and viruses are released. T helper cells (CD4$^+$ T cells) activate the cell they recognise. T_H1 (T helper 1) cells are important for controlling intracellular bacterial infections, by e.g. activating macrophages. T_H2 cells have a central role in the destruction of extracellular pathogens by activating B cells.

In all cases, T cells recognise their targets by detecting peptide fragments derived from the foreign proteins, after these peptides have been captured and by the host cell and displayed by them at the cell surcface. The molecules that display peptide antigen to the T cells are membrane glycoproteins encoded in a cluster of genes, called the major histocompatibility complex (MHC). There are two types of MHC molecules, called MHC class I and MHC class II. MHC class I molecules bearing viral peptides are recogised by cytotoxic T cells, which then kill the infected cell. MHC class II molecules bearing peptides derived from pathogens taken up into vesicle are recognised by T_H1 or T_H2 cells (Janeway, 2001). The antigen-specific activation of these T cells is aided by co-receptors that distinguish between the two classes of MHC molecules. Cytotoxic T cells express CD8 co-receptor, which bind MHC class I molecules and T_H1 or T_H2 cells express CD4 co-receptor with specificity for MHC class II molecules. Maintaining the proper number and proportion of CD4$^+$ and CD8$^+$ T cells is essential for optimal host defence.

1.2.1 T cell development

Committed lymphoid progenitors arise in the bone marrow and migrate to the thymus. Early committed T cells lack expression of T cell receptor (TCR), CD4 and CD8 and are termed double-negative (DN) thymocytes. These cells can give rise to the major population of αβ T cells and the minor population of γδ T cells. γδ T cells are involved in immunoregulation and tumour surveillance, and have been implicated in wound healing

DN thymocytes are further subdivided into four stages of differentiation according to their cell surface marker expression: DN1, $CD44^+CD25^-$; DN2, $CD44^+CD25^+$; DN3, $CD44^-CD25^+$; DN4, $CD44^-CD25^-$ (Godfrey et al., 1993). The precise DN stage at which $\gamma\delta$ lineage commitment occurs is currently unclear. Subsequent expression of TCR$\gamma\delta$ in $\gamma\delta$ committed progenitors selects those cells for $\gamma\delta$ T cell maturation, whereas expression of the pre-T cell antigen receptor (pre-TCR) in $\alpha\beta$ committed cells selects for $\alpha\beta$ T cell maturation (Pennington et al., 2005). As cells progress through DN2 to DN4 stages, they express the pre-TCR, which is composed of the non-rearranging pre-Tα chain and a rearranged TCR-β chain. Successful pre-TCR expression leads to substantial cell proliferation and differentiation into DN4 and $CD4^+CD8^+$ double-positive (DP) cells. Successful rearrangement of TCRα genes leads to the expression of a complete $\alpha\beta$ TCR. The $\alpha\beta$-$TCR^+CD4^+CD8^+$ (DP) thymocytes then interact with cortical epithelial cells that express a high density of MHC class I and MHC class II molecules associated with self-peptides. The fate of the DP thymocytes depends on signalling that is mediated by interaction of the TCR with these self-peptide MHC-ligands (Robey and Fowlkes, 1994). The appropriate, intermediate level of TCR signalling initiates effective maturation (positive selection). Thymocytes that express TCRs that bind self-peptide-MHC-class I complexes become $CD8^+$ single posititive (SP) T cells, whereas those that express TCRs that bind self-peptide-MHC class II ligands become become $CD4^+$ T cells. These cells are then ready to be exported from thymus to peripheral lymphoid sites (Germain, 2002).

1.2.2   The role of B cells in adaptive immunity

B cells are small (6-10 μm in diameter) and have a dense nucleus and little cytoplasm. Early DNA-labelling studies of bone marrow, using ^{3}H-thymidine and autoradiography, demonstrated a large-scale local production and emigration of small lymphocytes that expressed surface IgM molecules and other B-cell-related receptors and were thus identified as "B-lymphocytes" (Osmond and Everett, 1964; Osmond and Nossal, 1974; Osmond et al., 1998; Yang et al., 1978). B cells develop in the fetal liver, the bone marrow, and in a specialised organ called bursa of Fabricius in birds. B cells take their name from "bursa", which has been widely used as a model system (Glick, 1991).

Each B cell carries a unique receptor (B cell antigen receptor, BCR), in the form of surface-bound immunoglobulin M (IgM) that determines the antigen specificity of a given B cell. Each BCR contains heavy and light chains, both of which are composed of variable V and constant C portions. The need for a large repertoire of antigen-specific B cells carrying distinct receptors is served by the mechanism of gene rearrangement that during development brings together gene segments coding for different parts of the receptor proteins. The great diversity stems partly from the possibility of different gene-segment combinations and partly from the variability of the junctions, including the non-exact positions of crossovers and the addition of extra, non-germline-encoded nucleotides. Ontogenically the first step is the recombination of a given D_H (diversity gene of the heavy chain locus) segment with one of the four J_H (joining) segments. The rearrangement process only becomes entirely B lineage specific at the next step, when a V_H (variable) segment is chosen to join a DJ_H pair. If the assembly happens in the correct

reading frame, a heavy chain protein can be synthesised and tested. Light chain generation follows that of heavy chain, by a similar mechanism involving joining of V_L and J_L segments (Max, 1993). V_H segments in the mouse have been grouped into 14 families, altogether consisting of potentially more than 1000 genes, followed by 13 D_H genes in three families and 4 J_H segments. There are two light chain genes: κ and λ. The κ locus contains multiple V region families to be recombined with one of the four $J_κ$ segments, whereas the λ locus contains only 3 $V_λ$ genes with 3 $J_λ$ segments.

During development cells are eliminated if they express self-reactive receptors or fail to express a functional receptor. Antigen binding is required for the final stages of development. Following antigen recognition, B cells become activated with or without T cell help. Once activated, B cells proliferate; moreover, following selection, some B cells differentiate into antibody-secreting plasma cells and later into memory B cells. Two types of genetic changes occur during this differentiation. First, somatic mutations change the antigen-binding properties so that B cells can bind antigens more avidly. Second, class switching, which results from a change in the heavy chain constant region alters the way that immunoglobulins on B cells are recognised by effector cells (for example cells from the innate immune system) (Ollila and Vihinen, 2005).

During B cell activation, the cells become enlarged, especially their cytoplasm. Subsequent cell division leads to the expansion of specific clonal lineages. Terminally differentiated B cells, the antibody-producing plasma cells, have a very large rough endoplasmic reticulum and Golgi apparatus, which are necessary for the production and secretion of immunoglobulins. The expression of immunoglobulin mRNAs increases 6-

12 fold while the expression of surface immunoglobulin decreases, since plasma cells produce only soluble antibodies.

1.3 B cell development

In the mouse, B cells are generated throughout life by differentiation from haematopoietic stem cells (HSC) in the liver before birth and in the bone marrow of adult animals (Hardy, 2003). This differentiation can be viewed as a progression from very primitive progenitors with multiple lineage potentials (e.g. B, T, NK, myeloid and erythroid) through more restricted progenitors, such as common lymphoid progenitor (CLP), finally to a B cell-restricted stage. Lineage-restricted cells execute a programmed development, first rearranging immunoglobulin heavy chains at the pro-B cell stage, then undergoing multiple rounds of clonal expansion at the pre-B cell stage and finally rearranging the light chain to develop into newly formed surface IgM^+ B cells.

1.3.1 Early stages of B cell development

B cell development can be divided into several stages based on the rearrangement status of the immunoglobulin genes and the expression of specific cell surface markers (Fig. 1.1). Immediately prior to B lineage commitment is a subset of cells called common lymphoid progenitors (CLP) (Kondo et al., 1997). While these cells are negative for lineage markers (including B220, Mac-1, Ter119, GR-1), they express c-kit and IL-7

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receptor α chain. Individual cells with this surface phenotype give rise to mixed B, T and NK progeny, but not to other hematopoietic lineages, such as myeloid or erythroid cells.

	Pre-BCR signal		BCR signal
Philadelphia	A B C	C' D	E
	pro	pre	Immat
Basel	pro-B Pre-BI	PreBII large Pre-BII small	Immat

	A	B	C	C'	D	E
c-kit	+	+	+	−	−	−
SLC	+	+	+	+	−	−
CD43	+	+	+	+	−	−
HSA	−	+	+	++	++	++
BP1	−	−	+	+	−	−
CD25	−	−	−	+	+	+
CD2	−	−	−	+/-	+	+
CD19	−	+	+	+	+	+
Rag1+2	+	+	+	+/-	+	−
HC	DJ	VDJ	VDJ	VDJ	VDJ	VDJ
LC	G	G	G	G	G	VJ
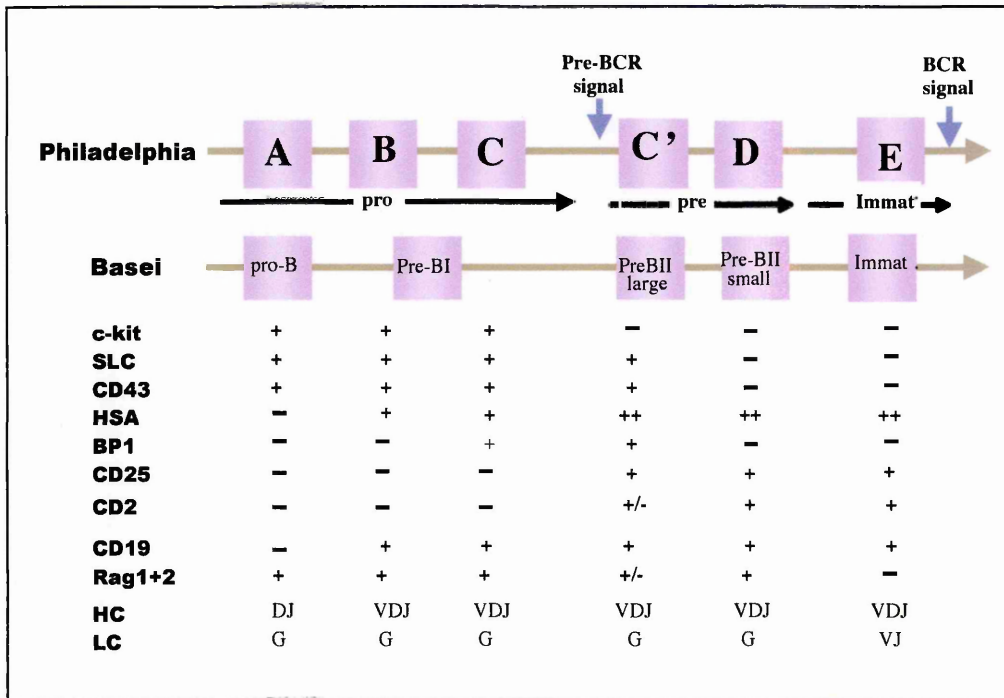
Figure 1.1. Early stages of B cell development

The development of B cells proceeds through a number of stages marked by the rearrangement of the immunoglobulin genes and expression of specific cell surface markers. Currently two nomenclatures are used, the Philadelphia and Basel nomenclature (Hardy and Hayakawa, 2001; Martensson et al., 2002; Osmond et al., 1998). SCL, surrogate light chain; HC, Immunoglobulin heavy chain; LC, Immunoglobulin light chain; G, germline; D, diversity; J, joining; V, variable; immat, immature; HC and LC rows show heavy chain and light chain rearrangement status in different B cell subsets.

Immediately following the CLP stage, B lineage restriction can be recognised by expression of the tyrosine phosphatase B220 (an isoform of CD45) (Allman et al., 1999; Li et al., 1996) on the surface of pro-B cells. Currently two nomenclatures are used to define the various stages of B cell development: the Hardy (Philadelphia) and the Rolink/Melchers (Basel) classifications (Alarid et al., 1999; Hardy and Hayakawa, 2001; Martensson et al., 2002; Osmond et al., 1998). Here, I will use the Hardy (Philadelphia) nomenclature. These earliest B220$^+$ pro-B cells are also CD43$^+$ CD24 (HSA)^{low} AA4.1$^+$ and is known as Fraction A (Li et al., 1996), whereas the AA4.1$^-$ subset consists of non-B cell lineage cells. AA4.1$^+$ cells can be further subdivided into half that express low levels of CD4 antigen (Fraction A$_1$) and half that do not (Fraction A$_2$). Fraction A$_1$ is less efficient in generating B cells than Fraction A$_2$ (Hardy, 2003; Li et al., 1996). Both A$_1$ and A$_2$ cells have little or no immunoglobulin gene rearrangement.

Next, in fractions B and C of the pro-B cell stage, all cells have recombined D$_H$ to J$_H$ gene segments on both Immunoglobulin heavy chain (IgH) gene alleles and have upregulated CD19 expression. These cells express the VpreB and λ5 genes encoding surrogate light chain (SLC) (Abbas, 2000; Karasuyama et al., 1993; Kudo and Melchers, 1987; Sakaguchi and Melchers, 1986).

Several transcription factors are important for lineage restriction during hematopoiesis and early B cell development. PU.1, E2A and early B cell factor (EBF) play critical roles in B cell development, as mice lacking PU.1 fail to generate B lineage cells at all (Scott et al., 1994), while E2A$^{-/-}$ and EBF$^{-/-}$ mice show very early blocks in B cell development (Bain et al., 1997; Lin and Grosschedl, 1995). PAX5 is a key transcription factor for B cell commitment, as the inactivation of PAX5 allowed the generation of

multiple hematopoietic cell types from cells that would normally be B lineage restricted (Nutt et al., 1999).

1.3.2 The pre-BCR

If the rearrangement of the IgH genes in pro-B cells is successful, the resulting μ heavy chain associates with SLC resulting in surface expression of a complex known as the pre-BCR. Signals from the pre-BCR induce proliferation and differentiation into pre-B cells characterised by a loss of c-kit and CD43 expression, and gain of CD25 and CD2 expression (Rolink et al., 2000). B cell development in mutant mice unable to generate rearranged heavy chains, such as SCID (severe combined immunodeficiency) mice, which have a defect in the DNA-PK repair complex and consequently fail to rejoin DNA efficiently during Ig H rearrangement is arrested prior to the pre-B cell stage (Reichman-Fried et al., 1990). Likewise, targeting of the recombination-activating genes, Rag-1 and Rag-2 that are required to generate double strand breaks in DNA during immunoglobulin rearrangement result in a similar phenotype (Spanopoulou et al., 1994). Analysis of Rag expression in pre-BCR^{+}pro-B cells fraction C' shows a downregulation of both Rag-1 and Rag-2 in this rapidly cycling fraction. In addition to this transient downregulation of Rag-1 and Rag-2 genes, terminal deoxynucleotidyl transferase (TdT) expression, which is expressed by cells during V_H rearrangement, is permanently downregulated (Wasserman et al., 1997). TdT adds untemplated nucleotides to protein coding segment ends exposed by activity of the Rag-1 and Rag-2 proteins. The level of

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kappa light chain germline transcription, which precedes rearrangement is also upregulated in fraction C' pro-B cells.

The pre-BCR assembles with the imunoglobulin accessory proteins Igα and Igβ on the surface of the cell and plays a critical role in cell differentiation. In μMT mice, generated by a targeted disruption of the μ heavy chain membrane exon, the heavy chain locus undergoes rearrangement, but the protein product cannot be inserted into the membrane. The result of this is a developmental block at the pro-B cell stage (Kitamura et al., 1991). Elimination of other components of the SLC of the pre-BCR complex, λ5 or Vpre-B1 and/or Vpre-B2 also partially blocks development at this stage (Gong and Nussenzweig, 1996; Kitamura et al., 1992; Martensson et al., 2002).

Signalling initiated through the pre-BCR induces clonal expansion of pre-B cells, whereby interleukin (IL)-7 acts as a crucial pre-B cell specific proliferation factor (Hendriks and Middendorp, 2004). As a result, cells from fraction B and C differentiate into the next compartment of large, cycling cells of fraction C'. As mentioned above, in these cells Rag-1 and Rag-2 are transiently downregulated to terminate further IgH chain rearrangement, thus ensuring that only one functional Igμ is synthesized, a phenomenon referred to as allelic exclusion. After 2-5 rounds of cell division, the large cells from fraction C' stop cycling and differentiate into small, resting pre-B cells (fraction D). This transition is associated with reactivation of the rearrangement machinery for the initiation of V_L and J_L rearrangements and significant changes in cell surface marker profiles. Cell from fraction D have downregulated CD43 and BP-1 expression but now express CD2, CD25 and CD22 on their cell surface.

After clonal expansion of cells with successful heavy chain rearrangement, pre-B cells stop cycling. Rag-1 and Rag-2 and Igκ germline transcripts are expressed and pre-B cells undergo light chain gene recombination. Successful light chain gene rearrangement leads to the assembly of the BCR and replacement of the SLC of the pre-BCR with Igκ or Igλ.

Productive rearrangement at the immunoglobulin light chain (IgL) loci is an important event that ultimately allows cell surface immunoglobulin expression and developmental progression towards B cell maturity. As metioned above, the light chains exist in two forms, κ or λ. The κ locus rearranges first and excludes λ locus from recombination – a process called isotype exclusion (Corcoran, 2005). If rearrangement of the first κ allele is not productive, rearrangement and expression of the second allele occurs. If this rearrangement is not productive either, rearrangement and expression of λ locus starts. In the mouse, Igλ recombination only occurs in 10% of cells, when rearrangements on both κ alleles have been unsuccessful. Unproductive rearrangements on both IgH alleles or on all IgL alleles, lead to apoptosis.

1.3.3 Receptor editing

Immature B cells that express unique antigen receptors are tested for reactivity against self-antigen, with autoreactive B cells becoming deleted (clonal deletion), anergised or modified (Bergman and Cedar, 2004) This latter process occurs mostly through continuous rearrangement at the IgL locus, known as receptor editing (Gay et al., 1993).

During this process, secondary IgL rearrangements replace existing light chains, generating receptors with altered specificity.

Immature B cells are the first B lineage cells to express surface BCRs, mainly surface IgM. B cells remain in the immature compartment for an average of 3.5 days (Meffre et al., 2000; Osmond, 1993). It is in this compartment that self-reactive B cells that fail to edit their receptor are deleted or anergised (Sandel and Monroe, 1999).

1.3.4   Clonal deletion

Immature B cells differ from mature B cells in that they are particularly susceptible to BCR-induced apoptosis. B cells bearing self-reactive antibodies are efficiently eliminated in large numbers (Nemazee and Burki, 1989), and the degree of B cell elimination is dependent on the strength of receptor cross-linking. High-affinity interactions with membrane-bound antigen result in deletion, whereas lower-affinity interactions and soluble antigens allow editing or result in anergy (Hardy and Hayakawa, 2001). Deletion of self-reactive B cells during development is considered to be one way of establishing tolerance (Lederberg, 1959; Meffre et al., 2000).

1.3.5   Anergy

Anergy is the third mechanism by which self-tolerance is induced in immature B cells. This phenomenon was observed in cultures of developing B cells exposed to different

concentrations of anti-μ. B cells cultured in high concentrations of anti-μ did not develop and underwent deletion. In contrast, intermediate levels of BCR crosslinking by lower concentrations of anti-μ allowed B cells to develop but abrogated normal B cell function, as determined by decreased proliferation and antibody production upon mitogen exposure (Pike et al., 1982). Anergic B cells are short-lived and have difficulty developing from the immature to the long-lived B cell compartment in the spleen.

Anergy preserves the cells, but they are rendered inactive (e.g. do not secrete antibody) after they meet their cognate ligand. Using the HEL (hen egg lysozyme) system, where anti-HEL BCR transgenic cells meet their cognate ligand in soluble (sHEL) form, IgM expression levels are reduced on anergic B cells (while IgD expression is maintained) as a sign of previous antigen encounter. The life span of these cells is much shorter (Cyster and Goodnow, 1995; Goodnow et al., 1988).

1.4 Late stages of B cell development

Of the 2×10^7 BCR$^+$ (IgM$^+$) B cells that develop in the murine bone marrow daily, only around 10% exit to the periphery (Osmond, 1993). Of these cells, about a third progresses to the mature B cell pool.

1.4.1 Transitional B cells

The emigrating immature B cells, now called transitional B cells, retain key phenotypic characteristics of their bone marrow counterparts, such as susceptibility to negative selection and the expression of defining cell surface markers. All transitional B cells are heat-stable antigen $(HSA)^{high}$ when compared to their mature counterparts and express AA4.1. Also, their IgM levels are higher than in mature B cells, whereas the surface levels of CD22 and B220 are lower (Chung et al., 2003). Although BCR stimulation of mature B cells leads to activation and proliferation, the same signals on developing B cells lead to arrest in an unresponsive state or cell death (Goodnow et al., 1988; Norvell et al., 1995).

On exiting the bone marrow, the early transitional B cells enter the peripheral circulation. The open circulation of the spleen leads to the entry of newly emerging transitional B cells into the red pulp through the terminal branches of the central arterioles. From the red pulp, these B cells penetrate the marginal zone sinuses to reach the outer zone of the periarteriolar lymphoid sheath (PALS) (Liu, 1997). Although transitional B cells migrate to the spleen, they are excluded from the lymph nodes, perhaps lack of surface expression of CD62L (Flaishon et al., 2000; Loder et al., 1999).

Separation of splenic transitional B cells into two distinct populations, termed transitional 1 (T1) and transitional 2 (T2), was first proposed by Loder et al., based on their surface phenotype and functional characteristics (Loder et al., 1999). T1 B cells are distinguished from the other subsets by their expression of the following surface markers: they are $IgM^{high}IgD^-CD21^-CD23^-$, whereas T2 B cells retain high levels of surface IgM, but are also $IgD^+CD21^+CD23^+$ (Fig.1.2). Using the AA4.1 antibody,

Allman et al. described a third non-proliferating population, termed T3, which resembles T2 with the exception of a lower level of surface IgM (Allman et al., 2001).

The transitional B cell subsets differ in their localization within the spleen. T1 B cells appear to be limited to the outer PALS, whereas T2 B cells are located in B cell follicles (Loder et al., 1999).

	Immat.	T1	T2	MRF	MZ	B1a	B1b
CD21	-	-	+	+	++	-	-
CD23	-	-	+	+	-	-	-
AA4.1	+	+	+	-	-	-	-
IgM	++	++	++	+	++	++	++
IgD	-	-/+	+	++	+/-	+/-	+/-
CD5	-	-	-	-	-	+	-
Mac-1	-	-	-	-	-	+	+
Anatomic location	BM sinusoid	Red pulp, outer PALS	Splenic B cell follicles	Splenic and LN follice. blood	Splenic MZ	PEC	PEC

Figure 1.2. Late stages of B cell development

Figure shows the late stages of B cell development from the immature to the mature stage. Different surface markers expressed on the different cells and their anatomic locations are indicated. T1, T2 = transitional 1, 2; MRF = mature recirculating follicular; PALS = periarteriolar lymphoid sheath, MZ = marginal zone; PEC = peritoneal cavity; LN = lymph nodes; BM = bone marrows;

Based on reconstitution and adoptive transfer studies, T1 B cells were classified as the progenitors of T2 B cells, while T2 B cells were classified as the progenitors of both follicular and marginal zone B cells (Srivastava et al., 2005; Su and Rawlings, 2002). Although T1 B cells fail to proliferate and instead undergo apoptosis on BCR-cross linking, there is disagreement in the literature regarding the response of T2 B cells to the same signals. One study indicates that T2 B cells also do not proliferate and are susceptible to BCR-induced apoptosis (Allman et al., 2001). However, another study suggests that T2 B cells are able to proliferate and are resistant to apoptosis (Loder et al., 1999).

Only a small percentage of T1 B cells proceed to the T2 stage, which indicates that most T1 B cells undergo apoptosis. Some of the B cells that are generated in the bone marrow might cross-react with self-antigens that are expressed only in peripheral tissues and so need to be deleted. In addition, the fact that T1 B cells express the pro-apoptotic molecule Fas (CD95), and not the anti-apoptotic protein BCL-2, is consistent with them being highly sensitive to negative selection (Carsetti et al., 1995). T1 B cells that successfully enter the splenic B cell follicles become T2 B cells.

BCR signalling pathways are different in T1 and T2 B cells. After BCR cross-linking, T1 B cells express little cyclin D2 or A1, and a reduced amount of active AKT, whereas T2 B cells express high levels of cyclin D2 and A1 and active AKT (Su and Rawlings, 2002).

T-cell help signals, such as CD40-CD40 ligand (CD40L) interactions and IL-4 rescue T2 B cells from BCR-induced apoptosis and promote vigorous proliferation (Chung et al.,

2002). This is consistent with the idea that T2 cells are located in the splenic follicles, where they can receive T cell signals.

1.4.2 Mature peripheral B cell populations

Subsets of mature B lymphocytes have been described based on phenotypic, topographic and functional characteristics, which have different generation and maintenance requirements. After maturation, B cells either recirculate through secondary lymphoid organs as part of a long-lived pool (mature recirculating follicular (MRF) or B2 B cells), or join more static compartments enriched in specific locations, in the marginal zone of the spleen (MZ B cells) or in the pleural and peritoneal cavities (B1 cells). These compartments are present in germ-free mice and rats, suggesting that they are indeed part of the pre-immune repertoire (Martin and Kearney, 2000a).

In studies of B cell homeostasis, Rag-2 was conditionally deleted at specific times in adult mice (Hao and Rajewsky, 2001). In these mice, splenic naïve follicular B cells were gradually lost over a year of observation. By contrast, the pool of MZ B cells in the spleen and B1 cells in the peritoneal cavity were kept at normal levels. This study might suggests that MZ and B1 cells represent self-renewing populations.

1.4.3   Mature recirculating follicular (MRF) B cells

MRF B cells have low levels of surface IgM and high levels of surface IgD. MRF B cells are naïve B cells and are by far the largest B cell subset. They are present primarily in the follicles of secondary lymphoid organs and in the circulation, and in the bone marrow as recirculating cells. They are also known as B2 cells and respond to T-dependent antigens, undergo germinal center reactions and give rise to memory B cells. The differentiation and maintenance of MRF B cells clearly require signals derived from the BCR, as incompetent BCR signalling causes the arrest of B cell differentiation at the transitional stage and the loss of a BCR by mature B cells results in cell death (Lam et al., 1997).

Tonic signalling is ligand-independent signalling generated by the expression of the BCR. It has been demonstrated that the cytoplasmic domains of Igα and Igβ in the absence of antigen binding are sufficient to drive B cell differentiation into a splenic B cell, arguing that a basal BCR tonic signal is sufficient for B cell maturation (Bannish et al., 2001). However, it is not clear if the resting splenic B cells from this experiment are MRF B cells. By contrast, over the years evidence has been accumulated supporting the role for ligand-mediated positive selection in MRF B cell maturation: CD45-deficient B cells are arrested at the immature B cell stage. $CD45^{-/-}$ HEL-specific B cells are also arrested, but become mature if soluble HEL is available (Cyster et al., 1996).

Furthermore, transitional B cells can be induced to differentiate into MRF-like B cells upon BCR ligation *in vitro* (Su and Rawlings, 2002). It has also been demonstrated that B cells expressing a single V_H segment, V_H12, are developmentally arrested at a transitional stage, but can be induced to become MRF B cells *in vivo* upon treatment with a low dose of anti-Igβ antibody (Wang and Clarke, 2003).

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Taken together, these data suggest that the BCR tonic signal alone is not enough to fully convert transitional B cells into MRF B cells, and that weak ligand stimulation is required for MRF B cell maturation.

1.4.4   Marginal zone (MZ) B cells

MZ B cells are $IgM^{hi}CD23^{lo}CD21^{hi}$ and are non-recirculating cells enriched primarily in the marginal zone of the spleen. MZ B cells are considered to be self-renewing (Hao and Rajewsky, 2001; Pillai et al., 2004). MZ B cells are thought to be critical for T-independent responses to blood-borne pathogens (Martin et al., 2001). MZ B cells express higher levels of B7-1 (CD80) and B7-2 (CD86) than MRF B cells and they might also present blood-borne antigens to naïve T cells and possibly contribute to T-dependent responses (Martin and Kearney, 2000a; Pillai et al., 2005).

1.4.5   B1 cells

The B1 cells make up the major B cell subset of the peritoneal cavity (PEC) and a minor population in the spleen. B1 cells can be distinguished from B2 cells by their surface phenotype, which is similar to MZ B cells. They have an $IgM^{hi}IgD^{lo}CD23^{-}B220^{lo}$ phenotype, with Mac-1 (CD11b, C3) expression on peritoneal but not on splenic B1 cells. B1 cells are absent from peripheral lymph nodes and variably make up about 5% of splenic B cells (Berland and Wortis, 2002).

Originally, B1 cells were identified by their expression of CD5. Subsequently, a population of peritoneal CD5⁻ B cells was identified whose surface phenotype was in other respects identical to that of B1 cells. By consensus, CD5⁺ B1 cells are referred to as B1a cells and CD5⁻ B1 cells as B1b cells.

Splenic B1a cells have not been studied extensively, because of their low frequency. They differ, however, from peritoneal B1a cells in two important aspects. Peritoneal B1a cells contain constitutively active STAT-3, an inducible transcription factor, while splenic B1a cells do not (Rothstein et al., 2000). Splenic B1a cells, unlike those isolated from the peritoneal cavity, flux calcium normally upon BCR ligation (Chumley et al., 2000).

B1 cells are responsible for the majority of non-immune serum IgM and contribute substantial amounts of resting IgA (Kroese et al., 1993; Rothstein, 2002). B1 cell-derived immunoglobulin generally adheres more closely to the germline state than B2 immunoglobulin, as a result of diminished somatic mutations and reduced length of nontemplated N-insertions (Rothstein, 2002). Transfer experiment studies have revealed that B1 cells have self-renewal capacities, which also distinguishes them from conventional B2 cells (Herzenberg, 2000). B1 cells are thought to be key players in early humoral response against pathogens and are thought to be the primary antibody producers in response to T-cell-independent type 2 (TI-2) antigens along with MZ B cells.

Two main hypotheses (lineage versus differentiation) have been proposed for the generation of B1 cells. Early work suggested that B1 cells exist as a separate lineage, distinct from conventional B cells. Studies comparing adoptive repopulation by fetal

omentum, fetal liver and adult bone marrow precursors demonstrated distinct tissue and developmental specificity in the location of progenitors for B1 (fetal omentum and fetal liver) and B2 cells (adult bone marrow) (Hardy and Hayakawa, 1991).

Bone marrow reconstitution studies also suggest that B1a and B1b cells derive from different progenitors (Herzenberg, 2000). In essence, while B1a cells are very poorly reconstituted from progenitors in the adult bone marrow, B1b cells are reconstituted to their normal frequency.

The lineage hypothesis discussed above proposes that certain B cell precursors are destined to become B1 cells. In contrast, the differentiation hypothesis proposes that every B cell has the same potential to become B1 cells. The B1 phenotype was proposed to be a consequence of TI-2 like activation. BCR crosslinking, in the absence of an innate antigen, T cell help, or CD40 ligation, resulted in the induction of CD5 expression of B2 cells. The combination of BCR crosslinking and IL-6 treatment induced two additional features of B1 cells, the downregulation of CD23 and IgD (Haughton et al., 1993; Wortis et al., 1995). It was subsequently shown that BCR crosslinking also induced in B2 cells the ability to proliferate in response to phorbol ester, another feature of B1 cells (Rothstein et al., 1991).

According to the differentiation model, encounters with natural occurring TI-2 antigens account for the appearance of B1 cells *in vivo*. Differences in the ability of precursors from adult and fetal sources to generate B1 cells are due to differences in the repertoires and hence specificities of B cells derived from these sources (Berland and Wortis, 2002). The fetal repertoire is skewed towards the expression of immunoglobulins that bind

frequently to TI-2 antigens. The adult repertoire rarely generates these specificities and therefore generates few B1 cells.

The importance of BCR specificity in the generation of B1 cells suggests that the requisite BCR signalling is ligand-dependent. Transgenic mice were made in one study expressing the SM6C10 heavy chain, which was derived from a B1-hybridoma, expressing an anti-T cell antibody (ATA) specific for the T cell glycoprotein Thy-1. These mice showed high titres of ATA, all of which was produced by transgene-expressing B1a cells (Hayakawa et al., 1999). The majority of these cells expressed an endogenous light chain identical to that in the original SM6C10 hybridoma. When SM6C10 μ transgenic mice were crossed onto a Thy1$^{-/-}$ background, ATA-producing B cells and transgene-expressing B1a cells were absent. Thus, engagement of the BCR by antigen, in this case Thy1, is necessary for B1a development.

B1 cell development and maintenance appear to depend critically on BCR signalling, because B1 cell numbers are reduced if there is insufficient BCR signalling. It is generally found that mutations in positive regulators of BCR signalling, including deficiency in CD19 costimulatory transmembrane protein (Inaoki et al., 1997), the BLNK/SLP-65 adaptor protein (Xu et al., 2000), the *Cr2* gene-encoded CD21 molecule that complexes with CD19 (Carroll, 1998), Vav1 (Gulbranson-Judge et al., 1999; Tarakhovsky et al., 1995) the PI3-kinase p85α (Suzuki et al., 1999) and Btk tyrosine kinase (Khan et al., 1995), all result in a greater reduction of B1 cells compared to peripheral B cells,.

BCR mediated signalling is also controlled by negative regulators, which also show altered B1 cell development. While SHP-1 mutant motheaten (Me) show an increase in

B1a cells, mice lacking CD22, lyn or PD-1 all show an increase in B1b cells (Chan et al., 1997; Nishimura et al., 2000; Sidman et al., 1986).

1.4.6 Generation of B1, MRF and MZ B cells – signal strength hypothesis

The analysis of mutant mice in which antigen receptor signalling in B cells is either attenuated or enhanced has revealed the existence of a follicular versus marginal zone B cell fate decision. These analyses indicate that weak antigen receptor-derived signals favour MZ B cell generation, and relatively strong signals favour the development of MRF B cells. Even stronger signals derived from the antigen receptor favour the generation of B1 cells (Pillai et al., 2004; Pillai et al., 2005).

The first studies proposing such a model came from the $Aiolos^{-/-}/Xid$ double mutant mice (Cariappa et al., 2001). Aiolos is a zinc-finger protein of the Ikaros family, expressed primarily in B cells. These data suggest that Aiolos is a negative regulator of the Btk pathway. In the absence of Aiolos BCR signal strength is enhanced, MRF B cell numbers are greater than in wild-type mice, but MZ B cells are lost. In mice deficient for both Aiolos and Btk, MZ B cells reappear (Cariappa et al., 2001).

Additional evidence supporting this signal-strength hypothesis, is that in the absence of CD21/CR2, a known positive regulator of BCR signalling, MZ B cell number is increased (Cariappa et al., 2001). Mice lacking CD22, a negative regulator of BCR signalling, are deficient in MZ B cells, whereas the number of B1 cells is increased (Samardzic et al., 2002).

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In addition, mice in which the Epstein-Barr virus LMP2A gene has been integrated into the IgH locus produce either B1 cells or MZ and MRF B cells, depending on the strength of the promoter used to drive LMP2A expression. When LMP2A expression was driven by a strong pormotor, B1 cells were mainly produces and when LMP2A was driven by a weak promotor, MZ and MRF B cell generation was increased. LMP2A is thought to act as a surrogate BCR in this model and therefore it was interpreted that signal strength might influence B cell development (Casola et al., 2004).

However, the hypothesis of the weakest BCR signals promoting MZ B cell development is inconsistent with a recent publication of Hayakawas group, where they found that, using a monoclonal BCR mouse line, specific for the self-Thy1/CD90 glycoprotein, an increase in BCR signal strength, induced by a low-dose self-antigen, directed naïve immature B cells to mature into MZ B cells, and not MRF B cells (Wen et al., 2005).

B1 and MZ B cells share many similarities:

It is of interest that MZ B cells are the only other B cells subset that seems to show similar properties to B1 cells. They share phenotypic similarities, since they are IgM^{hi}IgD^{lo}CD23$^{lo/-}$. They are the source of natural antibodies and are presumed to be critical for T-independent responses to blood-borne pathogens (Martin et al., 2001)

In contrast to MRF B cells, the generation of MZ and B1 cells appears to require ligand-induced positive selection through the BCR (Berland and Wortis, 2002; Martin and Kearney, 2002). A restricted repertoire that is weakly self-reactive is selected preferentially in these populations, which indicates that BCR signalling induced by self-ligands is required for the generation and maintenance of MZ and B1 cells.

Given the different anatomical locations of MZ B and B1 cells compared to MRF B cells, unique signals might affect the development of these B cell subsets. Microbial products that are recognised specifically through toll-like receptors (TLR), are sufficient to drive T2 cells into MZ B cells *in vitro*. In mice lacking the normal commensal intestinal microflora, the generation of MZ B and B1 cells is impaired (Niiro and Clark, 2002).

Although MZ and B1 B cells have many similarities, a direct developmental link has not been demonstrated. Several mouse models that have defects in B1 cells have altered development of MZ B cells. For example, the *aly* mutation, known to impair the MZ development, has been shown to inhibit B1 cell migration from the PEC to effector sites (Fagarasan et al., 2000). Mice deficient for CD19 are deficient in both B1 and MZ B cells (Martin and Kearney, 2000b; Rickert et al., 1995; Sato et al., 1996). This finding is inconsistent with the signal-strength hypothesis, as is the fact that mice lacking Aiolos, despite their hyperresponsive BCR signalling have reduced numbers of B1 cells (Wang et al., 1998).

Furthermore, Notch2$^{+/-}$ mice show a reduction in frequency and absolute number of both B1 and MZ B cells, suggesting firstly that Notch2 plays a role in determining the generation of MZ and B1 cells. Secondly this supports the notion that these two subsets share some common elements in their developmental pathways (Witt et al., 2003).

1.4.7 Plasma cells

After antigen encounter, plasma cells can develop from naïve mature B cells, from activated germinal centre B cells and from memory B cells. Which B cell subset becomes terminally differentiated depends on the nature of the antigen, its dose and form, and the location of the encounter.

Plasma cells are the sole producers of secreted antibody and are therefore crucial for an effective immune response. Numerous cell surface proteins change upon plasma cell differentiation. The expression of B220, CD19, CD21 and CD22 on the cell surface decreases, whereas Syndecan-1 (CD138) increases.

The first B cells to respond to foreign antigen by differentiating into plasma cells are MZ B cells and B1 cells (Pillai et al., 2005; Shapiro-Shelef and Calame, 2005). When B1 cells (mainly B1a) become activated in the pleural or peritoneal cavities, they migrate to the spleen or gut, lose expression of CD5 and become antibody-secreting plasma cells (Bikah et al., 1996). The unique location of the two B cell subsets facilitates early encounter with blood-borne antigens. Within a few hours of immunisation with a TI-2 antigen, MZ B cells move from the marginal zone to the bridging channel and the red pulp of the spleen, where they undergo a burst of proliferation. This occurs together with differentiation to form foci of plasmablasts, which secrete immunoglobulin but continue to proliferate (Lopes-Carvalho and Kearney, 2004). MZ B cells have a lower threshold for antigen activation than MRF B cells, and, when stimulated with LPS, proliferate to a greater extent (Oliver et al., 1997).

MRF B cells that both encounter antigen and receive help from T cells can also respond rapidly, albeit more slowly than MZ B cells. They undergo proliferation and plasmacytic differentiation to form extrafollicular foci of plasmablasts and plasma cells. Two days

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after immunisation with a T cell-dependent antigen, foci are observed along the periphery of the PALS. These foci expand until day 8 after immunisation and then diminish (Jacob et al., 1991).

Plasma cells formed from either MZ or MRF B cells in this early extrafollicular response do not have somatically mutated immunoglobulin genes and are short-lived, undergoing apoptosis *in situ* (Calame et al., 2003; Smith et al., 1996).

1.4.8   Germinal centre B cells

When MRF B cells both encounter antigen and receive T cell help, a second developmental possibility is the establishment of a germinal centre (Calame et al., 2003; Shapiro-Shelef and Calame, 2005). Germinal centres are specialised areas in the splenic follicle where B cells undergo rounds of proliferation, which is accompanied by affinity maturation and class-switch recombination of immunoglobulin.

Affinity maturation is the process that leads to increased affinity of antibodies for a particular antigen and is the result of somatic hypermutation of immunoglobulin genes, in and around the V-region of IgH and IgL genes. Somatic hypermutation is followed by selective survival of B cells producing antibody with the highest affinity (Jacobs and Bross, 2001). B cells in germinal centres can also modify their constant region by a genetic process called class-switch recombination. These B cells begin to express IgH chain classes other than μ and δ, for example γ, α or ϵ.

Antigen-specific T helper cells and follicular dendritic cells are important for the germinal centre response. Interactions between CD40-ligand and CD40, and inducible T cell co-stimulator (ICOS) and ICOS-ligand, which are expressed on T cells and B cells respectively, are important for the germinal centre response (Shapiro-Shelef and Calame, 2005). The germinal centre response peaks between day 10 and 14 after immunisation and then diminishes. Plasma cells and memory B cells, which mainly have somatically mutated, high affinity BCRs and express switched immunoglobulin isotypes, exit the germinal centre.

1.4.9 Memory B cells

Post-germinal-centre memory B cells retain high-affinity BCR at their surface, but do not secrete antibody. Memory B cells have the intrinsic ability to respond more rapidly than naïve B cells, and they show a proliferative burst on secondary encounter with antigen (Tangye et al., 2003).

1.5 B cell signalling

The B cell receptor has two main roles. The first role is to transmit signals that regulate B cell fate decisions. The second is to mediate antigen processing, leading to the presentation of antigen to T cells, which allows full activation of B cells in the effector phase (Niiro and Clark, 2002).

Signalling through the BCR is mediated by Igα and Igβ. The signalling capacity of both Igα and Igβ are dependent upon a specific motif, found within each cytosolic tail, known as the immunoreceptor tyrosine-based activation motif (ITAM) (Reth, 1989). The core of this motif (D/E(X)$_7$D/EXXYXXI/L(X)$_7$YXXI/L) comprises two tyrosine residues separated by 11 residues, each followed by leucine or isoleucine at the +3 position. Other receptors involved in antigen responses, including the TCR and many Fc receptors, also contain ITAMs (Samelson and Klausner, 1992). Mutational analysis has illustrated that the tyrosines, the 11 amino acid spacer between them and the +3 leucine/isoleucine residues, are all required for proper initiation of BCR-mediated signalling pathways (Sanchez et al., 1993).

Aspects of Igα and Igβ function in B cell development were studied in transgenic and gene-targeted mice. In Igβ knock-out mice, B cell development is completely blocked at the pro-B cell stage (Gong and Nussenzweig, 1996). Critical role of the cytoplasmic tails of Igα and Igβ were revealed in Igα$^{\Delta C/\Delta C}$ and Igβ$^{\Delta C/\Delta C}$ (Reichlin et al., 2001; Torres and Hafen, 1999). In both mutants, B cell development is severely impaired resulting in a drastic reduction of peripheral B lymphocyte numbers. Unexpectedly, Igα$^{\Delta C/\Delta C}$ yields an antigen receptor complex on immature B cells that signals constitutively, indicating a role for Igα in negatively regulating antigen receptor signaling during B cell development (Torres and Hafen, 1999).

Three families of protein tyrosine kinases (PTK) have been implicated in the earliest steps of BCR signalling. Members of the Src-family (in B cells mostly Lyn, Fyn and Blk), Syk from the Syk/Zap70-family and Btk from the Tec-family (Fig.1.3). The resting BCR is assembled with Src-family tyrosine kinases, which become activated following

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receptor ligation (Burkhardt et al., 1991; Wang and Clark, 2003). Syk can also be detected in the resting receptor complex. Upon BCR ligation the first kinases to be activated are the Src-family kinases. These in turn phosphorylate the tyrosine residues in the ITAMs of Igα and Igβ. The phosphorylated ITAMs serve as docking sites for the tandem Src-homology (SH2)-domain-containing PTK Syk, which is then activated by means of tyrosine phosphorylation by Src-family PTKs. The activation of Syk leads to the phosphorylation of several molecules, which in turn, activate signalling networks that lead to proliferation, actin cytoskeleton reorganization, gene transcription and cell motility.
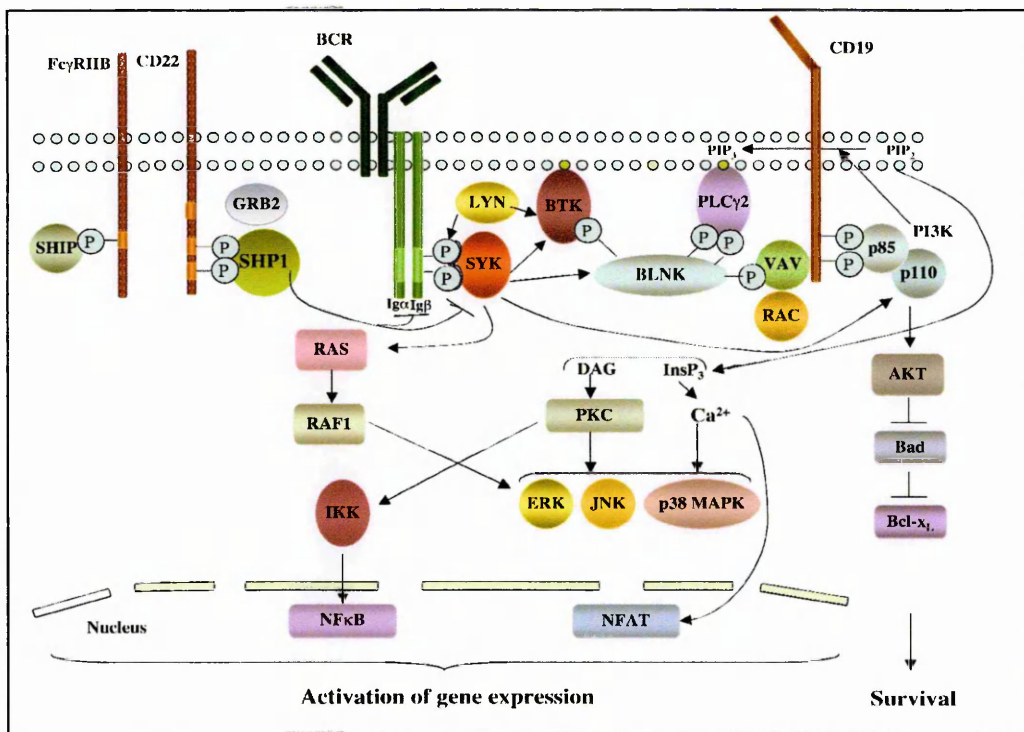
Figure 1.3.   Overview of the components of the BCR signalosome and their interactions.

Figure was adapted Niiro, et al. (Niiro and Clark, 2002)

1.5.1 Src-family kinases

The primary Src-family PTK in B cells is Lyn, although B cells also express other Src family kinases, including Fyn, Blk, Hck and Fgr. Mice deficient for individual Src-family PTKs have little effect on B cell function (Hibbs et al., 1995; Lowell and Soriano, 1996; Nishizumi et al., 1995; Stein et al., 1992; Texido et al., 2000). However, mice deficient for Blk, Fyn and Lyn, showed reduced numbers of pre-B cells followed by substantial reduction in peripheral B cell numbers (Saijo et al., 2003). Furthermore, $Blk^{-/-}Fyn^{-/-}Lyn^{-/-}$ mice have completely abrogated pre-BCR-mediated NF-κB activation. Src-family PTKs seem to be connected to NF-κB activation through the atypical protein kinase C (PKC)-λ, which is hypophosphorylated in the absence of the three Src family PTKs and if activated can bypass the requirement for Src-family PTKs in pre-BCR-mediated NF-κB activation.

Lyn is not absolutely required for phosphorylating Igα/β ITAMs. B cell development in the bone marrow of Lyn-deficient mice is normal suggesting that in the absence of Lyn this function can occur through other Src-family kinase members as mentioned before (Chan et al., 1997). While the positive role of Lyn is redundant, Lyn also plays a role as a negative regulator of BCR signalling. Its negative function was first suggested by the analysis of Lyn-deficient mice in which peripheral B cells exhibit enhanced activation of downstream effectors, intracellular Ca^{2+} flux, proliferative responses *in vitro*, and B cell hyperactivity *in vivo* that leads to increased levels of autoantibodies (Dal Porto et al., 2004). Lyn's inhibitory function depends on its ability to phosphorylate FcγRIIB and CD22, two immunoreceptor tyrosine-based inhibitory motif (ITIM) containing

inhibitory receptors, which recruit tyrosine phosphatases and suppress the BCR response, as discussed later.

1.5.2   Tec-family kinases

The Tec-family kinases consist of 5 members: Btk, Tec, Itk/Emt/Tsk, Etk/Bmx and Rlk/TXK. A crucial role for the non-receptor tyrosine kinase, Bruton's tyrosine kinase (Btk) in B cell development and function was illustrated by the B cell immunodeficiencies X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (*Xid*) in mice (Lewis et al., 2001; Satterthwaite et al., 1998). XLA is caused by loss of Btk expression or more subtle functional changes in any Btk subdomain including the pleckstrin homology (PH), Tec homology (TH), SH3, SH2 or kinase motifs. It is characterized by a block in B cell development at the pro- to pre-B cell stage. *Xid* mice, which have a point mutation in the PH domain of Btk, and Btk-deficient mice have a milder phenotype than XLA patients. *Xid* and Btk-deficient mice have decreased numbers of mature B cells and lack B1a cells. IgM and IgG3 levels are low and response to TI-2 antigens is impaired.

Activation of Btk downstream from antigen receptors requires two steps: membrane localization followed by phosphorylation by a Src-family kinase. Membrane localization occurs by virtue of the interaction of an intact PH domain of Btk with phosphatidylinositol-(3,4,5)-triphosphate (PIP_3), a product of phosphoinositide-3-kinase (PI3K). Once at the membrane, Btk can be phosphorylated at the activation loop of the kinase domain by Src-family kinases, which fulfills the second requirement for

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activation. Subsequently the SH3 domain is autophosphorylated, rendering Btk fully activated (Lewis et al., 2001; Satterthwaite et al., 1998). Analysis of mutant cells suggests that Btk is required for full activation of Phospholipase Cγ (PLCγ) (Scharenberg and Kinet, 1998).

1.5.3 Vav-family

The Vav-family of guanine-nucleotide exchange factors (GEF) consists of three structurally related Vav proteins. Vav1 is expressed primarily in cells of the heamatopoietic system, whereas Vav2 and Vav3 have broader pattern of expression (Turner and Billadeau, 2002). The DBL homology domain containing GEFs promote the activation of RHO-GTPases by catalysing the exchange of GDP for GTP. Vav1 is primarily a GEF for Rac1, Rac2 and RhoG, whereas Vav2 and Vav3 act primarily on RhoA, RhoB and RhoG GTPases (Crespo et al., 1997; Movilla and Bustelo, 1999; Schuebel et al., 1998). The recruitment of Vav proteins to activated receptors on lymphocytes and their subsequent tyrosine phosphorylation requires an intact SH2 domain. Several tyrosine kinases, including Fyn and Syk, have been shown to phosphorylate Vav (Deckert et al., 1996; Schuebel et al., 1998). Furthermore, Vav1 and Vav2 have been shown to interact, by means of their SH2 domain, with the tyrosine phosphorylated Tyr391-Glu-Glu-Pro motif in the cytoplasmic tail of CD19 (O'Rourke et al., 1998). It is thought that CD19 activates Vav-family members through the recruitment of Lyn. The significance of Vav binding to CD19 is highlighted by the observation that CD19 signal transduction is less efficient in B cells that lack Vav1, and

that the Tyr391Phe mutation of CD19 abolishes both Vav1 binding and CD19-regulated responses (Fujimoto et al., 2000; O'Rourke et al., 1998).

Studies of $Vav1^{-/-}Vav2^{-/-}$ mice showed clearly that Vav proteins have a crucial role in B cell signalling. In $Vav1^{-/-}$, $Vav2^{-/-}$ and $Vav1^{-/-}Vav2^{-/-}$ mice, normal numbers of immature B cells are found in the bone marrow, which indicates that the early developmental transitions occur independent of these proteins. However, $Vav1^{-/-}Vav2^{-/-}$ immature B cells fail to complete their maturation into recirculating B cells. They remain immature expressing high levels of IgM and low levels of IgD (Doody et al., 2001; Tedford et al., 2001). Furthermore, in $Vav1^{-/-}$, $Vav2^{-/-}$ and $Vav1^{-/-}Vav2^{-/-}$ mice, peripheral B cells are impaired in their *in vitro* proliferative and Ca^{2+} responses after BCR engagement, which provides evidence for the role of Vav proteins in the regulation of Ca^{2+} mobilization. A target of Vav1, Rac1 has been indicated to play a role in the activation of phosphatidylinositol-4-phosphate 5-kinase (PIP5K) and therefore regulating the level of PIP_2, the substrate for PLCγ proteins (O'Rourke et al., 1998; Tolias et al., 1995). Interestingly, mice deficient for Rac1 and Rac2 GTPases, the substrates of Vav1, have defective BCR signalling and B cell development is arrested at the immature-T1 transition (Walmsley et al., 2003).

1.5.4   Phosphoinositide-3-kinase (PI3K)

PI3Ks are a family of proteins that regulate diverse biological functions in every cell type by generating lipid second messengers. The PI3K family can be subdivided into three classes, class I, class II and class III. Whereas little is known about the role of class

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II and class III PI3Ks in lymphocytes, class I PI3K are well characterised (Okkenhaug and Vanhaesebroeck, 2003). Class I PI3Ks are further subdivided into class IA and class IB. Class IA PI3Ks are activated by tyrosine kinase-associated receptors, such as antigen receptors and co-stimulatory receptors (e.g CD19). Upon BCR stimulation, the co-receptor CD19 is phosphorylated on tyrosine residues and provides binding sites for PI3Ks, which are important for activation of the enzyme. Class IA PI3Ks are heterodimeric enzymes consisting of a regulatory subunit (p85α, p85β or p55γ) and a catalytic domain (p110α, p110β or p110δ). Class IB PI3Ks are activated by G-protein-coupled receptors. The function of class I PI3Ks is to convert phosphatidylinositol-(4,5)-bisphosphate (PIP$_2$) to phosphatidylinositol-(3,4,5)-trisphosphate (PIP$_3$) at the inner leaflet of the plasma membrane. PIP$_3$ acts as a binding site for numerous intracellular proteins that contain PH domains with selectivity for this lipid, such as Btk and the serine/threonine kinase Akt.

Knock-out studies have shown that B cell development is partially blocked at the pro-B to pre-B cell transition in mice that lack the catalytic domain p110δ or the regulatory subunit p85α (Clayton et al., 2002; Koyasu, 2003). Furthermore, *p110$\delta^{-/-}$* mice have reduced numbers of MZ B cells and B1 cells and an impaired antibody production in response to TD and TI antigens. In addition, the splenic B cells of mice deficient in p110δ or p85α undergo poor proliferative responses following stimulation at the BCR indicating that the activation of PI3K is required for BCR-driven proliferation (Clayton et al., 2002).

The key downstream signal of PI3K is Akt, which is recruited by PIP$_3$ to the plasma membrane via its PH domain. Akt is a serine/threonine protein kinase that promotes cell

survival by a variety of mechanisms including phosphorylating and inhibiting pro-apoptotic proteins such as Bad (Brazil et al., 2002).

1.5.5 Phospholipase Cγ (PLCγ)

Two PLCγ isoforms are expressed in B cells, PLCγ1 and PLCγ2, with the latter generally being most abundant. Both isoforms appear to participate in BCR signal transduction, however it is not clear whether they play distinct functional roles (Marshall et al., 2000). Upon BCR ligation, Syk associates and phosphorylates B-linker protein (BLNK), leading to binding of PLCγ through its SH2 domain. This brings PLCγ into proximity with Syk, leading to its phosphorylation and partial activation (Fu et al., 1998; Kurosaki and Tsukada, 2000). Btk is recruited to the membrane by binding to PIP_3 generated by PI3K, and becomes tyrosine phosphorylated, probably by Lyn. Activated Btk then may also phosphorylate PLCγ leading to its full activation (Rodriguez et al., 2001). Alternatively the adapter protein BLNK, phosphorylated by Syk, provides docking sites for Btk as well as PLCγ2, thus bringing Btk into close proximity with PLCγ2. The activated Btk then phosphorylates PLCγ2, which leads to its full activation (Hashimoto et al., 1999). PLCγ hydrolyzes, PIP_2, to produce soluble inositol-1,4,5-trisphosphate (IP_3) and membrane-anchored diacylglycerol (DAG). IP_3 can then bind to IP_3 receptors on the endoplamatic reticulum and induces the release of calcium from intracellular stores. The elevation of cytosolic calcium levels activates downstream signalling molecules, including calcineurin and conventional protein kinase C (PKC) isoforms (α, β, γ). By contrast, DAG activates all phorbol ester-sensitve isoforms of

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PKC (α, β, γ, δ, η, ε, θ), as well as other proteins such as the Ras guanine nucleotide releasing protein (RasGRP) family of Ras GEFs. Calcium flux and PKC activation induce activation transcription factors, including nuclear factor-κB (NF-κB) and nuclear factor of activated T cells (NF-AT).

1.5.6   Erk MAPK pathway

Erk MAPK activation in B cells has been associated with a variety of cellular responses as diverse as proliferation and apoptosis. The Erk MAPK pathway is activated primarily via the adaptor protein Grb2, which can associate with the BCR complex via Shc or BLNK (Harnett et al., 2005). The SH3 domains of Grb2 bind to guanine-nucleotide exchange factor SOS (son of sevenless), which activates the small GTPase Ras by exchanging GDP for GTP. Ras can also be activated by RasGRPs (Oh-hora et al., 2003). Ras-GTP can bind and activate serine/threonine protein kinase Raf1, leading to the stimulation of the dual-specificity protein kinases MEK1 and MEK2, which activate Erk by phosphorylating the threonine-glutamate-tyrosine (TEY) motif of its activation loop.

1.5.7   NF-κB pathway

NF-κB, a family of dimeric transcription factors, has been shown to protect many cells from pro-apoptotic signals (Barkett and Gilmore, 1999). In mice that are deficient for inhibitor of NF-κB (IκB) kinase-α (IKKα), IKKβ and the regulatory subunit NF-κB

essential modulator (NEMO), which are upstream molecules of NF-κB activation, B cell apoptosis is increased markedly (Kaisho et al., 2001; Pasparakis et al., 2002). PLC-γ2-induced Ca^{2+} flux and PKC activation are both required for NF-κB activation, which indicates that the activation of calcium-dependent PKC isoforms might regulate the activation of NF-κB. And indeed, it has been shown that PKCβ is required for activation of IKKα/β (Saijo et al., 2002). NF-κB induces expression of anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-x_L and A1. These molecules counteract the effect of pro-apoptotic Bcl2 family members, thereby protecting cells from BCR-induced cell death. Bcl-x_L was reported to block expression of IP_3-receptor in a B cell line, which indicates that Bcl-x_L might also regulate BCR-induced cell death by inhibiting the release of calcium through the IP_3-receptor (Li et al., 2002).

1.5.8 Positive modulator: CD19

Although Igα/β are the primary signal transducers for BCR complexes, the signal quality and quantity can be significantly influenced by co-receptors.

BCR-associated CD19 molecules positively contribute to Igα/β signalling by enhancing the recruitment and activation of Lyn, PI3K, Btk and Vav (Fujimoto et al., 2000). Genetic ablation of CD19 has revealed a role for this molecule in B cell development as well as the importance of CD19 to the B cell response to TD antigens (Rickert et al., 1995). Studies in mice expressing CD19 molecules incapable of binding to PI3K exhibit a phenotype identical to that of mice deficient in CD19, indicating that an essential contribution of CD19 to B cell responses is the activation of PI3K (Wang et al., 2002).

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However, the CD19 tyrosines mutated in these mice are also required for amplification of Lyn activity following BCR ligation (Fujimoto et al., 2000).

CD19 is recruited to the BCR via two mechanisms. First, CD19 form a complex with the tetraspanin molecule CD81 and the receptor for the C3d component of complement, CD21 (Cr2). This complex can then associate with the BCR through the co-recognition of antigen, which have affixed complement (Fearon and Carter, 1995). The second mechanism is a direct association of CD19 with the BCR, and is supported by the finding that CD19 molecules become phosphorylated and transduce signals upon ligation of surface BCR alone (Carter et al., 1997).

1.5.9 Negative modulator: CD22

CD22 is a sialic acid-binding immunoglobulin-like lectin superfamily member that binds α2,6-linked sialic acid residues and is tyrosine-phosphorylated upon BCR ligation (Dal Porto et al., 2004). CD22 contains three ITIMs (Immunoreceptor Tyrosine-based Inhibitory motifs) in its cytoplasmic domain, which are phosphorylated by Lyn upon BCR ligation. CD22-deficient mice display increased sensitivity to BCR aggregation, as measured by calcium mobilization and proliferation and have a reduction of MZ B cells (Nitschke et al., 1997; Samardzic et al., 2002). CD22 exerts its negative effect in part by recruiting SH2-containg tyrosine phosphatase-1 (SHP-1). Possible SHP-1 substrates include Igα, Igβ, Syk, Vav and BLNK (Mizuno et al., 2000).

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1.5.10 Negative modulator: FcγRIIB

The low affinity receptor for IgG, FcγRIIB, is a potent transducer of signals that block antigen-induced B cell activation. Coligation of FcγRIIB with BCR causes premature termination of phosphoinositide hydrolysis and calcium mobilization and inhibits proliferation. Similar to CD22, the inhibitory signal transduction by FcγRIIB is mediated primarily by its ITIM region, phosphorylated in a Lyn-dependent fashion. Tyrosine phosphorylation of FcγRIIB recruits the SH2-containing inositol 5'-phosphatase (SHIP), which prevents PIP_3 accumulation, by hydrolysing PIP_3 to PIP_2.

1.6 The role of Syk-family kinases in B cell signalling

1.6.1 Structure of Syk-family tyrosine kinases

The Syk-family of tyrosine kinases consists of two members: Syk and Zap70. These kinases contain a C-terminal kinase domain and tandem N-terminal SH2 domains that bind phosphorylated ITAMs (Fig.1.4) (Turner et al., 2000). A linker region, designated interdomain B that contains multiple tyrosines separates SH2 domains from the kinase domain. These tyrosines, once phosphorylated act as docking sites for proteins such as PLCγ1 and 2 (Law et al., 1996; Takata and Kurosaki, 1996) and the Vav family of GEFs (Deckert et al., 1996), which might be substrates for Syk and Zap70.

Though similar in overall structure, there are important differences between Syk and Zap70. In particular, the most abundant form of Syk contains a 23 amino acid insert in the linker region, which is not found in Zap70. A less abundant, alternatively spliced form of Syk, SykB, does not contain this insert and is thus more similar to Zap70. Experiments have shown functional consequences of this difference. Syk couples the FcεRI receptor of mast cells to downstream intracellular signalling pathways much more efficiently than SykB (Latour et al., 1998). This difference correlates with a higher binding affinity of Syk to phosphorylated ITAMs compared to SykB. According to these findings, Zap70, which also lacks the linker region, behaved much more like SykB. It was inefficient at coupling FcεRI signalling and bound less avidly to phosphorylated ITAMs than Syk.
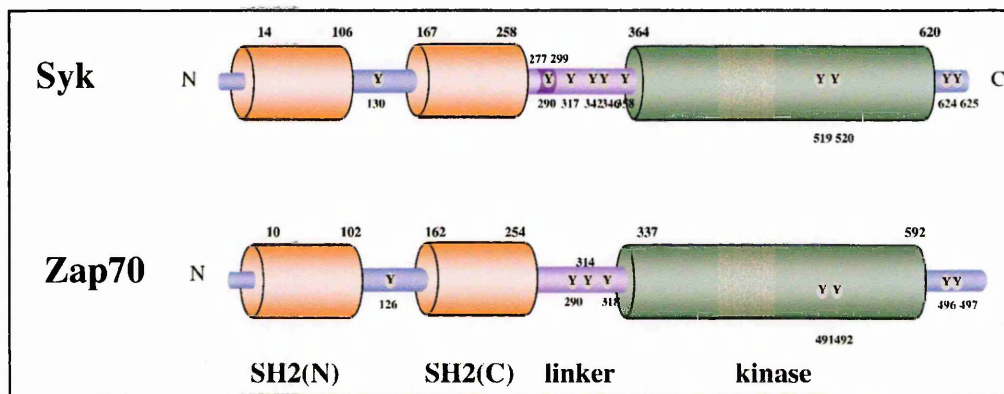
Figure 1.4. Structure of Syk and Zap70

The tandem SH2 domains are shown as orange cylinders and the kinase domain in green. Interdomain A connecting SH2-SH2 is shown in a blue tube, and Interdomain B connecting SH2-kinase domain is shown in purple. The tyrosines, which have been shown to undergo phosphorylation are indicated. Numbers indicate beginning and ends of domains and positions of tyrosine residues. Syk contains a 23 amino acid insert in its linker domain, which is missing in Zap70. Figure was adapted from a review (Turner et al., 2000).

Many tyrosines have been shown to undergo phosphorylation and may be important in regulation of enzymatic activity or recruitment of other signalling proteins. Phosphorylation of Y317, Y342 and Y346 in Syk is dependent on Lyn, whereas phosphorylation of Y130, Y519 and Y520 is dependent on Syk itself and thus these may be sites of auto-phosphorylation. Tyrosines 518 and 519 were found to be major autophosphorylation sites on Syk and *in vivo* they are important for signal propagation (Kurosaki et al., 1995). Since Syk with mutated tyrosines 518 and 519 is still almost fully kinase-active, these sites seemed to be more important for protein-protein interaction, e.g. Src-family kinase or PLCγ2 binding (Couture et al., 1997). Tyrosines 342 and 346 within the linker region of Syk are important for interaction with Vav1 (Deckert et al., 1996). The phosphorylation of Y342 and Y346 enhances the phosphorylation and activation of PLCγ and calcium release. However, phosphorylation of Y317 seems to play an inhibitory role, because in cells lacking Lyn, the phosphorylation of Y317 is suppressed leading to elevated production of IP_3 and amplified calcium signal (Hong et al., 2002). Furthermore, Y317 is a putative Cbl binding site (Deckert et al., 1998). In addition, phosphorylated Y130 causes conformational changes in the inter-SH2 domain, altering both SH2-SH2 interface interactions and the interactions between the inter-SH2 region and the kinase active site. Phosphorylation of Y130 negatively influences the binding of Syk to the antigen receptor and at the same time allows increased access of the catalylic site to protein substrates (Keshvara et al., 1997)

Zap70 has homologous tyrosines, although Y290 and Y358 are missing. Among the best-studied phosphorylation sites in Zap70 are Y492 and Y493, analogous to Y519 and Y520 in Syk. A model has been proposed, whereby Y493 becomes phosphoryated by

the Src-family PTK Lck. This phosphorylation is then followed by auto-phosphorylation at Y492. Y292 in the interdomain B, analogous to Y317 in Syk, is proposed to play a negative regulatory function by recruiting the E3 ubiquitin ligase c-Cbl. Y315 and Y319 have been proposed to be positive regulatory sites and have been demonstrated to be phosphorylated upon TCR-crosslinking, either by Zap70 itself or by Lck (Brdicka et al., 2005).

Several studies suggest that Syk is less dependent upon Src-family kinases for its function than Zap70. T cell hybrids expressing a CD16-Zap70 chimeric receptor required co-crosslinking of the receptor with Src-family kinases Fyn and Lck to activate their cytotoxic potential. By contrast, crosslinking of CD16-Syk alone activated cytotoxicity (Kolanus et al., 1993). Furthermore, Jurkat T cells that lack Lck or the tyrosine phosphatase CD45, which is implicated in the activation of Lck, show defective TCR-induced Zap70 activation and no TCR-induced gene expression (Chu et al., 1996). Interestingly, introduction of Syk into either mutant line restores TCR-mediated signalling. In a related experiment, Zap70 expressed in COS cells required co-expression of Lck or Fyn for optimal activity (Chan et al., 1992). By contrast, Syk was phosphorylated when expressed by itself in COS cells, although this phosphorylation was strongly enhanced by co-expression of Src-family kinases (Kurosaki et al., 1994). Furthermore, expression of Lyn, BCR and Syk in Drosophila S2 Schneider cells revealed that Syk can interact with the BCR independently of Lyn (Rolli et al., 2002). Taken together, these results suggest that the activation of Zap70 by immune receptors requires Src-family PTKs, whereas Syk can function in a Src-family kinase-independent

manner. This difference might be due to the ability of Syk, but not Zap70, to induce

phosphorylation of ITAMs independently of Src-family kinases (Zoller et al., 1997).

1.6.2 Syk and Zap70 in αβ T cells

Syk was initially characterized as an abundant PTK in thymus and spleen. During T cell

development, Syk is expressed by DN and DP thymocytes, but expression levels are

significantly reduced in CD4$^+$ and CD8$^+$ SP thymocytes and peripheral T cells (Chan et

al., 1994). By contrast, Zap70 is expressed throughout thymocyte development and at

high levels in pheripheral T cells (Chan et al., 1994). The different expression profile of

Syk and Zap70 suggests that these two tyrosine kinases play distinct roles in T cell

development. In radiation chimeras reconstituted with Syk-deficient fetal liver cells,

mature CD4$^+$ and CD8$^+$ T cells bearing αβ-TCRs develop in normal numbers and

proliferate normally *in vitro* to TCR-signals. By contrast, Zap70-deficient mice display

a profound block in T cell development at the DP to SP thymocyte transition, Zap70

appears to be necessary for positive and negative selection of T cells (Chan et al., 1994;

Negishi et al., 1995).

Forced overexpression of Syk in the thymi of *Zap70$^{-/-}$* mice rescues the block in positive

selection and restores peripheral T cell numbers to normal levels, showing that

overexpression of Syk can compensate for the lack of Zap70 in T cell development

(Gong et al., 1997). However in wild-type mice it appears that only Zap70 transduces

these positive selection signals because Syk is not expressed at sufficiently high levels

(Chu et al., 1999).

Mice deficient for either Syk or Zap70 develop DP thymocytes, however, in mice bred to be deficient for both Syk and Zap70, development was arrested at the DN stage (Cheng et al., 1997). This suggests that although Syk is not essential for TCR-mediated positive selection, it does play a role in pre-TCR signalling during the maturation of DN to DP thymocytes.

1.6.3 Syk and Zap70 in γδ T cells

Syk-deficient mice do not have a defect in αβ T cell development, but show a deficit in specific populations of γδ T cells, namely the dendritic epidermal γδ T cells of the skin and the intraepithelial γδ lymphocytes of the intestine (Mallick-Wood et al., 1996; Turner et al., 1995). A similar defect in intraepithelial γδ lymphocytes development is also seen in Zap70-deficient mice (Gong et al., 1997; Kadlecek et al., 1998). Although normal numbers of γδ T cells are found in the spleen and lymph nodes of $Zap70^{-/-}$ mice, dendritic epidermal γδ T cells are decrease in numbers and show an abnormal morphology in these animals. Thus, in certain γδ T cell subpopulations, Syk and Zap70 cannot fully compensate for each other in case of a deficiency of either kinase. Notably, expression of a Syk transgene in Zap70-deficient mice did not restore development of the intestinal intraepithelial γδ T cells (Gong et al., 1997).

1.6.4 Syk and Zap70 in natural killer (NK) cells

NK cells are BM-derived lymphocytes that do not express rearranged TCRs or BCRs. NK cells mediate cytotoxic killing without prior sensitization. Evidence that Syk is not required for the differentiation of NK cells comes from studies of $Rag2^{-/-}/\gamma_c^{-/-}$ radiation chimeras reconstituted with $Syk^{-/-}$ fetal liver. $Rag2^{-/-}/\gamma_c^{-/-}$ host are completely depleted of all mature lymphoid cells, including NK cells, and their progenitors (Colucci et al., 1999a). Phenotypically mature $Syk^{-/-}$ NK cells develop naturally and demonstrate normal natural killing (Colucci et al., 1999b). However, NK cells lacking Syk are partially defective in antibody-dependent cell mediated cytotoxicity. In contrast, NK cells from $Zap70^{-/-}$ mice kill NK-susceptible targets and demonstrate normal antibody-dependent cell mediated cytotoxicity (Negishi et al., 1995). It is likely that Syk and Zap70 are functionally redundant in NK cell development and peripheral NK cell function. However, in mice deficient in both kinases, NK cells still develop normally and Syk and Zap70 seem to be dispensable for natural cytotoxicity, although they seem to be essential for triggering NK cells via ITAM-bearing receptors (CD16 and Ly49D), because crosslinking of these receptors in the absence of both kinases leads to decreased cytotoxicity (Colucci et al., 2002).

1.6.5   Syk and Zap70 in myeloid cells

Activation of mast cells by antigen-IgE complexes stimulates the release of inflammatory mediators in form of secretory granule contents, cytokines and arachidonic acid metabolites. The receptor for antigen-IgE complexes, FcεRI, contains an IgE binding α subunit complexed with a β chain and a dimer of γ chains that each contains

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an ITAM. The γ chain of FcεRI (FcRγ) binds Syk, whereas the ITAM of the β chain

binds Lyn (Lin et al., 1996). Stimulation of leukotriene production and granule release

was inhibited in permeabilized rat basophilic leukaemia (RBL-2H3) cells by a mutant

form of Syk, consisting only of the SH2 domains (Taylor et al., 1995). The development

of a variant of RBL-2H3 cells lacking Syk further demonstrates that Syk is essential for

degranulation. In the absence of Syk, the antigen-receptor could not activate PLCγ and

hence intracellular calcium release (Zhang et al., 1996). Studies using murine mast cell

cultures established from $Syk^{-/-}$ fetal livers further demonstrated that degranulation,

leukotriene production and cytokine release were all dependent on Syk, as was NF-AT

activation and stimulation of ERK and JNK protein kinase pathways (Costello et al.,

1996).

Syk also associates with the tyrosine phosphorylated FcRγ chain of FcγRI and FcγRIII.

Reconstitution experiments using COS cells indicate that the FcRγ chain and Syk are

essential for FcγR-mediated phagocytosis, because co-transfection of Syk and FcγRI into

COS cells results in enhanced phagocytosis (Indik et al., 1995). Furthermore, clustering

of a Syk-FcγRIII fusion protein in COS cells leads to phagocytosis that is abrogated by a

point mutation in the kinase domain of Syk (Greenberg et al., 1996). In addition to these

findings, macrophages cultured from $Syk^{-/-}$ fetal liver are defective in their ability to

phagocytose antibody-coated sheep red blood cells, but retain the ability to phagocytose

latex beads, bacteria and complement-coated zymosan (Kiefer et al., 1998).

Syk is also an essential component of integrin signalling in neutrophils. It has been

reported that $Syk^{-/-}$ neutrophils fail to undergo respiratory burst, degranulation, or

spreading in response to pro-inflammatory stimuli while adherent to immobilised

ligands or when stimulated by direct crosslinking of integrins (Mocsai et al., 2002). Furthermore, signalling from the β1, β2 and β3 integrins, which leads to activation of neutrophil effector functions, is also defective in $Syk^{-/-}$ neutrophils.

Syk is also involved in differentiation and function of osteoclasts. When cultured in the presence of receptor activator of NF-κB-ligand (RANK-L), a tumor necrosis factor-related cytokine, wild-type bone marrow cells form large multi-nucleated osteoclasts, which are able to resorb bone *in vitro* (Teitelbaum, 2000). However, $Syk^{-/-}$ osteoclasts do not go through such a morphological differentiation and they also fail to resorb bone (Mocsai et al., 2004).

Furthermore, Syk is required for monocyte/macrophage chemotaxis to CX3CL1, which is the only member of the δ/CX3C subclass of chemokines. It has been reported that treatment of monocyte/macrophage RAW cell line with CX3CL1 elicits a rapid and transient increase in F-actin and the formation of F-actin-enriched cell protrusions (Gevrey et al., 2005). CX3CL1 also triggers tyrosine phosphorylation of proteins localised in this protrusion. Syk is activated upon CX3CL1 treatment, and reduction of Syk expression using RNA-mediated interference results in an impairment of RAW cell migration to CX3CL1 (Gevrey et al., 2005). In contrast, Zap70 was not found to be expressed in myeloid cells.

1.6.6   Syk and Zap70 in platelets

Analysis of $Syk^{-/-}$ platelets has allowed a direct assessment of the role of Syk in signalling from a variety of receptors. Signalling through GPVI collagen receptor was shown to require Syk, because $Syk^{-/-}$ platelets fail to respond to collagen (Poole et al., 1997). In contrast, signalling by the thrombin receptor, a G-protein coupled receptor, appears to be intact in $Syk^{-/-}$ platelets. The absence of Syk had no effect on fibrinogen-induced signalling from the integrin $\alpha_{IIb}\beta_3$ (Law et al., 1999).

The most striking feature of Syk deficiency in mice was the lethal phenotype associated with petechiae, diffuse haemorrhage and chylous ascites (Cheng et al., 1995; Turner et al., 1995). Interestingly, the bleeding phenotype is transferable when Syk-deficient bone marrow is transplanted into wild-type mice suggesting that it is due to a defect in haematopoietic cells (Poole et al., 1997). The haemorrhagic diathesis could be a consequence of platelet dysfunction, but classical tests of platelet function such as bleeding time are normal in radiation chimeras reconstituted with $Syk^{-/-}$ platelets and mice deficient for platelets do not show this fetal haemorrhaging (Poole et al., 1997). More recently the reason for the haemorrhaging phenotype in $Syk^{-/-}$ embryos was found to be a failure to separate emerging lymphatic vessels from blood vessels (Abtahian et al., 2003).

1.6.7   Syk and Zap70 in B cell development

Radiation chimeras reconstituted with $Syk^{-/-}$ fetal liver show a partial block in B cell development at the pro-B to pre-B cell transition. A few immature B cells develop, but there is a second complete block at the immature to mature B cell transition. In contrast,

Zap70^{-/-} mice develop mature B cells (Negishi et al., 1995). Although a very small number of bone marrow-derived immature *Syk^{-/-}* B cells migrate to the red pulp and the outer T zone of the spleen, they fail to enter B cell follicles (Turner et al., 1997). Furthermore, they are completely blocked in their ability to mature into MRF B cells. Reconstituting radiation chimeras with *Syk^{-/-}* fetal liver containing a 3-83μδ BCR transgene, revealed that B cell development was also completely blocked at the immature B cell stage suggesting that this defect is due to the role of Syk in the B cells themselves. However, radiation chimeras reconstituted with *Syk^{-/-}* fetal liver containing a 3-83μδ BCR transgene on a deleting background showed that Syk-deficient and Syk-sufficient chimeras had very few 3-83$^+$ B cells in the bone marrow and no 3-83$^+$ B cells in the spleen or lymph nodes, indicating that negative selection proceeds even in the absence of Syk (Meade et al., 2004).

Radiation chimeras reconstituted with *Syk^{-/-}Zap70^{-/-}* fetal liver show a complete block of B cell development at the pro-B to pre-B cell transition, indicating that Zap70 plays a role in B cell development at this first checkpoint (Schweighoffer et al., 2003). In addition, radiation chimeras reconstituted with *Syk^{-/-}Zap70^{-/-}* fetal liver containing a 3-83μδ BCR transgene, demonstrated that in the absence of both kinases, B lineage cells do not shut off heavy chain rearrangement despite the presence of a rearranged BCR, resulting in the generation of cells expressing two different functional heavy chains, thus revealing a failure of heavy chain exclusion (Schweighoffer et al., 2003). Furthermore, chimeras reconstituted with *Syk^{-/-}* fetal liver containing a 3-83μδ BCR transgene showed low levels of endogenous heavy chain rearrangement and therefore a partial failure of allelic exclusion. This finding revealed that Syk and Zap70 play a role in heavy chain

allelic exclusion. In addition, Syk is also necessary for light chain allelic exclusion, as in radiation chimeras reconstituted with $Syk^{-/-}$ fetal liver containing a 3-83$\mu\delta$ BCR transgene on a deleting and non-deleting background the frequency of λ-expressing cells was dramatically increased when Bcl-2 was overexpressed (Meade et al., 2004). Furthermore, Zap70 expression was shown in pro-B cells, pre-B cells and splenic B cells in the mouse. Zap70 was also reported to be expressed in splenic and tosillar human B cells, and it was found to be phosphorylated upon BCR stimulation (Nolz et al., 2005).

1.6.8 Zap70 in B cell chronic lymphocytic leukaemia (B-CLL)

Zap70 is also expressed in some B-CLL. B-CLL cells can be segregated into two major subsets based upon the mutational status of the expressed IgH chain variable region (IgV_H) (Chen et al., 2005; Chen et al., 2002; Fais et al., 1998; Hamblin et al., 1999). Patients with B-CLL cells that have unmutated IgV_H tend to have a relatively aggressive clinical course. Patients rapidly need intensive treatment and their median survival after diagnosis ranges between 79 and 119 months (Wiestner et al., 2003). On the other hand, patients, who have B-CLL cells that have rearranged IgV_H genes with somatic mutations have a less aggressive disease. Patients might not need therapeutic interventions at all and their median survival after diagnosis reaches 293 months (Oscier et al., 2002; Wiestner et al., 2003). Microarray-based gene expression analysis has shown that Zap70 is the gene whose expression best distinguishes the B-CLL subtypes. Zap70 expression was 5.54-fold higher in unmutated IgV_H B-CLL cells than in mutated IgV_H B-CLL cells and distinguishes the subtypes with a statistical significance of $P<10^{-21}$ (Wiestner et al.,

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2003). The existence of these B-CLL subsets, differing in whether or not their IgV_H genes are mutated and in the expression of Zap70, suggests that the may have different origins. B-CLL cells expressing unmutated IgV_H genes may originiate from antigen-inexperienced B cells, whereas B-CLL cells expressing mutated IgV_H genes may come from antigen-experienced B cells. Furthermore, it is interesting that unmutated IgV_H B-CLL cells show a higher degree of tyrosine phosphorylation than IgV_H B-CLL cells, and Zap70 can associate with the BCR in unmutated IgV_H B-CLL cells (Chen et al., 2005; Chen et al., 2002). Further work will be needed to define the functional role of Zap70 in B-CLL *in vivo*.

1.7 Aims of the work in this thesis

I wanted to further understand the role of Syk-family of PTKs in B cell development and function. It has been shown that Zap70 is expressed in B cells, and not only in T cells and NK cells. I wanted to investigate whether Zap70 is expressed differentially in distinct developing and mature B cell subsets and to study the role of Zap70 in B cell development, by analysing B cell populations in the lymphoid organs in *Zap70*$^{-/-}$ mice. In addition, I analysed B cell function, by investigating whether *Zap70*$^{-/-}$ B cells were able to mount TD- and TI-immune responses and whether BCR-induced calcium release was normal in *Zap70*$^{-/-}$ B cell subsets. Furthermore, I wanted to explore whether Zap70 and Syk perform similarly during B cell development or whether these two kinases have distinct functions, by overexpressing Zap70 in *Syk*$^{-/-}$ fetal liver cells to see whether they were able to develop into mature B cells. Finally, I was interested in the role of Syk in mature B cell development and tried to establish a model, with an inducible form of Syk that would allow this question to be addressed.

2. Materials and Methods

2.1 Solutions and media

TE	10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0
ACK lysis buffer	0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4
FACS buffer	PBS, 0.1 % Sodium Azide, 0.5 % BSA

For Western blotting:

4 x Lower Tris Buffer	1.5 M Tris-HCl, 0.4 % SDS, pH 8.8
4 x Upper Tris Buffer	0.5 M Tris-HCl, 0.4 % SDS, pH 6.8
10 x Running Buffer	3.03 M Tris-HCl, 1 % SDS, 14.42 % Glycine, pH 8.3
2 x RSB	20 % glycerol, 6 % SDS, 1x Upper Tris Buffer, 0.005% bromophenol blue
10 % polyacrylamide gel	10 % Acrylamide/Bis-acrylamide, 1x Lower Tris Buffer, 0.033 % ammonium-persulphate, 0.066 % TEMED
Stacking gel	3.75 % Acrylamide/Bis-acrylamide, 1x Upper Tris Buffer, 0.05 % ammonium-persulphate, 0.1 % TEMED
CAPS buffer	2.21 % CAPS (3-[cyclohexylamino]-1-propanesulphonic acid) in H_2O, pH 11.0
Stripping buffer	62.5 mM Tris.Cl, pH 6.7, 100mM 2-mercaptoethanol,

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2 % SDS

Blot wash, ELISA wash	0.05 % Tween-20 in PBS

For Southern blotting:

50 x TAE	40 mM Tris-acetate, 2 mM $Na_2EDTA.2H_2O$
20 x SSC	3 M NaCl, 0.3 M Na_3citrate.$2H_2O$, pH 7.0
Denaturing Solution	0.5 M NaOH, 1.5 M NaCl
Pre/Hybridization solution	0.2 M $NaPO_4$, 1 mM EDTA, 1 % BSA, 7 % SDS, 15 % formamide
DT40 medium	RPMI-1640 (Sigma), 10% heat inactivated FCS, 2% heat inactivated chicken serum, 100 U/ml Penicillin, 0.1mg/ml Streptomycin, 2mM glutamine, 50μM 2-Mercaptoethanol
PlatE / HSC medium	DMEM (Life Technology), 10% FCS, 100U/ml Penicillin, 0.1mg/ml Streptomycin, 1x non-essential amino acids, 5μg/ml Glutamine, 2mM glutamine, 50μM 2-Mercaptoethanol
Lockes Buffer	1.54M NaCl, 56mM KCl, 22mM $CaCl_2$, 10mM $MgCl_2$, 60mM $NaHCO_3$, 100mM Glucose, 20mM HEPES; pH7.4
NP40-solution	1% NP-40, 150mM NaCl, 50mM Tris

ELISA

coating buffer	50mM $NaHCO_3$
blocking buffer	PBS, 3 % BSA

ABTS substrate   0.3mg/ml ABTS in 0.1M citric acid, pH 4.35

2.2 Antibodies

Company	Antibody	Clone/Cat.Number
Becton-Dickinson	B220-CyChr	RA3-6B2
Becton-Dickinson	CD4-PerCP	L3T4, RM4-5
Becton-Dickinson	CD8-PerCP	Ly-2, 53-67
Becton-Dickinson	TCR-PE	H57-597
Becton-Dickinson	IgM-APC	11/41
Becton-Dickinson	IgD-FITC	11-26
Becton-Dickinson	CD2-PE	RM2-5
Becton-Dickinson	Ly5.1/CD45.1-bio	A20
Becton-Dickinson	Ly5.2/CD45.2-bio	104
Becton-Dickinson	Ly9.1/CD229.1-bio	30C7
Becton-Dickinson	Streptavidin-PerCP	554064
Becton-Dickinson	Streptavidin-PE	554061
Becton-Dickinson	huCD2-PE	555325
Becton-Dickinson	CD23-FITC	B3B4
Becton-Dickinson	CD21-PE	7G6
Becton-Dickinson	Ter119-bio	Ly-76

Becton-Dickinson	CD5-FITC	53-7.3
Caltag	B220-APC	RA3-6B2
Caltag	CD19-Tricolour	RM7706
Caltag	CD4-PETR	MCD0417
Caltag	B220-PETR	RM2617
eBiosciences	Mac1/CD11b-bio	MI/70
eBiosciences	AA4.1-APC	AA4.1
eBiosciences	Zap70-PE	1E7.2
Stratech	IgM-F(ab')$_2$-FITC	
Stratech	F(ab')$_2$ fragment Goat α-mouse IgM	115-006-075
Stratech	Goat anti-mouse IgG+IgM (H+L)	115-005-044
Transduction Lab.	Zap70	Z24820
DAKO	rabbit-α-mouse-Ig-HRP	PO260
Amersham Biosciences	Protein-A-HRP	NA9120V

For ELISA:

SouthernBiotech	Mouse Standard Panel kit	5300-01
SouthernBiotech	Clonotyping System-HRP-kit	5300-05

24G2, anti-FcR hybridoma was a gift from G.Stockinger.

M4, mouse anti-chicken IgM was a gift from T.Kurosaki.

2.3 Mice

Transgenic and gene targeted mutant mice were bred and maintained in the animal facilities of the National Institute for Medical Research according to Home Office regulations. *Syk*-mutant mice (Syk$^{tm1Tyb/tm1Tyb}$) were generated in our laboratory (Turner et al., 1995), *Rag-1* knock-out mice were provided by D. Baltimore (Spanopoulou et al., 1994), *Zap70*$^{-/-}$ mice by Art Weiss (Kadlecek et al., 1998), TCRα$^{-/-}$ mice (Mombaerts et al., 1992) by Gitta Stockinger and μMT mice (Kitamura et al., 1991) by Jean Langhorne. 129/Sv, B10.D2/o, BALB/c, C57BL/10, C57BL/6J, C57BL/6SJL-Ly5.1 and CBA/Ca were provided by the SPF (specific pathogen free) facility of the Institute.

2.4 Cloning pApuro-N-ER^{T2}

First a BamHI site was introduced into Syk between amino acids 8 and 9 using polymerase chain reaction (PCR). The primers used for this purpose were:

1. CCCAAGCTTGAATTCAGGAAGGTACTTCTCCAT

2. CGCGGATCCGCTGTCCACAGCACTTC

3. CGCGGATCCGCCAACCACCTGACCTAC

4. TCCCCGCGGCTCAAAGGGTCCGGTCTT

Fragment 1 (using primers 1+2) and fragment 2 (using primers 3+4) were cloned into pBluescriptII SK+/- (Stratagene) as an intermediate vector. Syk with introduced BamHI site (Syk-BamHI) was cloned back into pcDNA.

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The ER^{T2} was PCR-amplified from pCre-ER^{T2} (gift from P. Chambon) with flanking

BamHI –sites using the following primer combinations:

1. CGCGGATCCTCTGCTGGAGACATGAG

2. CGCGGATCCAGCTGTGGCAGGGAAAC

The new ER^{T2}-BamHI was introduced into the N-terminus of Syk using BamHI-digest.

Syk-N-ER^{T2} was then cloned into the pApuro-vector.

2.5 Statistical analysis

All statistical analyses were carried out using the non-parametric two-tailed Mann-

Whitney test, with levels of statistical significance as indicated.

2.6 Calcium Fluorimetry

DT40 cells (10^7 cells/ml) were loaded with Indo-1 acetoxymethylester (4μM: Molecular

Probes) in RPMI-1640 containing 0.5% bovine serum albumin (BSA) at 37°C for 30

min in the dark. Cells were then washed twice in Lockes Buffer supplemented with

0.05% BSA. The fluorescence ratio of Indo-1 emission at 405 nm and 485 nm was

measured in live cells on a BD-LSR flow-cytometer. M4 antibody was added to

crosslink the BCR. The ratio of fluorescence at 405nm to 485nm was used as a measure

of intracellular calcium concentration.

Splenocytes and peritoneal cells were cell surface stained as described and then 10^6

cells/ml loaded with 4μM Indo-1 AM as above. Cells were washed with RPMI-1640

containing 0.5% BSA and fluorescence detected as above. B cells were stimulated with F(ab')$_2$ fragment Goat α-mouse IgM using 0.5μg, 1μg or 2.5μg of antibody per 10^6 cells.

2.7 DNA precipitation

DNA to be electroporated was precipitated by adding 2.5 volumes of 100% Ethanol and 1/10 volume NaAc (3M, pH 4.6), kept at -70°C for 1 hr and centrifuged for 30 min at 13000 rpm. The pellet was then washed with 70% Ethanol and re-centrifuged. The supernatant was removed in a laminar flow hood and the pellet was left to air dry, before resuspending the DNA in sterile-filtered TE at a final concentration of 1μg/μl.

2.8 Electroporation of DT40 cells

Wild-type and $Syk^{-/-}$ DT40 cells were cultured in DT40 medium. Prior to electroporation, cells were harvested and washed twice in serum-free RPMI-1640. Cells were resuspended in serum-free RPMI-1640 at 8×10^7 cells/ml and 450μl of the cell suspension was used for each electroporation in an elelectroporation cuvette (0.4cm, BioRad). Plasmid DNA was precipitated (as described above). 10μg DNA/450μl cells were used for each electroporation.

Electroporation was carried out in a BioRad Gene Pulser at 350V, 500μF. Cells were then let stand at room temperature for 5-10 min, before reculturing them in flasks containing 20ml DT40 media. Puromycin (0.5μg/ml) was added to each flask 24 hr later.

Two days later no live cells were found in the mock-transfected (no DNA) cultures (data not shown). To obtain stably transfected clones, cells were plated out on 96-well plates in limiting dilutions (20000 – 10000 – 5000 – 1000 cells/well).

2.9 Purification of M4 monoclonal antibody

Supernatant of M4 hybridoma (from T. Kurosaki) was harvested and precipitated overnight by dialysing against water. The precipitate was resuspended in PBS and protein concentration was determined by measuring optical density at 280nm on a spectrophotometer.

2.10 Western Blotting

Cytoplasmic lysates were prepared using Triton Lysis Buffer (NEB), mixed with equal amounts of 2xRSB (reducing sample buffer) and denatured at 100°C for 5 min. Samples were loaded onto a 10% polyacrylamide gel, then transferred onto a PVDF membrane by electroblotting in CAPS solution. Blots were blocked in 10% dry milk in PBS/Tween overnight or for at least 1 hr, incubated in the primary antibody (in PBS/Azide/Milk) for 1 hr, washed extensively, incubated with the secondary antibody for 1 hr and washed again. Signal was revealed by ECL (Amersham) using Kodak XB200 film.

Before reprobing blots, they were stripped for 20 min at 55°C in Western Stripping Buffer, washed extensively and blocked again.

2.11 Southern Blotting

Genomic DNA was prepared from 129/Sv or C57BL/6J tails. Tails were incubated overnight in $500\mu l$ tail-lysis buffer containing 0.1mg/ml Proteinase K (Boehringer Mannheim) in a water-bath at 55°C. Next day tubes were spun down for 20 min at 13000 rpm and supernatant transferred to fresh tubes. Genomic DNA was precipitated, by adding 0.7 volume isopropanol, then centrifuged 30 min at 13000rpm. The pellet was washed in 70% Ethanol, air-dried and resuspended in appropriate amounts of TE. Genomic DNA was digested overnight with the appropriate restriction enzymes. Digests were separated on a 0.8% agarose gel overnight. Gel was then denatured, using 1x Denaturing solution and DNA transferred onto Hybond N^+ nylon membrane (Amersham) via capillary action in 20xSSC. DNA was fixed onto the membrane with 0.4M NaOH. Probes were labelled with ^{32}P-CTP using the Ready-To-Go DNA Labelling Beads (Amersham) according to the manufacturer's instructions.

Hybridization was carried out at 65°C for 16 hr. Blots were washed in 2xSSC/0.5%SDS twice for 20min, then in 0.1xSSC/0.5%SDS twice for 20 min. Blots were placed into phosphoimager cassettes and scanned 1 to 3 days later. Alternatively, blots were placed into autoradiograph cassettes with Kodak Xomat AR films. Films were developed 2 to 3 days later.

2.12 Flow cytometric analysis and sorting

a.) Bone marrow, spleen, lymph nodes, peritoneal cavity and thymus

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Bone marrow (BM) cells were obtained by flushing out cells from femur and tibia of both legs. Spleens, lymph nodes and thymi were pressed through a 75μm nylon mesh to obtain cell suspensions. Peritoneal cells were obtained by washing the peritoneum with 5ml of PBS, 5 mM EDTA using a 27G needle to introduce and a 19G needle to recover injected medium. Cell suspensions from BM and spleen were spun down and resuspended in 1ml ACK lysis buffer to lyse red blood cells. 1 to 2 min later, when hemolysis was visible, cells were washed in FACS-buffer. 10^6 cells were stained in 50μl FACS-buffer, containing the appropriate, pre-titered antibodies. To avoid non-specific binding through Fc receptors, cells were pre-treated with 24G2 antibody, before adding specific antibodies. Flow cytometric analysis was done on FacsCalibur (BD) or on BD-LSR flow-cytometer and sorting on MoFlo (Cytomation), and data was analysed using Flowjo (Treestar) software.

b.) Blood

Peripheral blood was collected using heparinized capillaries. Blood was treated with 24G2 for 5 min before FACS-buffer containing the appropriate antibodies was added and incubated for 20 min on ice. Then 1ml ACK lysis buffer was added to lyse red blood cells. After 2 min (hemolysis visible) cells were washed with FACS buffer and analysed on FacsCalibur (Becton Dickinson).

2.13 Intracellular Zap70 staining

Surface staining of samples was carried out as described above. After two washes in PBS, cells were fixed in 3% paraformaldehyde on ice for 20 min. Samples were washed twice with PBS, then autofluorescence from paraformaldehyde was quenched by incubation with 50mM NH_4Cl. After another wash with PBS, cells were permeabilised using 0.1% NP-40 solution for 3 min, followed by a wash with PBS. Cells were then stained with Zap70 in FACS-buffer over night, then washed and analysed on the FacsCalibur (BD).

2.14 Harvesting and freezing fetal livers

Timed matings were set up, and vaginal plugs checked every morning. The day the plug is found is considered day 0.5. Embryos were sacrificed at day 12.5 or 16.5 of gestation, fetal liver was removed, made into cell suspension in RPMI medium, using 70μm cell strainer (Falcon), and spun down for 5 min at 1400 rpm. Cells were frozen at -70°C in 900 μl FCS and 100 μl DMSO per liver.

2.15 Infection of fetal liver cells

Frozen fetal liver (from embryos at E15.5) was thawed, washed once and cultured overnight in PlatE/HSC medium containing 6 μg/ml IL-3, 10 μg/ml IL-6, 100 μg/ml Stem cell factor (SCF), 100 μg/ml trombopoietin (TPO) and 20 μg/ml Flt3L. Cells were infected (see below) twice in the next two days, as described below.

2.16 Generation of infectious retrovirus

The day before transfection, PlatE cells (packaging cell line) were split and plated out on 6-well plates ($2x10^5$/well). The following day, the cells were transfected using 6μl Genejuice (Novagen) and 2μg DNA/well according to the Manufacturer's protocol. The vector used for the transfections was pMSCV-IREShuCD2 or pMSCV-IRES-GFP (a gift from Owen Williams). 48 – 72 hr later, retroviral supernatant was harvested and filtered through a 0.45μm filter unit to remove all cells. Retrovirus was concentrated by centrifugation for 1 hr at 13000 rpm at 4°C in a microcentrifuge and resuspension of the pellet in a smaller volume of PlatE / HSC-medium.

2.17 Retroviral infection of fetal liver cells

48 or 96-well plates were coated with Retronectin (recombinant human fibronectin fragment, Takara Bio Ing.; 25μg/ml) and incubated for 2 hr at room temperature. Retronectin was then removed, plates were blocked in PBS containing 2% BSA and washed with PBS before aliquoting the concentrated viral supernatant into each Retronectin-coated well. Cultured fetal liver cells were harvested and added to wells and incubated overnight at 37°C. Infection procedure was repeated the next day and cells were injected the day after that.

2.18 Infection of 3T3 cells for viral titre determination

3T3 cells were split the day before infection (2×10^5 cells/well) and plated out on 6-well

plates. Next day, medium was removed and 1 ml medium containing polybrene (8μg/ml)

and different dilutions of the concentrated viral supernatant (1:100 or 1:200) was added.

Cells were incubated at 37°C for 3 hr, before adding an additional 1 ml of PlatE / HSC-

medium. Cells were then incubated overnight. 3T3 cells were analysed the next day for

surface expression of either GFP or huCD2 using flow cytometry and viral titres were

calculated as follows: %huCD2$^+$/100 x number of cells plated x dilution factor.

2.19 Generation of Radiation Chimeras

On the day of the transfer, recipient mice received two doses of 475 rad (4.75 Gy) total

body irradiation from a ^{137}Caesium Source, administered 3 hr apart to minimise

gastrointestinal tract damage. Donor fetal liver (infected or uninfected) or BM cells were

washed twice in serum-free medium and filtered through a 75μm nylon mesh before

injecting cells in 0.2 ml volume intravenously (tail-vein) into irradiated recipients. Mice

were treated with 0.16% Neomycin-sulphate (Sigma) in their drinking water for at least 4

weeks post-transfer. Mice were analyzed 8-12 weeks after the transfer.

2.20 Immunisations

6-8 weeks old wt/μMT or *Zap70$^{-/-}$*/μMT chimeras were injected intraperitoneally with

TNP-KLH (100μg/mouse) in endotoxin-free PBS, TNP-Ficoll (25μg/mouse) or TNP-

LPS (50μg/mouse). Mice were bled one day before immunisation (day -1), at day 7 and

day 14 after immunisations. Mice immunised with TNP-KLH were re-immunised at day 21 and bled at day 28.

2.21 Serum

Peripheral blood was collected without the use of heparin and left for 10-20 min before spinning it down for 10 min at 13000 rpm. Serum was collected and respun. Serum was collected and immediately frozen at -70°C.

2.22 ELISA

Flat-bottom 96-well plates were coated with $50\mu l$ of either AffiniPure goat anti-mouse IgG+IgM (H+L) in coating buffer ($10\mu g/ml$) for the standard curves or with TNP-BSA (Biosearch Technmologies Inc.) ($33.3\mu g/ml$) in coating buffer. Plates were incubated overnight in a humid chamber at 4°C. 96-well plates were washed 2 x with ELISA-wash and then blocked in $200\mu l$/well Blocking Buffer. Plates were incubated at room temperature for at least 2 hours, then washed twice with ELISA-wash. $40\mu l$ of Standards (Mouse Standard Panel) was added to the standard wells (in a 2-fold dilution series from a concentration of 1000ng/well to 15.6ng/well) or $40\mu l$ of Serum-dilutions (1:100, 1:200, 1:500) was added to wells coated with TNP-BSA. Triplicates were prepared for each serum-dilution and Standards. Plates were incubated for 2 hours at room temperature and then washed twice with ELISA-wash. $40\mu l$ of HRP-conjugated secondary antibody (Clonotyping System-HRP) in a 1:500 dilution was added to the wells. Plates were

incubated for 1 hour at room temperature and washed twice with ELISA-wash. Then 40μl of ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), substrate was added to the wells. Plates were incubated for 10-20 min at room temperature in the dark, until the green colour developed. Finally, plates were read at λ = 405 on a 96-well-plate multi-scanEX.

2.23 Screening mouse genomic RPCI21-PAC Library

The mouse PAC library (RPCI21, UK HGMP Resource Centre) was provided by S. Ley. The source is female 129/SvevTACfBr mouse spleen genomic DNA. The library consists of 128 000 PAC clones in 336 microtiter plates. The average insert size is 147 kb. The library has been gridded in a 4x4 array on 22.2x22.2 cm Hybond N Nylon membrane. Each clone has been spotted twice. 7 filters cover the whole library.

7 filters were put into 2 hybridization tubes with meshes in between each filter. 100ml pre-hybridization buffer was added to each tube and they were incubated at 65°C for 4 hr, before the probe was added and incubated overnight at 65°C.

The probe is a 457 bp fragment of murine *Syk* gene, containing 275 bp of exon 2 obtained by cutting pcDNA3*Syk*ΔUTR with EcoRI and XcmI and gel purified using Qiagen gel purifying kit. Probe was labelled with ^{32}P-CTP using the Ready-To-Go DNA Labelling Beads (Amersham) according to the manufacturer's instructions.

Filters were washed in 2xSSC, 0.5%SDS twice for 20 min, then in 0.1xSSC, 0.5%SDS twice for 20 min. Then they were placed into autoradiograph cassettes with Kodak Xomat AR films. Films were developed the next day.

6 clones were identified (clone number: 539-K16, 560-L2, 401-L23, 501-N5, 452-H12 and 473-H3) and ordered from UK HGMP Resource Centre.

3. Role of Zap70 in B cells

In the absence of Syk, B cell development is partially blocked at the pro-B to pre-B cell transition. A few immature B cells develop, however there is a complete block at the immature to mature B cell transition (Turner et al., 1995). The partial block in B cell development at the pre-BCR checkpoint suggested the possibility of functional redundancy with another kinase substituting for Syk. The only other known Syk-family kinase is Zap70, which was reported to be expressed exclusively in T cells and NK cells, but not in B cells. However, in the absence of both kinases, Syk and Zap70, B cell development was found to be completely blocked at the pro-B to pre-B cell transition, confirming that in the absence of Syk, Zap70 indeed plays a compensatory role at the pre-BCR checkpoint (Schweighoffer et al., 2003). Zap70 has been reported to be expressed in pro-B cells, pre-B cells and splenic B cells in the mouse (Schweighoffer et al., 2003). Zap70 expression was also seen in human splenic and tonsillar B lymphocytes (Nolz et al., 2005). Furthermore, in tonsillar B cells, Zap70 was shown to be phosphorylated upon BCR stimulation.

Interestingly, Zap70 is also expressed in some B-CLL, where it could be used as a marker for the aggressive disease. However, the role of Zap70 in B-CLL is not clear.

All these findings clearly indicate that Zap70 is not only expressed in T cells and NK cells, but also in B lineage cells. Furthermore, Zap70 appears to compensate for the lack of Syk at the pro-B to pre-B cell transition and hence may transduce signals from the pre-BCR (Schweighoffer et al., 2003). However it remains unknown whether Zap70

may have a function at other points during B cell development or activation, other than the pre-BCR checkpoint.

3.1 Zap70 expression in different B cell populations

Zap70 expression has previously been shown in pro-B, pre-B and splenic B cells in the mouse (Schweighoffer et al., 2003) and recently also shown in human tonsillar and splenic B cells (Nolz et al., 2005). I wanted to find out whether all mature B cell populations expressed Zap70 and whether there were any subsets of B cells, which expressed higher levels of Zap70 than others.

My first choice of method was detecting Zap70 expression in different mature B cell subsets by intracellular staining and flow cytometry. I optimised the conditions for intracellular Zap70 staining using thymocytes, which express high levels of Zap70 (Fig.3.1). The histogram in Fig.3.1c shows intracellular Zap70 expression of $CD4^{+}CD8^{+}$-thymocytes of radiation chimeras reconstituted with wt or $Syk^{-/-}$ fetal liver or of a $Zap70^{-/-}$ mouse as a control. Zap70 expression levels in wt and $Syk^{-/-}$ DP thymocytes were easily distinguishable compared to $Zap70^{-/-}$ DP thymocytes. A radiation chimera reconstituted with $Syk^{-/-}$ fetal liver was included in the analysis as a control for the Zap70-specificity of the intracellular antibody used. I conclude that this staining method can clearly detect Zap70 and that the antibody does not detect the related Syk kinase.
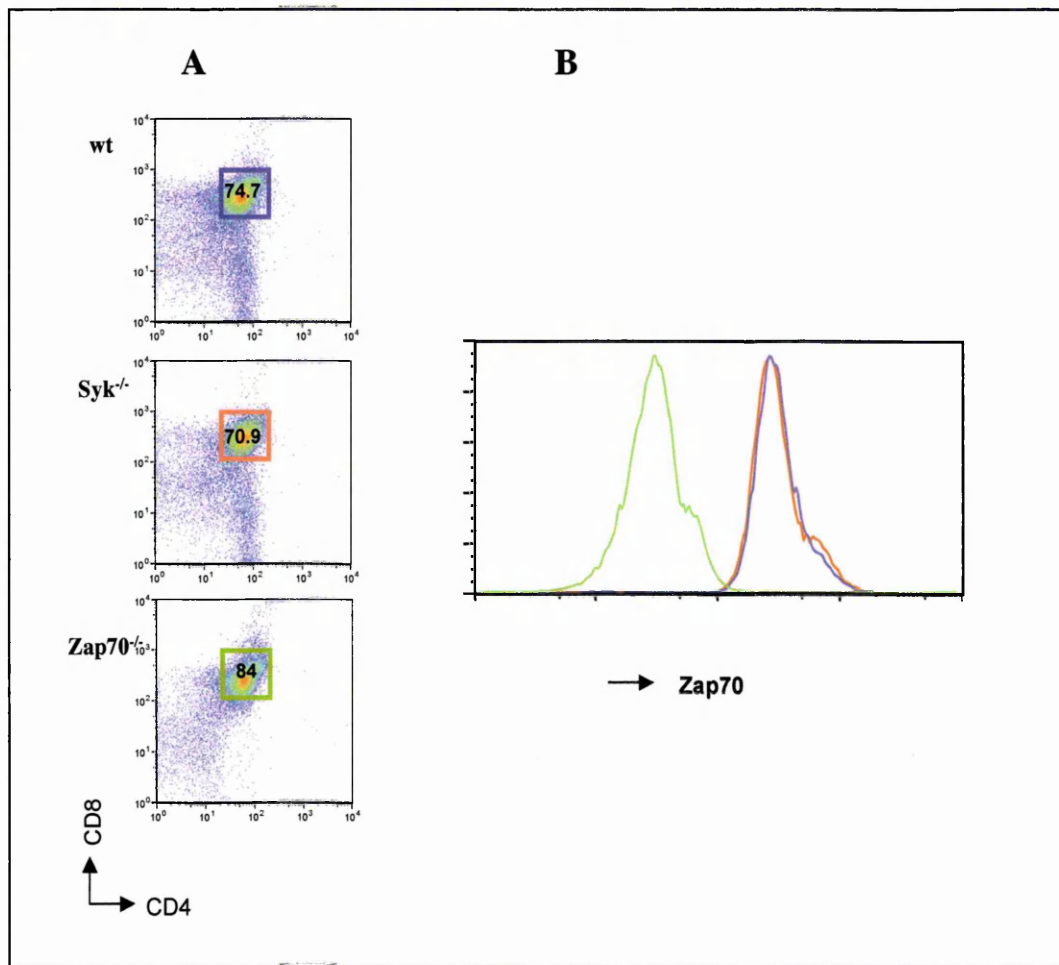
Figure 3.1. Zap70 expression in DP thymocytes

A) Flow cytometry analysis of CD4 and CD8 levels on thymocytes from radiation chimeras reconstituted with wild-type (wt) or *Syk*$^{-/-}$ fetal liver or from a *Zap70*$^{-/-}$ mouse. Percentage of cells falling into a CD4$^+$CD8$^+$ gate are indicated.

B) Histogram of Zap70 expression in CD4$^+$CD8$^+$ thymocytes, gated as in (A), from wild-type (blue), *Syk*$^{-/-}$ (red) or *Zap70*$^{-/-}$ mice (green).

Next I attempted to measure Zap70 expression levels in B cell subsets in the bone marrow using intracellular flow cytometry (Fig. 3.2). Bone marrow cells were stained to identify pro-B cells ($CD19^+CD2^-IgM^-$), pre-B cells ($CD19^+CD2^+IgM^-$) and immature/mature B cells ($CD19^+CD2^+IgM^+$) and stained for intracellular Zap70 using the same method that had been optimised for thymocytes. Whereas Zap70 expression was readily detected in thymocytes, no Zap70 expression was detectable in pro-B, pre-B or immature/mature B cells.

In conclusion, Zap70 expression could not be detected in B lineage cells in the bone marrow using intracellular staining methods and flow cytometry. The same result was achieved in B lineage cells from the spleen and peritoneal cavity (data not shown). Because Zap70 expression levels appear to be much lower in B cells compared to T cells, it is likely that this method was not sensitive enough to detect Zap70 in B lineage cells.
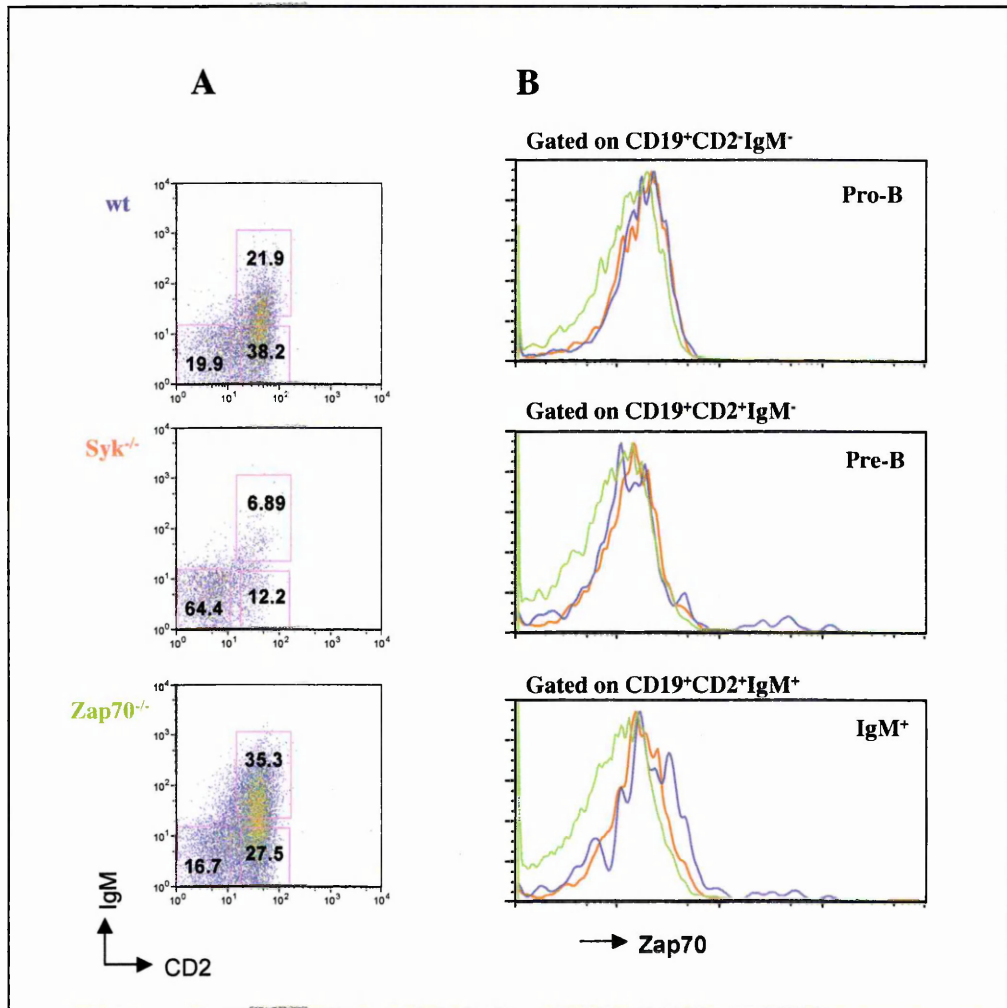
Figure 3.2. Zap70 expression in bone marrow B cells.

A) Flow cytometric analysis of CD2 and IgM levels on CD19$^+$ cells from the bone marrow of radiation chimeras reconstituted with wild-type (wt) or $Syk^{-/-}$ fetal liver or from the bone marrow of $Zap70^{-/-}$ mice. Numbers show percentages of cells falling into the indicated gates.

B) Histograms of Zap70 expression in pro-B (CD19$^+$CD2$^-$IgM$^-$), pre-B (CD19$^+$CD2$^+$IgM$^-$) and immature/mature B cells (CD19$^+$CD2$^+$IgM$^+$) from the bone marrow of radiation chimeras reconstituted with wild-type fetal liver (blue), $Syk^{-/-}$ fetal liver (red) or from a $Zap70^{-/-}$ mouse (green). Each set of histograms was gated on gates shown in (A).

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Next I examined Zap70 expression in B lineage cells using immunoblotting. I could indeed detect Zap70 in cytoplasmic lysates from sorted $Zap70^{+/+}$ splenic B cells (CD19$^+$) (Fig.3.3a). In contrast, no Zap70 was found in sorted $Zap70^{-/-}$ splenic B cells, confirming that the antibody used was specific for Zap70.

I then examined Zap70 expression in cytoplasmic lysates of different B cell subsets in the spleen. Zap70 could be detected in sorted MRF B cells (CD19$^+$CD21$^+$CD23$^+$), MZ B cells (CD19$^+$CD21^{hi}CD23$^-$) and also in NF B cells (CD19$^+$CD21$^-$CD23$^-$) (Fig. 3.3b). Zap70 expression could also be detected in cytoplasmic lysates from sorted B1a and B1b cells, which are the major B cell populations of the peritoneal cavity (Fig. 3.3c). All populations were sorted to 98-99% purity (data not shown).

To quantify Zap70 expression during B cell development from the bone marrow to the spleen and compare it to levels in thymocytes, I sorted pro-B (CD19$^+$CD2$^-$IgM$^-$) cells from C57BL/10-$Rag1^{-/-}$ bone marrow, pre-B cells (CD19$^+$CD2$^+$IgM$^-$) from bone marrow of C57BL/10-$Rag1^{-/-}$ mice containing a heavy chain transgene, and immature (CD19$^+$IgM$^+$IgD$^-$) and mature B cells (CD19$^+$IgM$^+$IgD$^+$) from bone marrow of C57BL/6J mice. MRF (CD19$^+$CD21$^-$CD23$^+$) B cells were sorted from the spleen of C57BL/6J mice. The same B cell populations were also sorted from $Zap70^{-/-}$ mice as a control for staining specificity. All B cell populations were sorted to a purity of 98-99% (data not shown). Unfortunately, I was unable to sort sufficient numbers of MZ and NF B cells in this particular experiment to include these populations in the analysis.
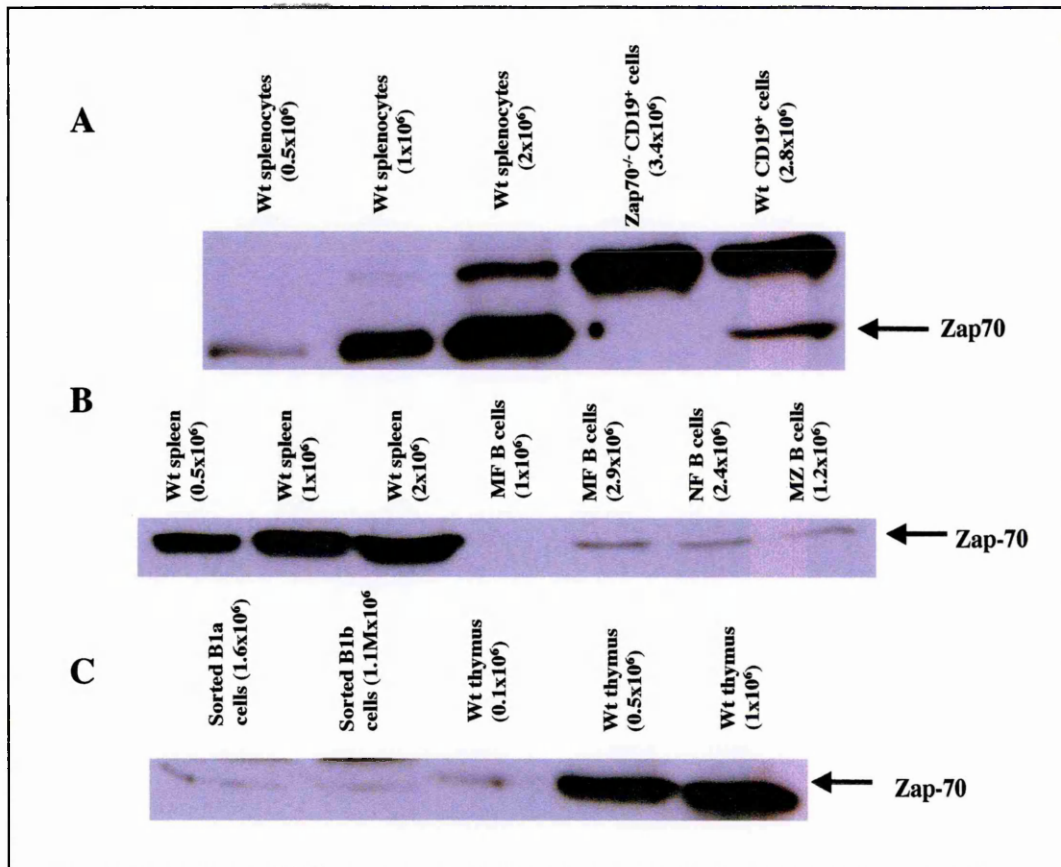
Figure 3.3. Zap70 expression in mature B cell subsets

A) Immunoblot of total cytoplasmic lysates wt splenocytes and of sorted CD19⁺ splenocytes from wt or *Zap70⁻/⁻* mice, probed with antibodies to Zap70. Arrow points to Zap70. The upper band is non-specific since it is seen in *Zap70⁻/⁻* cells.

B) Immunoblot of total cell lysates of total wt splenocytes and sorted wt mature B cell populations in the spleen: MZ, marginal zone B cells (CD19⁺CD23⁻CD21ʰⁱ), MF, mature recirculating follicular B cells (CD19⁺CD23⁺CD21⁺), NF, newly formed B cells (CD19⁺CD23⁻CD21⁻) probed with antibodies to Zap70.

C) Immunoblot of total cell lysates of wt thymocytes or sorted wt B1 B cells from the peritoneal cavity: B1a cells (CD19⁺Mac1⁺CD5⁺) and B1b cells (CD19⁺Mac1⁺CD5⁻) probed with antibodies to Zap70.

Cell numbers loaded are indicated in parenthesis. All cell populations were sorted to 98-99% purity (data not shown).

Immunoblot of cytoplasmic lysates of these B cell subsets in shown in Fig. 3.4 along with lysates of thymocytes from $TCR\alpha^{-/-}$ or $Zap70^{-/-}$ mice, both of which consist mainly (>80%) of $CD4^+CD8^+$ double positive cells. Zap70 expression could be detected in all sorted wt B cells populations, whereas no Zap70 expression was seen in sorted $Zap70^{-/-}$ B cell subsets. Quantification of Zap70 expression levels in sorted B cell subsets revealed that normalised to expression of ERK2 (Fig. 3.4a), pro-B cells express 30-fold less Zap70 than thymocytes, whereas pre-B, immature and mature B cells express around 60 to 100-fold less compared to $TCR\alpha^{-/-}$ thymocytes. However, ERK2 expression levels seemed to be lower in thymocytes compared to B lineage cells. If Zap70 expression in sorted B cell populations is normalised to the number of cells loaded, one can observe 8-fold less Zap70 expression in pro-B cells, and 30-fold less Zap70 expression in all other sorted B cell subsets compared to double positive thymocytes.

In conclusion, Zap70 could be detected in all developing and mature B cell populations. Furthermore, pro-B cells expressed 2 to 3 times more Zap70 that all other B cell subsets examined.
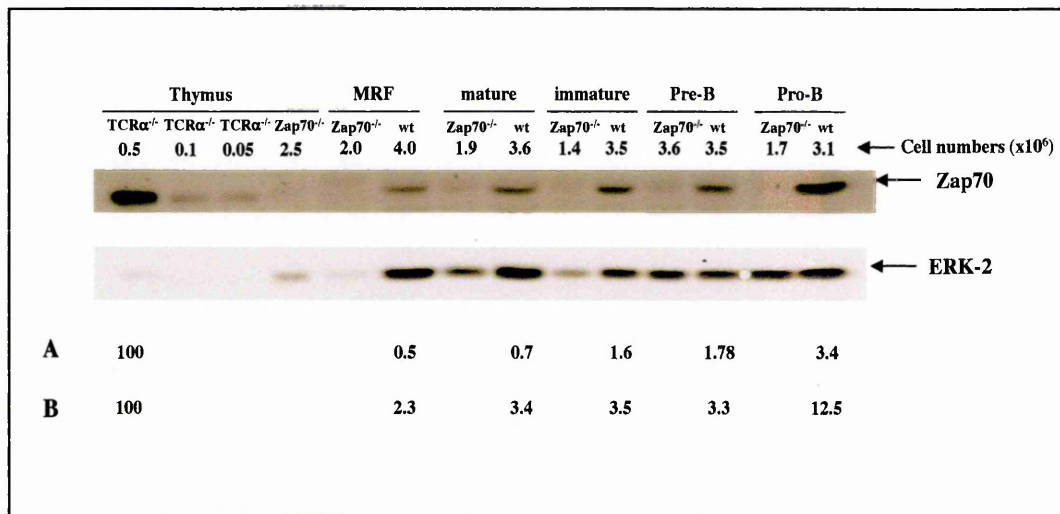
Figure 3.4. Zap70 expression in B cell subsets

Immunoblot of total cytoplasmic lysates of $TCR\alpha^{-/-}$ or $Zap70^{-/-}$ thymocytes and wt or $Zap70^{-/-}$ sorted proB cells (CD19⁺CD2⁻IgM⁻), preB cells (CD19⁺CD2⁺IgM⁻), immature B cells (CD19⁺IgM⁺IgD⁻) and mature B cells (CD19⁺IgM⁺IgD⁺) from bone marrow and MRF B cells (CD19⁺CD21⁺CD23⁺) from the spleen probed with antibodies to Zap70. The same blot was also probed with antibody to ERK2 as indicated. Number of cells loaded into each lane (x10⁶) is shown. All cell pupolations were sorted to 98-99% purity (data not shown).

(A) Densitometric analysis of Zap70 expression in B cell populations normalised to levels of ERK2, setting expression of $TCR\alpha^{-/-}$ thymocytes to be 100.

(B) Densitometric analysis of Zap70 expression in B cell populations normalised to the number of cells loaded, setting Zap70 expression in $TCR\alpha^{-/-}$ thymocytes to be 100.

3.2 Role of Zap70 in B cell development

I set out to explore whether Zap70 plays a role at distinct stages of B cell development other than the pre-BCR checkpoint. To address this question, I examined B cell populations from the bone marrow, spleen, lymph nodes and peritoneal cavity of *Zap70⁻/⁻* mice and compared them to wild type mice. *Zap70⁻/⁻* mice have no CD4⁺ or CD8⁺ mature T cells (Negishi et al., 1995; Wiest et al., 1997). Although αβ T cells and epithelial γδ T cells are severely affected by the lack of Zap70, some γδ T cells in the peripheral tissues are still present in *Zap70⁻/⁻* mice (Gong et al., 1997; Kadlecek et al., 1998). To control for whether any differences found in *Zap70⁻/⁻* B cell populations were due to the absence of Zap70 in these cells or due to the lack of αβ T cells in these mice, I decided to also compare B cell subsets from *Zap70⁻/⁻* mice to *TCRα⁻/⁻* mice, which lack all αβ T cells but still have γδ T cells. In order to match genetic backgrounds, *Zap70⁻/⁻*, which were two times backcrossed to C57BL/6J, and *TCRα⁻/⁻* mice, which were fully backcrossed to CBA/Ca, were compared to C57BL/6J and CBA/Ca mice respectively.

The following data is a summary of 3 subsequent experiments using 4-5 *Zap70⁻/⁻* and 4-5 C57BL/6J mice per experiment or 2 individual experiments with 4-5 *TCRα⁻/⁻* mice and 4-5 CBA/Ca mice as their control.

3.2.1 Bone Marrow

First, I examined B cell populations in the bone marrow. I distinguished B cell subsets at different stages of B cell development based on their cell surface marker expression: pro-B cells were defined as $CD19^+CD2^-IgM^-IgD^-$; pre-B cells were $CD19^+CD2^+IgM^-IgD^-$; immature B cells were $CD19^+CD2^+IgM^+IgD^-$ and mature B cells were defined by their expression of CD19, CD2, IgM and IgD (Fig.3.5). Counts of cell numbers showed that compared to wt controls, $Zap70^{-/-}$ bone marrow had a slight reduction in lymphocytes (as defined by forward and side scatter) and in total numbers of $CD19^+$ B lineage cells (Fig.3.5b). Furthermore, examining different populations within the B cell lineage in the bone marrow, a significant reduction in pre-B cells and immature B cells was observed. However, the number of mature B cells from $Zap70^{-/-}$ mice compared to control mice was similar. Examining the percentage of each B cell population in the bone marrow, only a reduction in percentages of lymphocytes could be observed (Fig.3.6a). The percentage of $CD19^+$ cells and of pro-B, pre-B and immature B cells, was similar between $Zap70^{-/-}$ and wild-type mice. However a slight increase in percentage of mature B cells could be seen in $Zap70^{-/-}$ mice.

The number of lymphocytes in the bone marrow of $TCR\alpha^{-/-}$ mice was similar to their wild-type control animals. However, they had more $CD19^+$ cells, and therefore more pro-B cells, pre-B cells and immature B cells. Surprisingly the number of mature B cells was reduced (Fig. 3.5b). These findings correlated with the percentages of B cell subsets in the bone marrow (Fig.3.6b). A higher percentage of $CD19^+$ cells was observed in $TCR\alpha^{-/-}$ bone marrow compared to wild-type. The percentage of pro-B, pre-B and immature B cells was similar in $TCR\alpha^{-/-}$ compared to wt. The percentage of mature B cells however was decreased.
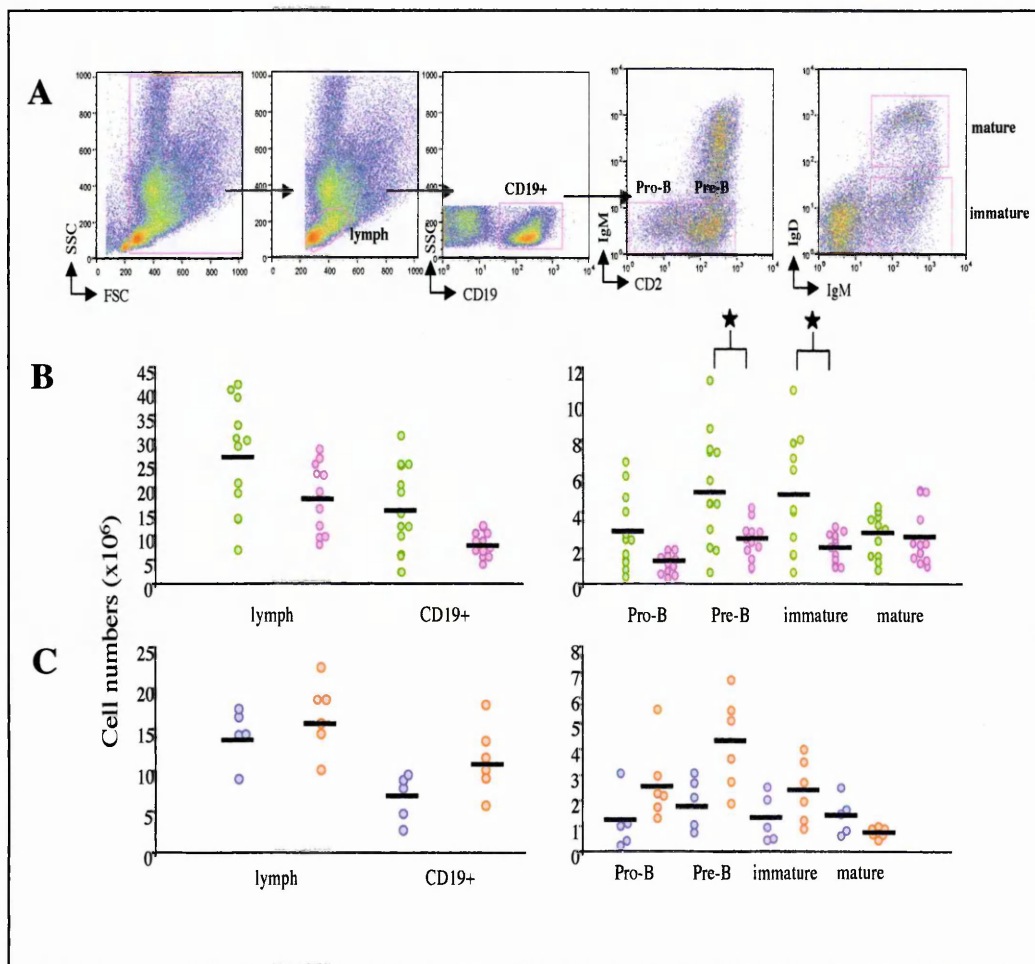
Figure 3.5. Zap70 in B cell development : bone marrow (I)

Graphs show numbers of total lymphocytes (lymph, defined by forward and side scatter), CD19+ cells, pro-B cells (CD19$^+$CD2$^-$IgM$^-$IgD$^-$), pre-B cells (CD19$^+$CD2$^+$IgM$^-$IgD$^-$), immature B cells (CD19$^+$CD2$^+$IgM$^+$IgD$^-$) and mature B cells (CD19$^+$CD2$^+$IgM$^+$IgD$^+$).

(A) An example of the gated subsets is shown on wild-type bone marrow cells.

(B) *Zap70*$^{-/-}$ mice (n=11) (pink) compared to C57BL/6J mice (n=11) (green). Data are summaries of three independent experiments with 3-5 *Zap70*$^{-/-}$ and wt mice per experiment.

(C) *TCRα*$^{-/-}$ mice (n=6) (red) compared to CBA/Ca mice (n=6) (blue). Data is summaries of 1 experiment.

Each circle represents one mouse. Black lines show the mean value for each subset. All mice were between 8 and 10 weeks of age. Asterisk indicates statistically significant differences between samples (★, $p < 0.05$).
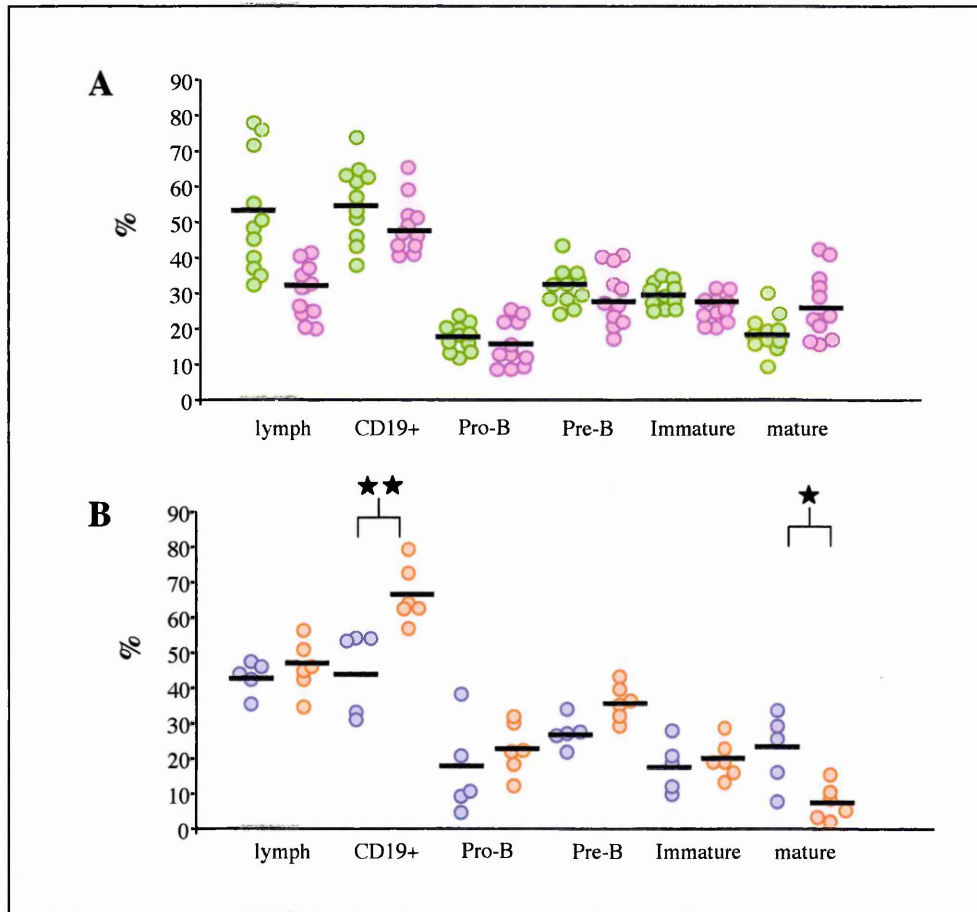
Figure 3.6. Zap70 in B cell development : bone marrow (II)

Graphs show percentages of B cell populations in the bone marrow defined as in Fig.3.1. (A) *Zap70*$^{-/-}$ mice (n=11) (pink) compared to C57BL/6J mice (n=11) (green). Data are summaries of three independent experiments with 3-5 *Zap70*$^{-/-}$ and wt mice per experiment.

(B) *TCRα*$^{-/-}$ mice (n=6) (red) compared to CBA/Ca mice (n=6) (blue). Data is summary of 1 experiment.

Each circle represents one mouse. Black lines show the mean value for each subset. Asterisk indicates statistically significant differences between samples (★ p<0.05, ★★ p<0.005). Data are summaries of three independent experiments.

3.2.2 Spleen

Next I examined B cells in the spleen. I divided the B cell populations in the spleen according to the cell surface expression of CD19, CD23, CD21 and IgM. I could distinguish between mature recirculating follicular (MRF) B cells ($CD19^+CD23^{hi}CD21^+$), also known as B2 cells, marginal zone (MZ) B cells ($CD19^+CD23^{-/lo}CD21^{hi}$) and Transitional 1 (T1) B cells ($CD19^+CD21^-IgM^+$) and Transitional 2 (T2) B cells ($CD19^+CD21^+IgM^+$).

Zap70$^{-/-}$ and wt mice had comparable numbers of lymphocytes, but increased numbers of $CD19^+$ cells (Fig.3.6a). Examining the B cell populations in more detail, revealed a slight increase in MRF B cell number and, surprisingly, a very significant increase in MZ B cells. The number of *Zap70$^{-/-}$* T1 and T2 B cells was increased to that in control mice. Similar results were found by examining the percentages of different B cell subsets in the spleen and also by using Allman staining method to distinguish between MRF and MZ B cells as well as T1, T2 and T3 cells (data not shown) (Allman et al., 2001).

TCRα$^{-/-}$ mice also had more $CD19^+$ cells and MRF cells in the spleen compared to wt mice (Fig.3.6b). However no increase in MZ B cells could be seen in *TCRα$^{-/-}$* mice. The number of T1 and T2 B cells was also increased, which was similar to the results of *Zap70$^{-/-}$* mice.

The increase in B cells, in particular the MRF B cell subset, which is the biggest B cell population in the spleen, was similar in *Zap70$^{-/-}$* and *TCRα$^{-/-}$* mice. The significant

increase in MZ B cells however, was specific to *Zap70*$^{-/-}$ mice, because no increase could

be seen in number of MZ B cells in *TCRα*$^{-/-}$ mice.
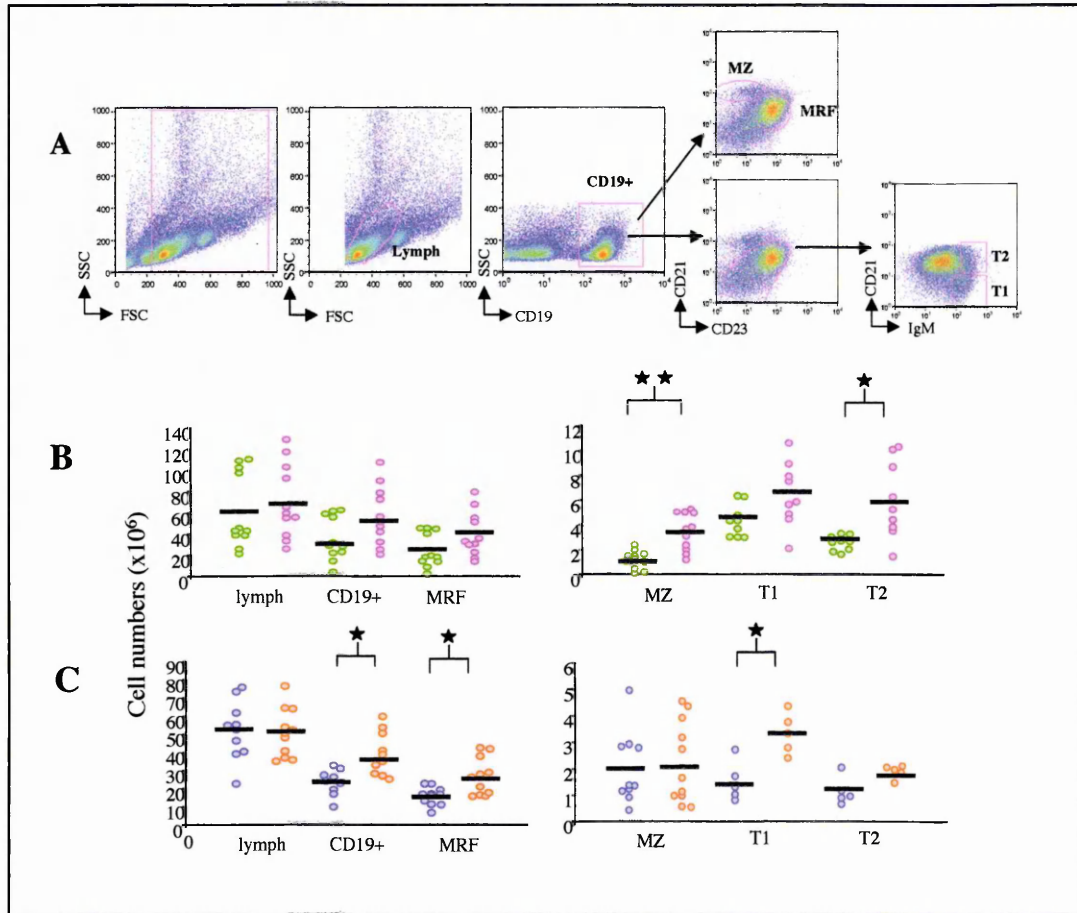
Figure 3.7. Zap70 in B cell development : spleen

Graphs numbers of lymphocytes (lymph, defined by forward and side scatter), all CD19$^+$ cells, mature recirculating follicular B cells (MRF, CD19$^+$CD23$^+$CD21$^+$), marginal zone B cells (MZ, CD19$^+$CD23^{lo}CD21^{hi}) and T1 B cells (CD19$^+$CD21$^-$IgM^{hi}) and T2 B cells (CD19$^+$CD21$^+$IgM^{hi}) in the spleen.

(A) An example of gated subsets is shown on wild-type splenocytes.

(B) *Zap70*$^{-/-}$ mice (n=11) (pink) compared to C57BL/6J mice (n=11) (green). Data are summaries of three independent experiments with 3-5 *Zap70*$^{-/-}$ and wt mice per experiment.

(C) TCRα$^{-/-}$ mice (n=10; T1,T2 n=5) (red) compared to CBA/Ca mice (n=11; T1,T2 n=5) (blue). Data are summaries of two independent experiments with 5 *TCRα*$^{-/-}$ and wt mice per experiment.

Each circle represents one mouse. Black lines show the mean value for each subset. Statistically significant differences are indicated (★, 0.01<p<0.05; ★★, p<0.0005).

1.2.3 Peritoneal cavity

Next I examined B cell populations in the peritoneal cavity, which has two B cell subsets: B2 cells and B1 cells, defined by their expression of CD19 and Mac1. B1 cells can further be subdivided into B1a and B1b cells, which are $CD5^+$ and $CD5^-$ respectively.

Similar numbers of B2 cells were observed in the peritoneal cavity of $Zap70^{-/-}$ and wt mice (Fig.3.8). Furthermore, no difference in numbers of B1a cells was found. However, the number of B1b cells was significantly increased in $Zap70^{-/-}$ mice (Fig.3.8a). $TCR\alpha^{-/-}$ mice also showed unchanged numbers of B2 and B1a cells, however the number of B1b cells was decreased compared to wt mice (Fig.3.8b). Similar results were seen in an analysis of percentages of B1 and B2 cells in the peritoneal cavity (Fig.3.9).

Therefore, the increase in B1b cells in $Zap70^{-/-}$ mice seems not to be a consequence of a lack of T cells, since this subset is decreased in $TCR\alpha^{-/-}$ mice.

Figure 3.8. Zap70 in B cell development: peritoneal cavity (I)

Graphs show numbers of lymphocytes (lymph, defined by forward and side scatter), all CD19$^+$ cells, B1a cells (CD19$^+$Mac1$^+$CD5$^+$), B1b cells (CD19$^+$Mac1$^+$CD5$^-$), and B2 cells (CD19$^+$Mac1$^-$CD5$^-$) in the peritoneal cavity.

(A) An example of gated subsets is shown on wild-type cells in the peritoneal cavity.

(B) Zap70$^{-/-}$ mice (n=13) (pink) compared to C57BL/6J mice (n=11) (green). Data are summaries of three independent experiments with 3-5 Zap70$^{-/-}$ and wt mice per experiment.

(C) TCRα$^{-/-}$ mice (n=11) (red) compared to CBA/Ca mice (n=10) (blue). Data are summaries of two independent experiments with 5-6 TCRα$^{-/-}$ and 5 wt mice per experiment.

Each circle represents one mouse. Black lines show the mean value for each subset. Significant differences are indicated (★, p<0.05).

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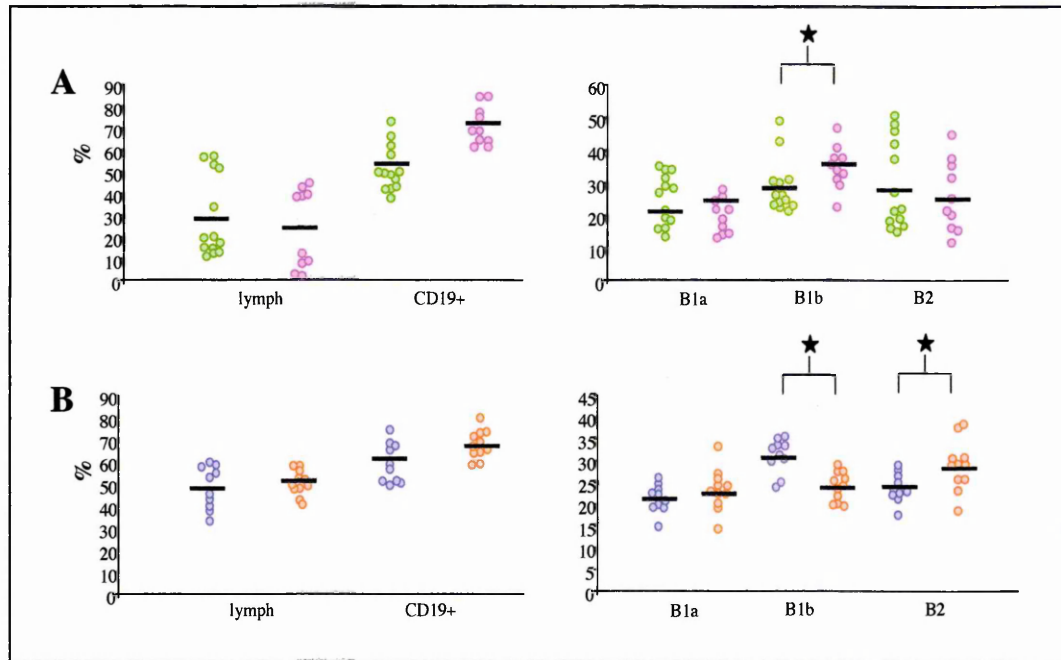
Figure 3.9. Zap70 in B cell development: peritoneal cavity (II)

Graphs show percentages of lymphocytes (lymph), all CD19$^+$ cells and B cell populations in the peritoneal cavity as described in Fig.3.8.

(A) $Zap70^{-/-}$ mice (n=13) (pink) compared to C57BL/6J mice (n=11) (green). Data are summaries of three independent experiments with 3-5 $Zap70^{-/-}$ and wt mice per experiment.

(B) $TCR\alpha^{-/-}$ mice (n=11) (red) compared to CBA/Ca mice (n=10) (blue). Data are summaries of two independent experiments with 5-6 $TCR\alpha^{-/-}$ and 5 wt mice per experiment.

Each circle represents one mouse. Black lines show the mean value for each subset. Significant differences are indicated ($\star$, p<0.05).

1.2.4 Lymph nodes

Finally, I examined B cells in the lymph nodes. Major B cell population in the lymph nodes are B2 cells (MRF). Lymph nodes of $Zap70^{-/-}$ mice had significantly reduced numbers of lymphocytes. However, the number of B cells was unchanged. No TCR^+

cells could be found in the *Zap70*^{-/-} lymph nodes, consistent with previous publications (Fig. 3.10a). *TCRα*^{-/-} mice gave a very similar picture: reduced numbers of lymphocytes, similar numbers of B cells and no T cells in *TCRα*^{-/-} mice (Fig. 3.10b).

This result contrast with the spleen, where in the absence of T cells, the number of B cells was increased.

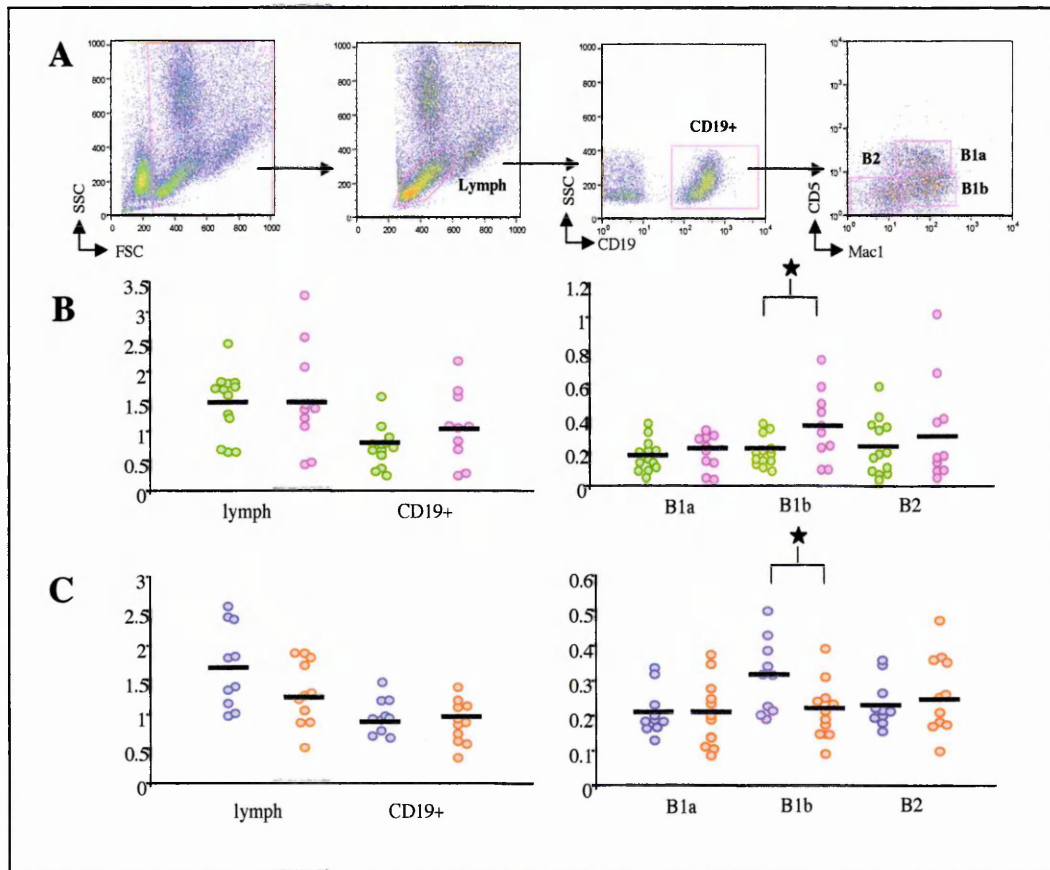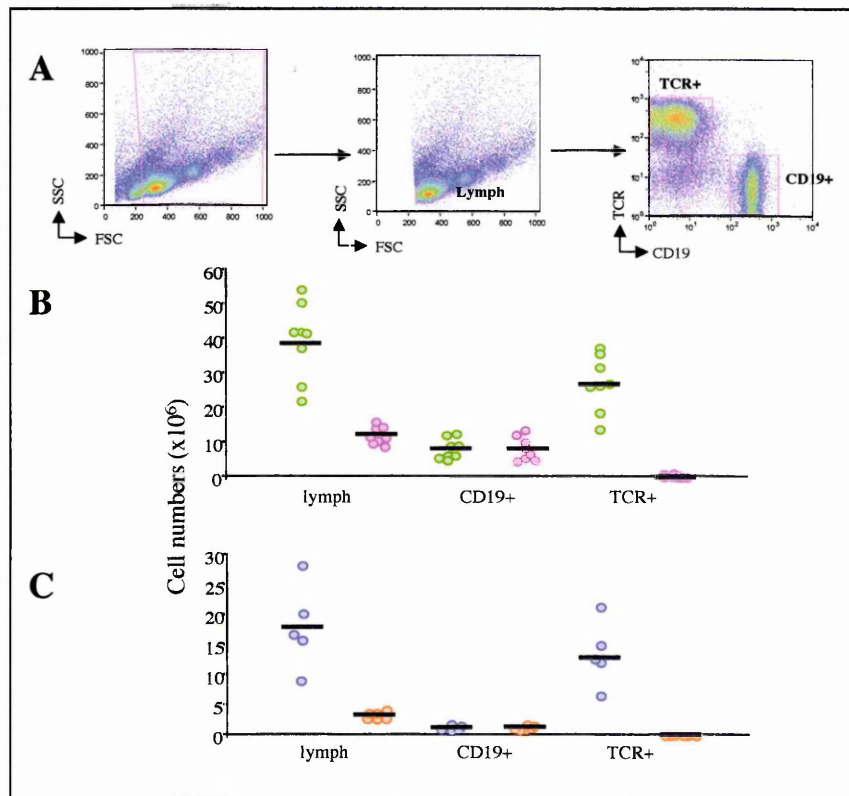**Figure 3.10. Zap70 in B cell development: lymph nodes**

Graphs show numbers lymphocytes (lymph, defined by forward and side scatter), all CD19^{+} and TCR^{+} cells in the lymph nodes.

(A) An example of gated subsets is shown on wild-type cells in lymph nodes.

(B) *Zap70*^{-/-} mice (n=8) (pink) compared to C57BL/6J mice (n=8) (green). Data are summaries of two independent experiments with 4 *Zap70*^{-/-} and 4 wt mice per experiment.

(C) *TCRα*^{-/-} mice (n=5) (red) compared to CBA/Ca mice (n=5) (blue). Data are summary of one experiment with 5 *TCRα*^{-/-} and 5 wt mice.

Each circle represents one mouse. Black lines show the mean value for B cells and T cells. Data are summaries of three independent experiments.

3.2.5 Mixed bone marrow radiation chimeras

To further explore whether differences observed in B cell populations in the spleen and peritoneal cavity in $Zap70^{-/-}$ mice, were due to the lack of Zap70 in these B cell subsets themselves or due to the lack of $\alpha\beta$ T cells in these mice, I examined mixed bone marrow chimeras with $Zap70^{-/-}$ bone marrow mixed in a 1:1 ratio with μMT BM ($Zap70^{-/-}/\mu$MT) or wt BM mixed in a 1:1 ratio with μMT BM (wt/μMT) as donors that reconstituted sublethally irradiated C57BL/10-$Rag1^{-/-}$ mice. Because $Zap70^{-/-}$ mice lack mature T cells and NK cells, differences in B cell subsets seen in $Zap70^{-/-}$ mice might not be due to the lack of Zap70 in B cells, but the lack of Zap70 in other populations affecting B cells.

To investigate whether changes in B cell populations in $Zap70^{-/-}$ mice are Zap70 intrinsic, I examined B cell subsets in mixed BM chimeras, where all B cells were Zap70-deficient and all T cells came from μMT mice, and were therefore not deficient for Zap70. As a control I examined mixed BM chimeras that received wt BM mixed with μMT BM as donors.

As shown in Fig. 3.11a, percentages in the spleen of $CD19^+$ cells were increased in $Zap70^{-/-}/\mu$MT radiation chimeras compared to wt/μMT chimeras. Examining B cells subsets in the spleen of these radiation chimeras, one can observe that the percentages of NF, MRF B cells and MZ B cells were similar in $Zap70^{-/-}/\mu$MT chimeras compared to

wt/μMT chimeras. A significant increase in numbers of lymphocytes, all CD19$^+$ cells,

MRF and MZ B cells could be observed (Fig.3.11b).
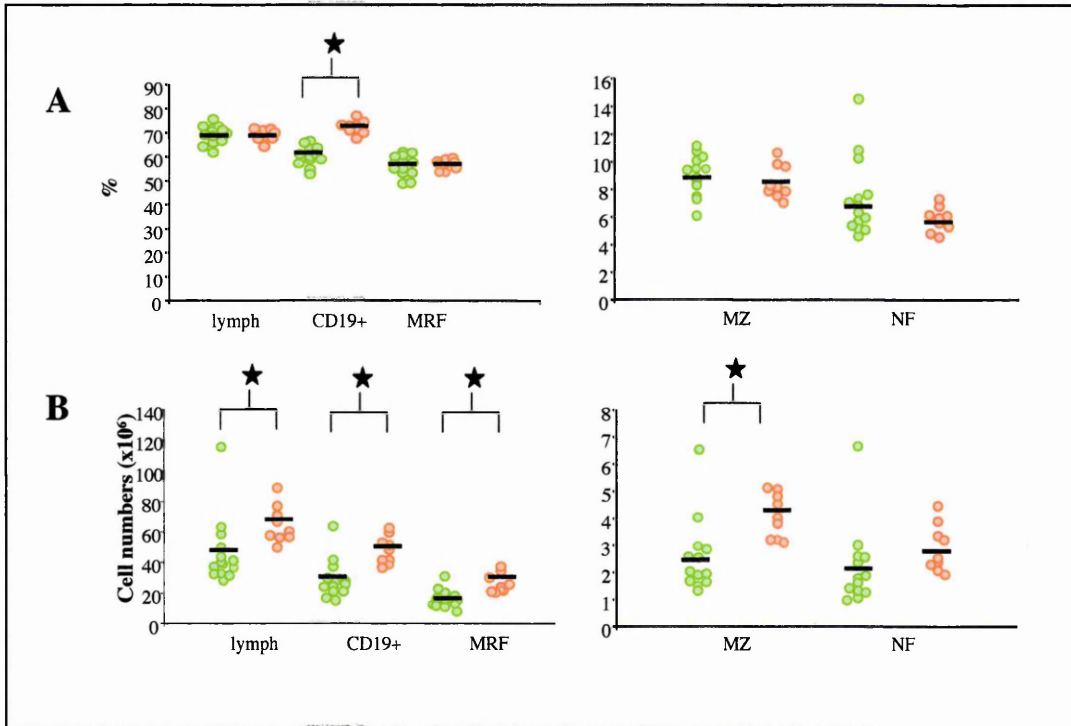
Figure 3.11.   Role of Zap70 in B cell development : mixed BM-chimeras, spleen

Mixed BM chimeras were made with *Zap70$^{-/-}$* BM mixed with μMT BM in a ratio of 1:1 (*Zap70$^{-/-}$*/μMT, red, n=9) and C57BL/6J BM and μMT BM in a ratio of 1:1 (wt/μMT, green, n=9) as donors and sublethally irradiated C57BL/10-*Rag-1$^{-/-}$* mice as recipients. Mice were analysed 11 weeks after reconstitution. Lymphocytes (lymph), all CD19$^+$ cells, MRF B cells, MZ B cells were defined as described in Fig.3.7, NF B cells were defined as CD19$^+$CD23$^-$CD21$^-$. Percentages (A) and cell numbers (B) of these populations are shown in graphs. Each circle represents one mouse. Black lines show the mean values. Significant differences are indicated ($\star$, 0.005<p<0.0001).

B cell subsets in the peritoneal cavity in these mixed BM chimeras confirmed the results

obtained in *Zap70$^{-/-}$* mice. Percentage of B1a cells in the peritoneal cavity was similar in

$Zap70^{-/-}/\mu$MT chimeras compared to wt/μMT chimeras (Fig.3.12). However, the percentage of B1b cells was significantly increased in $Zap70^{-/-}/\mu$MT chimeras. A tendency of the increase in B1b cells could also be observed examining cell numbers (Fig.3.12b), although this difference did not reach statistical significance.
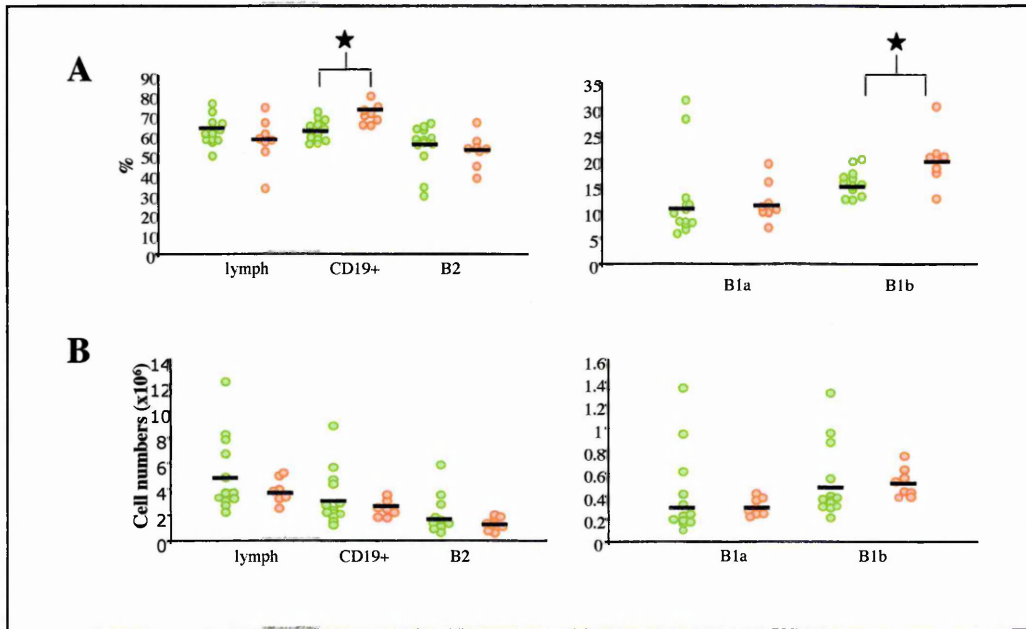
Figure 3.12.   Role of Zap70 in B cell development : mixed BM-chimeras, PEC

Mixed BM chimeras were made with $Zap70^{-/-}$ BM mixed with μMT BM in a ratio of 1:1 ($Zap70^{-/-}/\mu$MT, red, n=9) and C57BL/6J BM and μMT BM in a ratio of 1:1 (wt/μMT, green, n=9) as donors and sublethally irradiated C57BL/10-Rag-$1^{-/-}$ mice as recipients. Mice were analysed 11 weeks after reconstitution. Lymphocytes (lymph), all CD19$^+$ cells, B1a cells, B1b cells and B2 cells were defined as described in Fig.3.8. Percentages (A) and cell numbers (B) of these populations are shown in graphs. Each circle represents one mouse. Black lines show the mean values. Significant differences are indicated ($\bigstar$, 0.01<p<0.001).

In summary, an increase in MZ B cells and in B1b cells, both reaching statistical significance, could be observed in $Zap70^{-/-}$ mice compared to wt mice. No difference in

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these two B cell populations was seen in percentages or cell numbers in $TCR\alpha^{-/-}$ mice, indicating that these differences are due to the lack of Zap70 in these B cell populations themselves. Radiation chimeras reconstituted with $Zap70^{-/-}/\mu$MT bone marrow showed a significant increase in the number of MZ B cells in the spleen and in the percentage of B1b cells in the peritoneal cavity.

3.3   *The role of Zap70 in B cell function*

3.3.1 *Thymus-dependent and Thymus-independent immune responses*

It is of interest to find out whether or not Zap70 plays a role in the function of B cells. Are B cells deficient in Zap70 able to mount normal Thymus-dependent (TD) and Thymus-independent (TI) antibody responses?

Since in $Zap70^{-/-}$ mice, T cell development is blocked at the DP stage and no mature T cells develop, it is not possible to monitor TD immune responses in these mice. Therefore I decided to make mixed bone marrow chimeras with equal contribution of $Zap70^{-/-}$ bone marrow and μMT bone marrow used to reconstitute sublethally irradiated C57BL10-$Rag1^{-/-}$ mice. μMT mice have a mutation in the μ heavy chain and are unable to express a functional μ chain on the cell surface. B cell development is therefore blocked at the pro-B cell stage (Kitamura et al., 1991). In these chimeras all mature B cells come from the $Zap70^{-/-}$ bone marrow and all mature T cells were derived from the μMT bone marrow. As a control I made similar mixed bone marrow chimeras using wt

and μMT bone marrow. These radiation chimeras were then immunised eight weeks after reconstitution with TD or TI antigens and the sera of these mice were analysed for the presence of antigen-specific immunoglobulins.

3.3.2 TI-type 2 response

To examine TI-type 2 immune responses *in vivo*, I immunised the radiation chimeras with 2,4,6-trinitrophenol-Ficoll (TNP-Ficoll), a commonly used TI-type 2 antigen. TI-type 2 antigens activate B cells by strongly crosslinking the BCR (Fultz et al., 1989; Inman, 1975). The predominant isotype produced in this case is IgM, although some class switching can occur to IgG3. I measured TNP-specific antibody responses 7 and 14 days after immunisation.

Although analysis was performed for all Ig Isotypes (IgA, IgG1, IgG2a, IgG2b, IgG3 and IgM), only IgM contained TNP-specific antibodies (Fig.3.13). Radiation chimeras reconstituted with wt/μMT or $Zap70^{-/-}$/μMT bone marrow showed no significant TNP-specific response.

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Although analysis was performed for all Ig Isotypes (IgA, IgG1, IgG2a, IgG2b, IgG3 and IgM), only IgM contained TNP-specific antibodies (Fig.3.13). Radiation chimeras reconstituted with wt/μMT or $Zap70^{-/-}$/μMT bone marrow showed no significant TNP-specific response.

Figure 3.13. Immune response in mice immunised with TNP-Ficoll

Mixed radiation chimeras reconstituted with wt/μMT (green) or *Zap70$^{-/-}$/μMT* (red) bone marrow were immunised 8 weeks after reconstitution with 2,4,6-Trinitrophenyl (TNP)-Ficoll to elicit TI-type II responses. Concentration of TNP-specific IgM on the day before immunisation (day –1) and 7 and 14 days after immunisation with TNP-Ficoll (day +7, day +14) are shown for 7 radiation chimeras of each phenotype. Values for individual mice are shown as circles, and the line indicates the mean of each group. TNP-specific antibody levels for IgG1, IgG2a, IgG2b, IgG3 and IgA were also determined, but were undetectable. No significant difference could be detected (data not shown).

3.3.3 TI-type 1 response

To examine TI-type 1 responses, the radiation chimeras were immunised with 2,4,6-trinitrophenol-lipopolysaccharide (TNP-LPS) (Jacobs and Morrison, 1975). LPS, a major component of the cell walls of gram-negative bacteria, is a typical TI-type 1 antigen, activating B cells via Toll-like receptor (TLR) 4.

All mice showed significant TNP-specific IgM, IgG2b and IgG3 responses, which were similar between wt/μMT and *Zap70$^{-/-}$/μMT* radiation chimeras. Four out of seven

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chimeras of each genotype also showed an anti-IgG1 response at day 14, but again there

was no difference between the genotypes.

Figure 3.14. Antibody production in mice immunised with TNP-LPS

Mixed radiation chimeras reconstituted with wt/μMT (green) or $Zap70^{-/-}$/μMT (red) bone marrow were immunised 8 weeks after reconstitution with 2,4,6-Trinitrophenyl (TNP)-LPS to monitor TI-type I responses. Concentration of TNP-specific antibodies on the day before immunisation (day −1) and 7 and 14 days after immunisation with TNP-LPS (day +7, day +14) are shown for 7 radiation chimeras of each genotype. Values for individual mice are shown as circles, and the line indicates the mean of each group. (A) TNP-specific IgM response, which reached statistical significance ($0.001 < p < 0.01$); (B) TNP-specific IgG1 response; (C) TNP-specific IgG2b response, which reached statistical significance ($p < 0.01$) and (D) TNP-specific IgG3 response, which reached statistical significance ($p < 0.05$). TNP-specific antibody levels for IgG2a and IgA were also determined, but were undetectable (data not shown).

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3.3.4 TD-immune response

Finally, I wanted to examine the role of Zap70 in TD-immune responses *in vivo*. The response of B cells to TD antigens requires direct contact with T cells. Mixed bone marrow chimeras of both genotypes were immunised with a haptenated protein, 2,4,6-trinitrophenol-keyhole limpet haemocyanin (TNP-KLH) (Ludviksson et al., 1997) and anti-TNP immunoglobulin isotypes were measured. Immunisations were carried out without adjuvant, in order to maximise the chances of detecting subtle differences in response. Therefore radiation chimeras were immunised with TNP-KLH in PBS and primary TNP-specific immune responses were measured 7 days and 14 days after immunisation, re-immunised at day 21 and TNP-specific immune responses measured at day 28.

IgM and IgG2b contained TNP-specific antibodies. However, no significant response was obtained after immunisation (Fig. 3.15 a,c). Significant TNP-specific IgG1 and IgG3-responses were obtained only after re-immunisation (Fig. 3.15 b,d). However, no difference was observed between immune responses responses from wild-type or $Zap70^{-/-}$ mice.
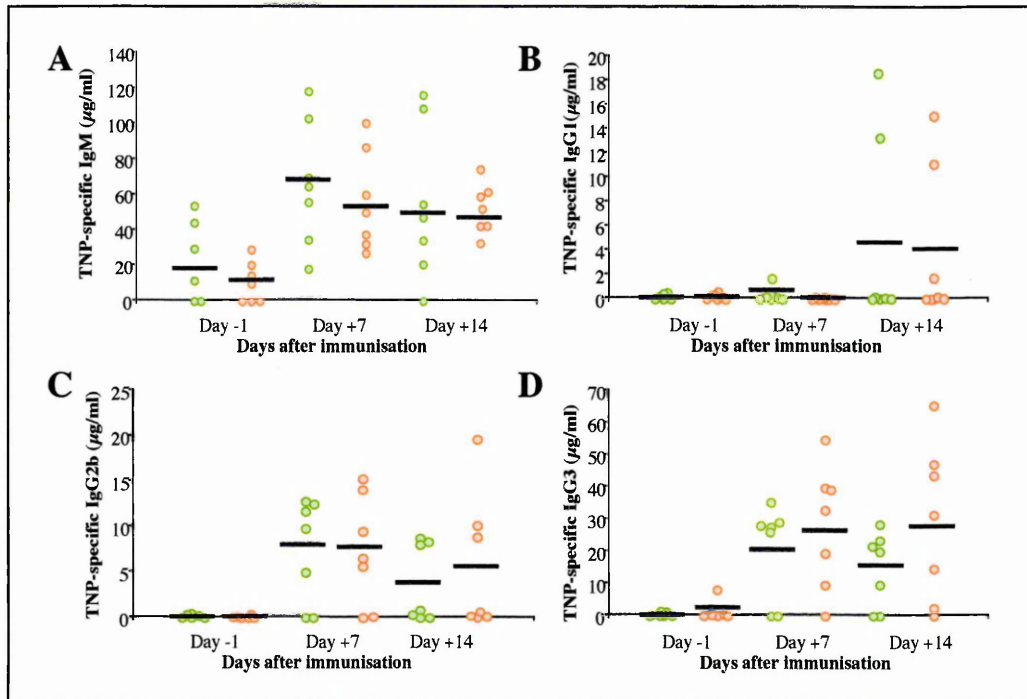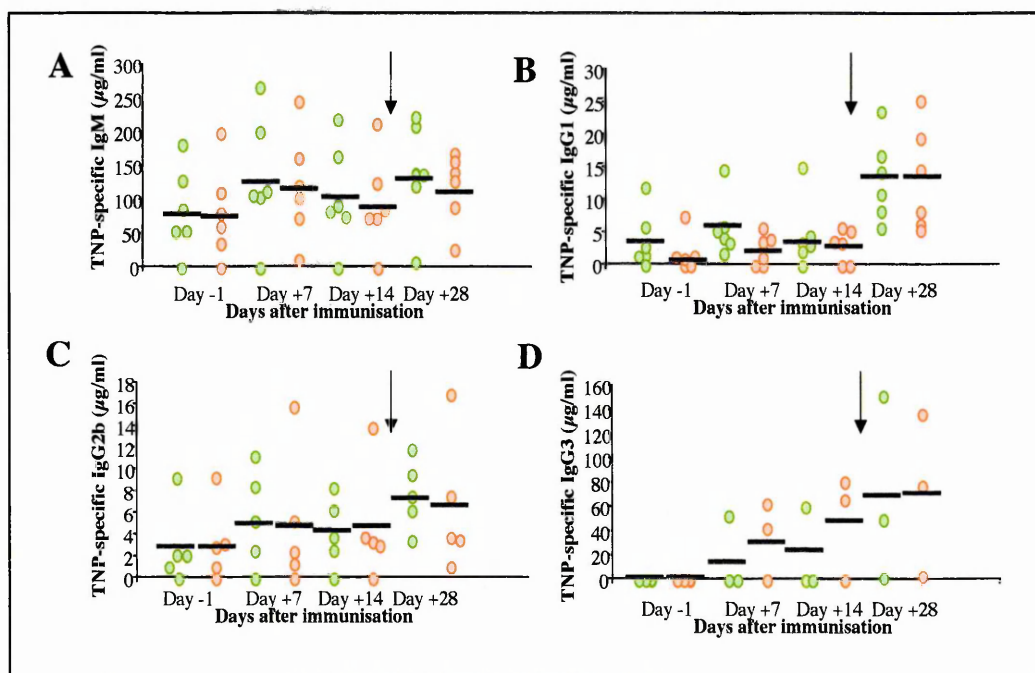
Figure 3.15. Antibody production in mice immunused with TNP-KLH

Mixed radiation chimeras reconstituted with wt/μMT (green) or $Zap70^{-/-}$/μMT (red) bone marrow were immunised 8 weeks after reconstitution with 2,4,6-Trinitrophenyl (TNP)-KLH to monitor TD responses. Concentration of TNP-specific IgM on the day before immunisation (day –1) and 7 and 14 days after immunisation with TNP-KLH (day +7, day +14) are shown for 7 radiation chimeras of each genotype. Responses against the second immunisation day 21 (black arrow) were measured at day 28 (day +28). Values for individual mice are shown as circles, and the line indicates the mean of each group. (A) TNP-specific IgM response; (B) TNP-specific IgG1 response, which reached statistical significance ($p<0.02$); (C) TNP-specific IgG2b response and (D) TNP-specific IgG3 response (for three mice of each phenotype). TNP-specific antibody levels for IgG2a and IgA were also determined, but were undetectable (data not shown).

In summary, I examined the role of Zap70 in the function of B cells by analysing TD and TI immune responses. To do this, I made mixed bone marrow radiation chimeras with $Zap70^{-/-}$ or wt bone marrow mixed with μMT bone marrow and reconstituted sublethally irradiated C57BL/10-$Rag1^{-/-}$ mice. In these mice B cells come from wt or $Zap70^{-/-}$ bone marrow and T cells from μMT bone marrow. Immunisation of these mice with TI antigens TNP-LPS and TNP-Ficoll and with TD antigen TNP-KLH showed no significant differences in the responses of wt and $Zap70^{-/-}$ B cells. However the TNP-Ficoll and TNP-KLH responses were very weak, so these studies will need to be repeated and extended.

I conclude that at least for the responses measured, Zap70 does not play an important role. In contrast Syk, the other member of this kinase family may play a dominant role.

3.3.1 Calcium flux in B cells

One of the earliest proximal signalling events after engagement of the BCR is the mobilization of intracellular calcium. It is known that intracellular calcium mobilization in B cells is completely dependent on Syk. In $Syk^{-/-}$ DT40 cells, which are chicken B lymphoma cells, calcium flux is impaired (Kurosaki et al., 1995).

I wanted to determine whether or not the absence of Zap70 affects BCR-induced calcium flux. Splenic cells were stained with antibodies to CD19, CD21 and CD23 and then labelled with Indo-1-AM. MZ B cells, MRF B cells and NF B cells were defined by CD19 expression and by their different expression of CD21 and CD23 (Fig. 3.16 a).

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Cells were stimulated with anti-IgM-F(ab')$_2$ and calcium mobilization was measured in splenic B cell subsets of wt or $Zap70^{-/-}$ mice. Calcium flux was observed in all B cell populations, whereas no calcium flux was seen in CD19$^-$ cells in the spleen (Fig. 3.16 b and data not shown). An overlay of calcium fluxes in the different B cell subsets of wt and $Zap70^{-/-}$ mice showed little or no difference between the genotypes (Fig. 3.16 c). In the experiment shown it appears that Zap70-/- MRF B cells have a lower BCR-induced calcium flux than wt MRF B cells, however this difference was not observed in a subsequent experiment.

Next I examined calcium fluxes in B cell populations of the peritoneal cavity (Fig. 3.17). Staining with antibodies to CD19, CD5 and CD23 was used to distinguish B2 cells (CD19$^+$CD5$^-$CD23$^+$) from B1a cells (CD19$^+$CD5$^+$CD23$^-$) and B1b cells (CD19$^+$CD5$^-$ CD23$^-$). Cells were stimulated through the BCR by using anti-IgM-F(ab')$_2$ and calcium flux was measured. Calcium flux was observed in all B cell subsets and once again no differences were observed between wt and $Zap70^{-/-}$ mice (Fig. 3.17b,c)

In summary, similar calcium fluxes were obtained in different B cell subsets of the spleen and peritoneal cavity in wt and $Zap70^{-/-}$ mice. Similar results were also obtained using more (2.5μg/10^6 cells) and also less (0.5μg/10^6 cells) anti-IgM to stimulate the cells (data not shown). These results suggest that Zap70 does not play an important role in mobilising calcium flux in response to BCR stimulation.
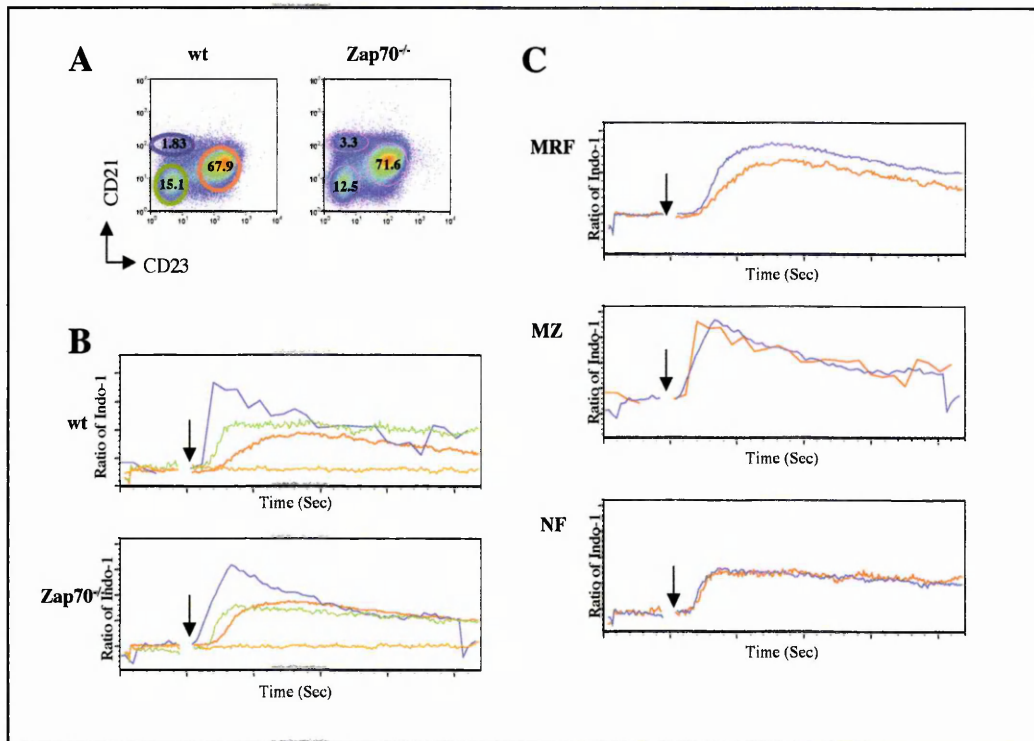
Figure 3.16. Calcium flux in B cell subsets in the spleen

Splenocytes were loaded with Indo-1AM and stained for different B cell subsets.

(A) Flow cytometry plots of wt or $Zap70^{-/-}$ splenocytes gated on $CD19^+$ cells, distinguishing MZ B cells ($CD23^-CD21^{hi}$), MRF cells ($CD23^+CD21^+$) and NF B cells ($CD23^-CD21^-$). Numbers are percentages of cells falling into this gate.

(B) Graphs show the mean ratio of Indo-1 fluorescence at 405nm/485nm as a measure of intracellular calcium in splenic B cell populations: MZ B cells (blue), MRF B cells (red), NF B cells (green) and CD19- cells (orange). Cells were stimulated with anti-F(ab')$_2$ IgM ($1\mu g/ 10^6$ cells) at the time indicated (arrow).

(C) Graphs show the mean ratio of Indo-1 fluorescence at 405nm/485nm as a measure of intracellular calcium in splenic B cell populations from wt (blue) or $Zap70^{-/-}$ (red) mice.

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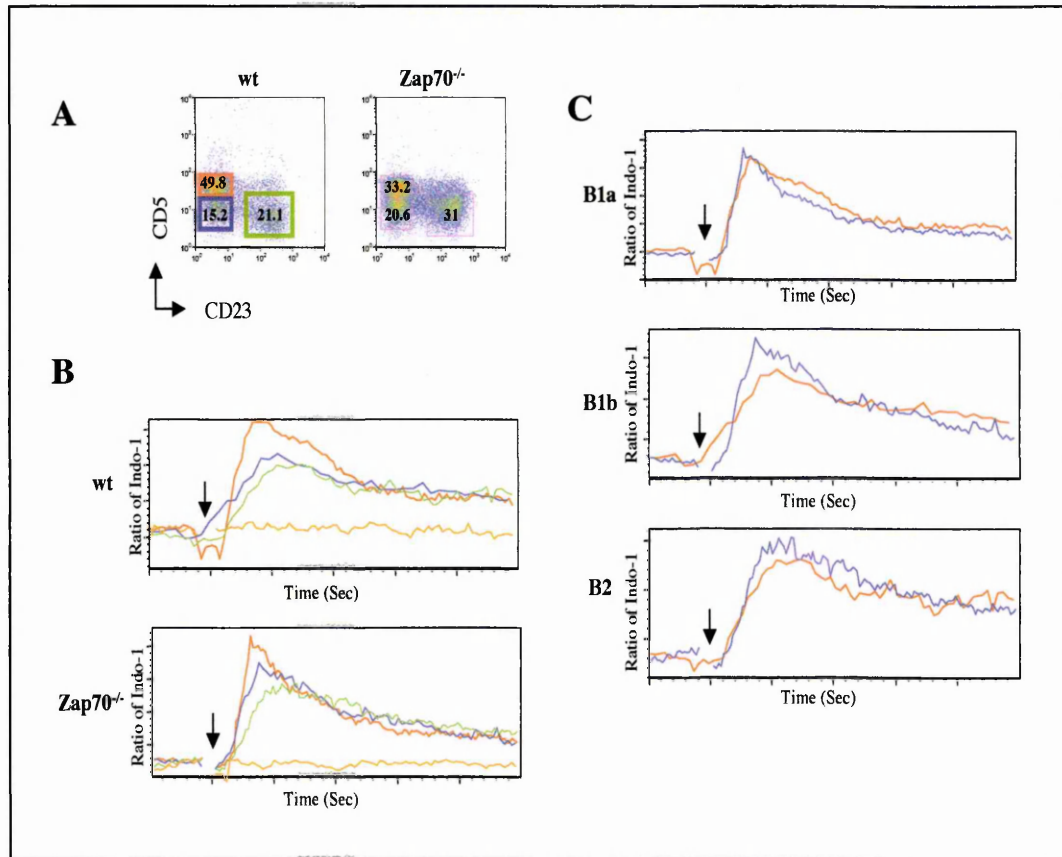
Figure 3.17. Calcium flux in mature B cell subsets in the peritoneal cavity

Peritoneal cavity cells were loaded with Indo-1AM and stained for different B cell subsets.

(A) Flow cytometry plots of wt or *Zap70*$^{-/-}$ cells gated on CD19$^+$ cells, distinguishing B1a cells (CD23$^-$CD5$^+$), B1b cells (CD23$^-$CD5$^-$) and B2 cells (CD23$^+$CD5$^-$). Numbers are percentages of cells falling into this gate.

(B) Graphs show the mean ratio of Indo-1 fluorescence at 405nm/485nm as a measure of intracellular calcium B cell populations: B1a cells (red), B1b cells (blue), B2 cells (green) and CD19- cells (orange). Cells were stimulated with anti-(Fab)$_2$ IgM (1μg/ 10^6 cells) at the time indicated (arrow).

(C) Graphs show the mean ratio of Indo-1 fluorescence at 405nm/485nm as a measure of intracellular calcium B cell populations of the peritoneal cavity of wt (blue) or *Zap70*$^{-/-}$ (red) mice.

4. Overexpression of Zap70 can rescue the *Syk*$^{-/-}$ phenotype

In the absence of Syk, B cell development is partially blocked at the pro-B to pre-B cell transition (Cheng et al., 1995; Turner et al., 1995). A few IgM$^+$IgD$^-$ immature B cells develop, but then there is a second complete block at the immature B to mature B cell transition.

In mice deficient in Zap70 or carrying a mutation that inactivates Zap70 kinase function, there is an arrest at the DP to SP stage in the thymus, due to a block of T cell development at the second checkpoint, i.e. positive selection (Kadlecek et al., 1998; Negishi et al., 1995; Wiest et al., 1997). *Zap70*$^{-/-}$ mice show no block in B cell development. Conversely, no defect in TCR $\alpha\beta$ thymocyte development is found in *Syk*$^{-/-}$ mice (Cheng et al., 1995; Turner et al., 1995).

Mice deficient in both members of the Syk family kinases, Syk and Zap70, show a complete block in B cell development at the pro-B to pre-B cell transition (Schweighoffer et al., 2003), confirming that Zap70 plays a role in early B cell development, most likely because both Syk and Zap70 can transduce signals from the pre-BCR. Furthermore, these mice also show a complete arrest in progression from the DN to DP stage in T cell development, indicating that Syk plays a role at the DN to DP developmental checkpoint, probably because both Syk and Zap70 transduce pre-TCR signals.

Thus while the pre-antigen receptors (pre-BCR and pre-TCR) can apparently signal through either Syk or Zap70, the mature antigen receptors (BCR and TCR) apparently only signal through either Syk or Zap70 alone, since the single knock-outs cause a complete arrest in development at these second checkpoints. This difference in the requirement for these kinases between the pre-antigen receptors and the mature antigen receptors may reflect differences in the expression levels of the kinases at the second checkpoint, or differences in the way these receptors signal. Thus mutation in Zap70 alone arrests T cell development at the DP thymocyte stage due to a failure in positive selection. To address whether this was because not enough Syk was expressed in DP cells to compensate for the lack of Zap70, or whether the TCR is unable to transduce positive selection signals through Syk, Chan and collegues overexpressed Syk in the T cell lineage of *Zap70*$^{-/-}$ mice. They found that this rescued the development of both CD4$^+$ and CD8$^+$ thymocyte and splenocyte populations (Gong et al., 1997). These thymocytes overexpressing Syk are fully functional as measured by their ability to increase [Ca^{2+}], to up-regulate early T cell activation markers, and to proliferate in response to TCR activation. Thus the TCR appears able to signal through both Syk and Zap70, and the developmental block in *Zap70*$^{-/-}$ mice results from insufficient expression of Syk in DP thymocytes.

A similar functional equivalence between Syk and Zap70 has also been suggested for BCR signal transduction. Expression of Zap70 in *Syk*$^{-/-}$ DT40 cells, which are chicken B lymphoma cells, reconstitutes B cell receptor signalling, as measured by induction of tyrosine phosphorylation of cellular proteins and mobilization of cytoplasmic [Ca^{2+}]

(Kong et al., 1995), suggesting that Zap70 is functionally equivalent to Syk in BCR signalling.

The same may be true for the transduction of pre-BCR and BCR signals during mouse development. While Zap70 can partially compensate for the lack of Syk in the pro-B to pre-B cell transition, the complete block at the immature to mature B cell stage in the absence of Syk may be due to the low expression level of Zap70, which is insufficient to drive cells through this second checkpoint.

Alternatively, Syk and Zap70 may have distinct functions at the immature to mature cell transition, and Zap70 may be unable to transduce the BCR positive selection signal. In favour of distinct roles for Zap70 and Syk are the observations that the specific kinase activity of Syk is 100-fold higher than that of Zap70 (Latour et al., 1996), which might lead to phosphorylation of a different set of downstream targets, and that the ability of Syk to be activated in an Lck-independent fashion is not shared by Zap70 (Chu et al., 1996; Latour et al., 1997; Noraz et al., 2000)

To address this issue, I decided to overexpress Zap70 in $Syk^{-/-}$ B lineage cells using retroviral gene transfer technology. $Syk^{-/-}$ embryos die around birth, so as a source of hematopoietic stem cells (HSC) I used fetal liver cells, that were infected with retrovirus *in vitro* and then used to reconstitute irradiated recipients. This method generates chimeric mice in which the hematopoietic system is derived from the transduced donor cells and hence expresses any genes carried by the retroviral vector.

4.1 Packaging cell lines, tranfection and infection conditions

The first step was to optimize retroviral gene transfer into HSC. A key requirement for

such infections is high titres of the retrovirus. To achieve optimal titres I examined

different packaging lines, transfection reagents and infection methods.

Packaging cell lines can efficiently produce large amounts of infectious, replication-

incompetent retrovirus that can transfer genes into mammalian cells. Three different

packaging cell lines were tested to determine which one gave the highest retroviral titres:

PlatE, Phoenix and LinxE (Carnero et al., 2000; Morita et al., 2000; Nolan).

The vector I used for optimising the transfection and infection conditions was the empty

vector, pMSCV-IRES-GFP (Fig.4.1a). The advantage of using this vector for opimising

experiments was that infected cells could be easily distinguished from uninfected cells

by their expression of GFP without the need of staining the cells with antibodies.

I transfected the three different packaging cell lines using different amounts of the

transfection reagents Fugene or Genejuice together with different DNA concentrations

of retroviral vector.
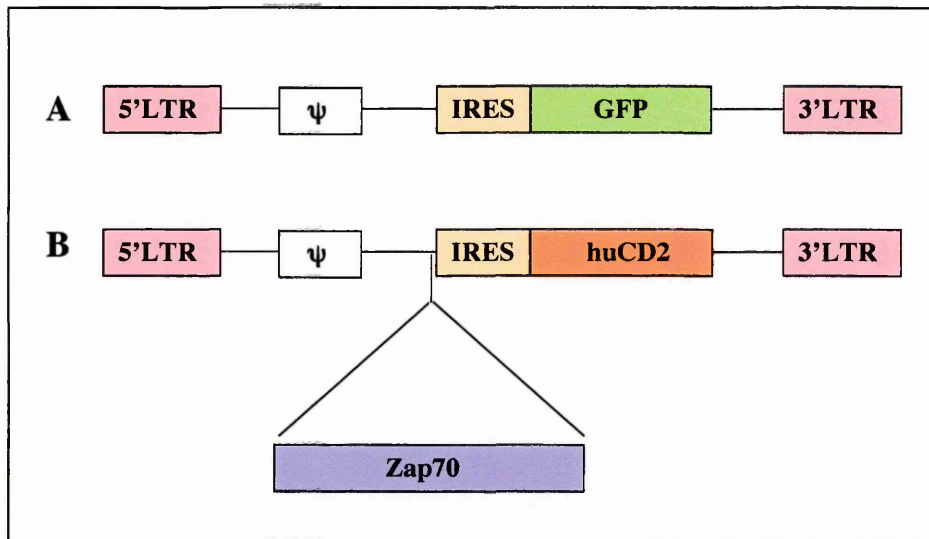
Figure 4.1. Schematic diagram of retroviral vectors
(A) pMSCV-IRES-GFP, (B) pMSCV-IRES-huCD2, Zap70 cDNA (blue) was inserted upstream of the IRES-huCD2, such that this vector expresses both ZAp70 and huCD2. Components of the vectors: IRES, internal ribosome entry site, creme; huCD2, tailless human CD2, red; 5'LTR and 3'LTR, long terminal repeats, pink; GFP, green fluorescence protein, green; ψ, psi, extended viral packaging signal.

Supernatant containing virus was harvested either 24 hours or 48 hours after tranfection and dilutions of it used to infect the mouse fibroblast cell line, NIH-3T3. To measure viral titres, the percentage of GFP⁺ 3T3 cells was determined 24 hours after infection.

Compared to Phoenix and LinXE, the highest titres were consistently achieved using the packaging cell line PlatE (Fig.4.2). Furthermore higher titres were obtained when the retroviral supernatant was harvested at 48 hours rather than 24 hours after transfection. However, no significant difference was observed between the two transfection reagents

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Fugene and Genejuice. I decided to use Genejuice for further experiments since it is more cost-effective.
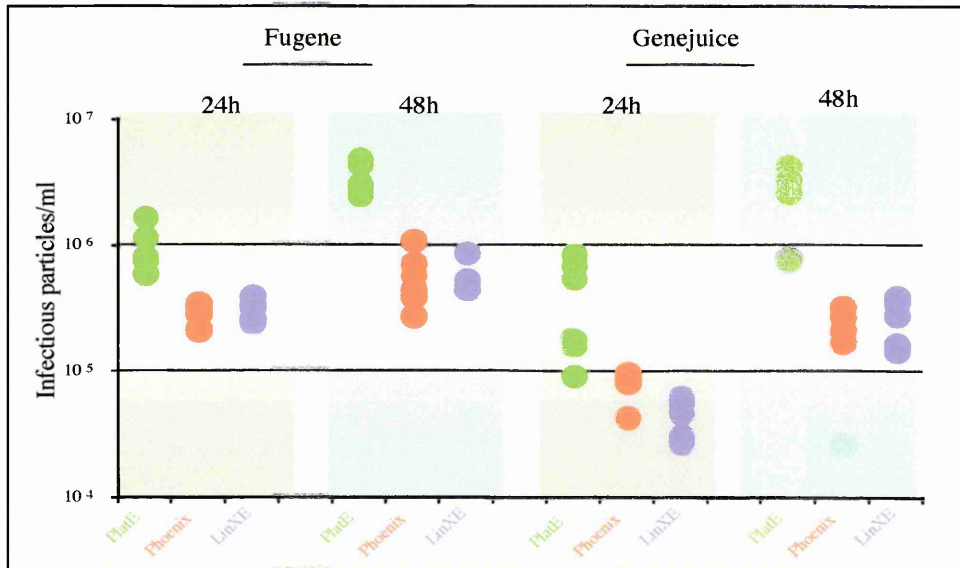
Figure 4.2.   Retrovirus production
Graph shows infectious retroviral particles in the supernatant of PlatE (green), Phoenix (orange) or LinXE (blue) cells transfected either 24 or 48 hours earlier with the pMSCV-IRES-GFP vector using either Fugene or Genejuice.

I achieved higher titres, when harvesting the supernatant containing virus at 48 hours rather than 24 hours after transfection. Next, I wanted to determine whether harvesting the virus 72 hours after transfection would give even higher viral titres. And indeed, the highest titres were achieved when harvesting the retroviral supernatant at 72 hours rather than 48 hours (Fig.4.3). Furthermore, in this experiment the highest titres were obtained using 6 μl transfection reagent and 0.5 μg DNA, although subsequent experiments did not confirm this (data not shown).

In summary, the highest titres were achieved using the packaging cell line, PlatE and harvesting supernatant containing virus 72 hours after transfection. Both of the transfection reagents gave similar results, but Genejuice is more cost-effective.
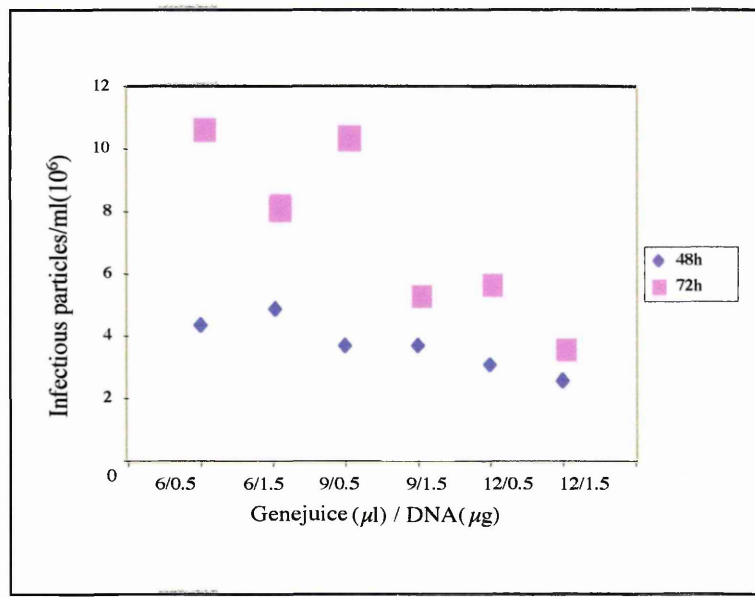
Figure 4.3. Determining optimal infection conditions
Graph shows the concentration of infectious retroviral particles in the supernatant of PlatE cells transfected 48 to 72 hours earlier with pMSCV-IRES-GFP using 6-12 μl Genejuice and 0.5-1.5 μg DNA as indicated.

4.2 Overexpression of Zap70 in Syk$^{-/-}$ mice via retroviral gene transfer

4.2.1 Radiation chimeras reconstituted with infected Syk$^{-/-}$ fetal liver

Is B cell development in Syk$^{-/-}$ mice completely blocked at the immature to mature B cell transition, because Zap70 cannot drive the cells through this selection checkpoint, or

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because Zap70 is not expressed at sufficiently high levels? To address this question I made radiation chimeras using $Syk^{-/-}$ fetal liver cells that had been infected *in vitro* with retrovirus expressing Zap70.

$Syk^{-/-}$ fetal livers (embryonic day 15.5 (E15.5), all Ly5.2$^+$) were cultured to induce the HSCs to divide and then infected twice with retroviral supernatant from PlatE cells that were either transfected with pMSCV-IRES-huCD2-Zap70 or the empty pMSCV-IRES-huCD2 (Fig.4.1). pMSCV-IRES-huCD2 expresses a "tailess" human CD2 (huCD2) composed of the extracellular and transmembrane domains of huCD2, but missing the cytoplasmic domain (Fig.4.1b). The extracellular domain of human CD2 does not bind the murine ligands of murine CD2. These features make the tailles huCD2 a useful marker for infection by the retrovirus, because is unable to signal into the infected cell and so is unlikely to perturb cellular physiology.

These infected fetal liver cells were used to reconstitute lethally irradiated C57BL/6.SJL (Ly5.1$^+$) hosts. A small number of infected $Syk^{-/-}$ fetal liver cells were kept in culture in order to determine the percentage of infected cells, by looking at the expression of huCD2 (Fig.4.4). Typically, 35-45% of cultured fetal liver cells were infected with the empty vector and 50-60% with retroviral vector expressing Zap70. This correlated with the retroviral titres obtained by infecting NIH-3T3 cells. Typically, retroviral titres in the supernatant from PlatE cells transfected with the empty vector were lower than titres achieved using pMSCV-IRES-huCD2-Zap70 (Fig.4.4 and data not shown).
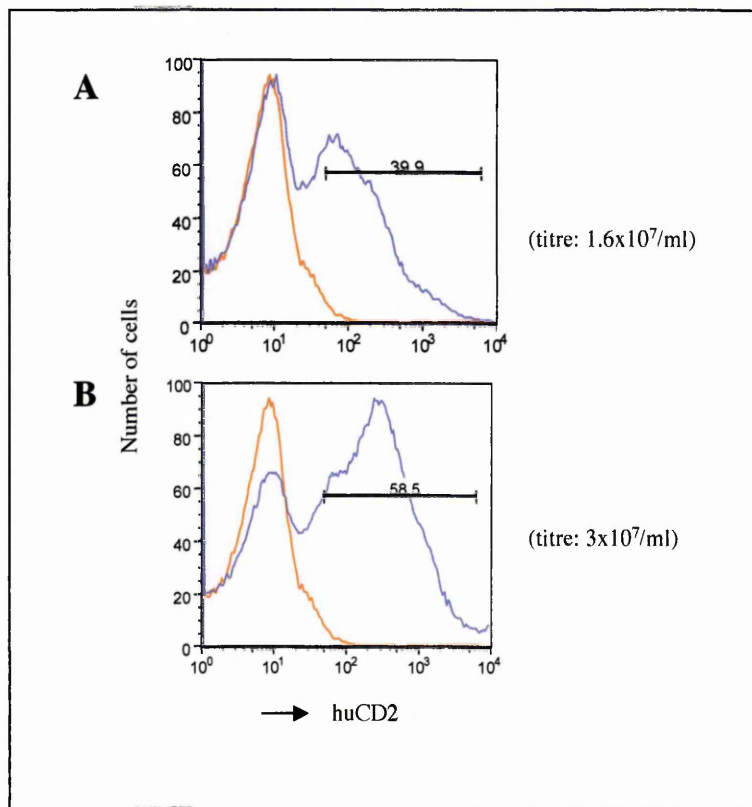
Figure 4.4. Efficiency of retroviral infection of fetal liver cells
Histograms showing expression of huCD2 on *Syk*$^{-/-}$ fetal liver cells that were uninfected (red line) or infected (blue line) with pMSCV-IRES-huCD2 (A) or pMSCV-IRES-huCD2-Zap70 (B). Marker indicates percentage of huCD2$^+$ cells; titres of retrovirus in the supernatants used to infect the cells are shown.

Survival of mice 9 weeks after reconstitution confirmed that the *ex vivo* culture protocol did not compromise the ability of cultured HSC to mediate long-term radioprotection.

Mice were sacrificed 9 weeks after transfer and organs were analysed. Donor cells, which expressed the marker CD45.2 (Ly5.2) could be easily distinguished from host cells, which were CD45.1 (Ly5.1) positive.

4.2.1.1 Bone marrow

First I examined the B lineage cells in the bone marrow of the radiation fetal liver chimeras (Fig.4.5). In radiation chimeras reconstituted with $Syk^{-/-}$ fetal liver cells that were infected with retrovirus expressing Zap70 ($Syk^{-/-}$*Zap70), over 60% of B lineage cells coming from the donor (CD19$^+$Ly5.2$^+$) were infected, as judged by expression of huCD2 (Fig.4.5a). Then, gating on infected B cells, I assessed whether these $Syk^{-/-}$ cells, presumably overexpressing Zap70, could overcome the developmental arrest and become mature IgM$^+$IgD$^+$ B cells. As shown in Fig.4.5a, only huCD2$^+$ cells could develop into IgM$^+$IgD$^+$ cells (7% of cells). In contrast, uninfected (huCD2$^-$) cells, were still completely blocked at the immature B cell stage (IgM$^+$IgD$^-$).

Examining the bone marrow of chimeras, which were reconstituted with $Syk^{-/-}$ fetal liver cells infected with the empty vector ($Syk^{-/-}$*empty vector) showed that once again about 60% of donor-derived B lineage cells were infected (huCD2$^+$). But neither the infected nor the uninfected B lineage cells developed into mature IgM$^+$IgD$^+$ B cells (Fig.4.5b), remaining at the immature IgM$^+$IgD$^-$ stage, typical of the developmental arrest previously seen in $Syk^{-/-}$ radiation chimeras.

Figure 4.5. Bone marrow of radiation chimeras reconstituted with infected $Syk^{-/-}$ fetal liver

Flow cytometric analysis of bone marrow cells from irradiated C57BL/6.SJL-Ly5.1 mice reconstituted with $Syk^{-/-}$ fetal liver cells (Ly 5.2$^+$) infected with pMSCV-IRES-huCD2-Zap70 (A) or pMSCV-IRES-huCD2 (B). Plots on the left show gated donor-derived B lineage cells (CD19$^+$Ly5.2$^+$), which are then separated into uninfected (huCD2$^-$) and infected (huCD2$^+$) cells in the middle plots. Finally, in each of these populations, B cell development was assessed by examining expression of IgM and IgD, as shown in the plots on the right. Numbers show percentages of cells falling into the gates or quadrants.

4.2.1.2 Spleen

Next, I examined the spleen of radiation chimeras for the development of mature B cells. Up to 90% of all donor-derived B cells (CD19$^+$Ly5.2$^+$) from radiation chimeras reconstituted 9 weeks earlier with $Syk^{-/-}$*Zap70 fetal liver cells, were infected, as identified by their huCD2 expression on the cell surface (Fig.4.6a). Furthermore,

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infected donor-derived B cells were able to develop into mature IgM⁺IgD⁺ B cells (Fig.4.6a).

In contrast, no donor-derived B cells could be found in the spleen of radiation chimeras reconstituted with $Syk^{-/-}$*empty vector fetal liver cells (Fig.4.6b). This mimics the phenotype seen in $Syk^{-/-}$ radiation chimeras. Radiation chimeras reconstituted with $Syk^{-/-}$ fetal liver have no detectable IgM⁺IgD⁺ conventional mature B cells (Turner et al., 1995). To prove that donor-derived cells were infected, I looked at non-B cells coming from the donor (Ly5.2⁺CD19⁻). Here I found over 20% of huCD2⁺ donor-derived cells (Fig.4.6b), confirming that the development of IgM⁺IgD⁺ B cells was dependent on the expression of Zap70.
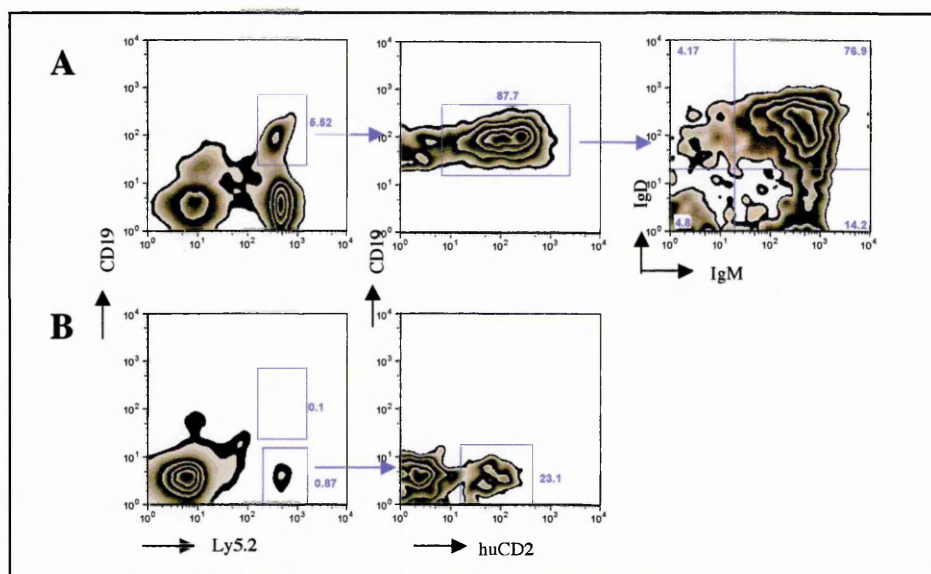
Figure 4.6. Spleen of radiation chimeras reconstituted with infected $Syk^{-/-}$ fetal liver
Flow cytometric analysis of spleen cells from irradiated C57BL/6.SJL-Ly5.1 mice reconstituted with $Syk^{-/-}$ fetal liver cells (Ly5.2⁺) infected with pMSCV-IRES-huCD2-Zap70 (A) or pMSCV-IRES-huCD2 (B), plots on the left show donor-derived B cells (CD19⁺Ly5.2⁺) or non-B cells (CD19⁻Ly5.2⁺). Plots in the middle show huCD2 expression on the indicated populations. Plot in the top right shows IgM and IgD expression in the infected donor-derived B cells (CD19⁺Ly5.2⁺huCD2⁺). Numbers show percentages of cells falling into gates.

4.2.1.3 Lymph nodes

The same was found in the lymph nodes of the radiation chimeras. Donor-derived B cells could only be detected in lymph nodes of chimeras reconstituted with $Syk^{-/-}$*Zap70 (Fig.4.7a), but not in radiation chimeras reconstituted with $Syk^{-/-}$*empty vector (Fig.4.7b). HuCD2$^+$ cells could be detected lymph nodes of both types of radiation chimeras. In chimeras reconstituted with $Syk^{-/-}$*Zap70, 80% of donor-derived B cells were infected and were able to develop into mature B cells (Fig.4.7a). In contrast, no donor-derived B cells could be detected in chimeras reconstituted with $Syk^{-/-}$*empty vector (Fig.4.7b).
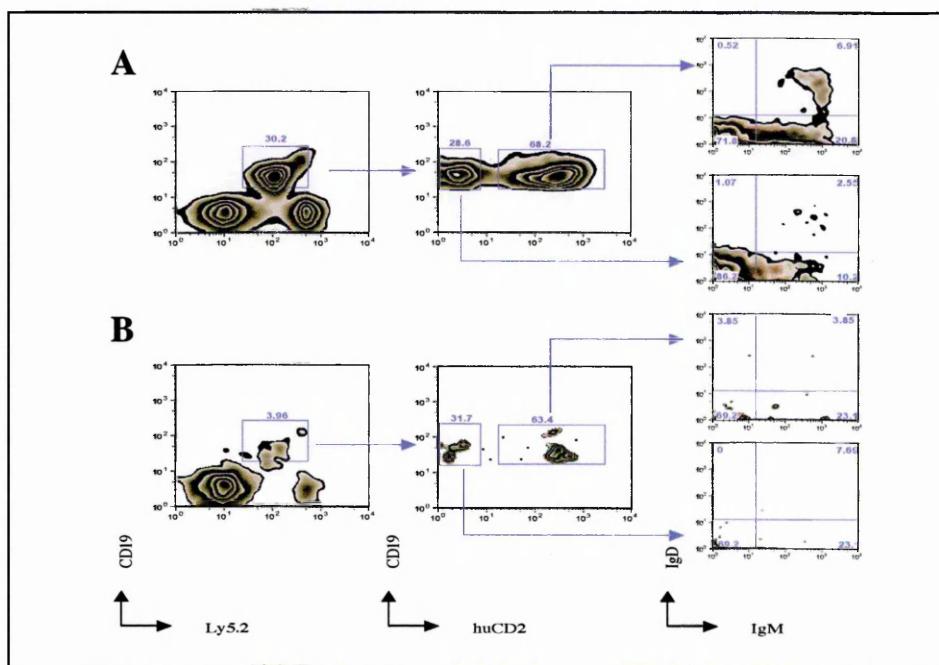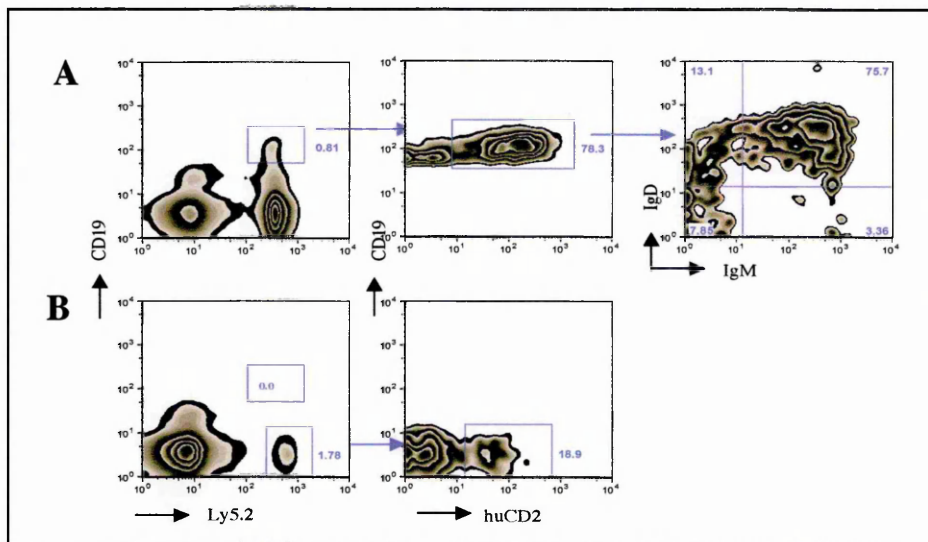

Figure 4.7. Lymph Nodes of radiation chimeras reconstituted with infected $Syk^{-/-}$ fetal liver

Flow cytometric analysis of lymph node cells from irradiated C57BL/6.SJL-Ly5.1 mice reconstituted with $Syk^{-/-}$ fetal liver cells (Ly5.2$^+$) infected with pMSCV-IRES-huCD2-Zap70 (A) or pMSCV-IRES-huCD2 (B), plots on the left show donor-derived B cells (CD19$^+$Ly5.2$^+$) or non-B cells (CD19$^-$Ly5.2$^+$). Plots in the middle show huCD2 expression on the indicated populations. Plot in the top right shows IgM and IgD expression in the infected donor-derived B cells (CD19$^+$Ly5.2$^+$huCD2$^+$). Numbers show percentages of cells falling into gates.

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4.2.1.4 Zap70 expression levels

Next, I determined Zap70 expression levels in donor-derived B cells from chimeras reconstituted with $Syk^{-/-}$*Zap70, compared to wild-type B cells from C57BL/6.SJL-Ly5.1 mouse as a control. Fig. 4.8 shows a typical example of Zap70 expression levels in lymph node cells of chimeras. Infected donor-derived B cells from chimeras reconstituted with $Syk^{-/-}$*Zap70 had higher intracellular Zap70 expression levels than a normal wild-type B cell, but their Zap70 expression levels were still lower than that found in T cells (Fig.4.8c).
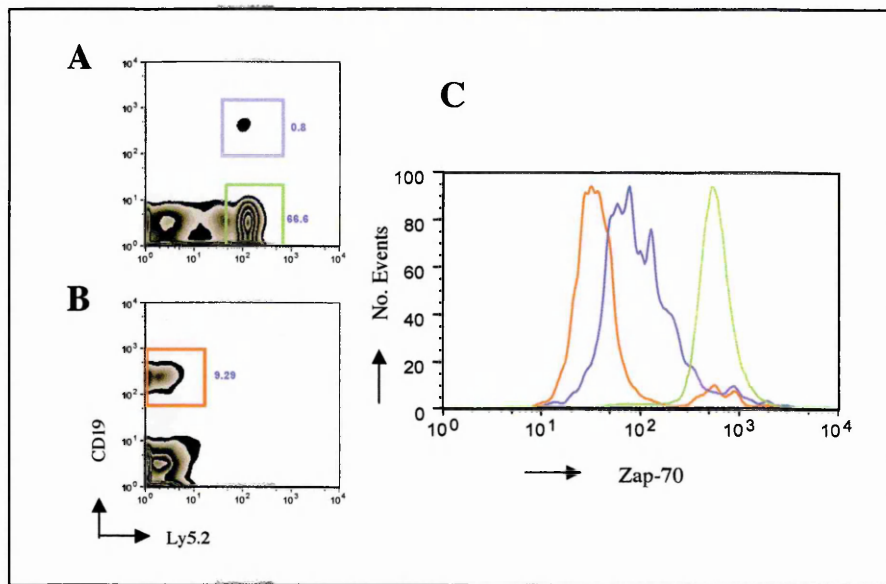
Figure 4.8.   Zap70 expression levels in a radiation chimera reconstituted with infected $Syk^{-/-}$ fetal liver
Flow cytometric analysis of CD19 and Ly5.2 expression on lymph node cells from a radiation chimera reconstituted with $Syk^{-/-}$*Zap70 fetal liver cells (A) or lymph node cells from a C57BL/6.SJL-Ly5.1 mouse (B). Gates show donor-derived B cells (blue), donor-derived non-B cells (green) and uninfected B cells (red). (C) Histogram of Zap70 expression levels in cells from gates in (A) and (B). Colours correspond to the colours of the gates. Numbers show percentages of cells falling within gates.

In summary, these sets of experiment showed that by overexpressing Zap70 in $Syk^{-/-}$ B lineage cells, the developmental block at the immature B cells stage could be overcome and cells could develop into $IgM^{+}IgD^{+}$ mature B cells in the spleen, lymph nodes and the bone marrow. Furthermore, these experiments demonstrate, that higher than endogenous levels of Zap70 were necessary for $Syk^{-/-}$ B cells to develop into mature B cells, but that the expression levels of Zap70 did not need to be as high as found in T cells.

4.2.2 Radiation chimeras reconstituted with infected wt, $Syk^{-/-}$ or $Syk^{-/-}Zap70^{-/-}$ fetal liver

$Syk^{-/-}$ B lineage cells still have endogenous Zap70, but $Syk^{-/-}Zap70^{-/-}$ B cells have no member of the Syk-family of tyrosine kinases, and are therefore a cleaner model to observe whether overexpression of Zap70 via retroviral gene transfer, can drive cells through both selection checkpoints (pro-B to pre-B cell and immature B to mature B cell).

In the next set of experiments, I looked at B cell development in chimeras reconstituted with either $Syk^{-/-}$ fetal liver infected with retrovirus expressing Zap70 ($Syk^{-/-}$*Zap70), $Syk^{-/-}Zap70^{-/-}$ fetal liver infected with retrovirus expressing Zap70 ($Syk^{-/-}Zap70^{-/-}$*Zap70) or wild type fetal liver infected with retrovirus expressing Zap70 (wt*Zap70) as a control. In the case of chimeras reconstituted with infected $Syk^{-/-}$ fetal liver or wt fetal liver, donor fetal livers were $Ly5.2^{+}$, $H2^{b}$ and the irradiated hosts were $Ly5.1^{+}$, $H2^{b}$. However, the only $Syk^{-/-}Zap70^{-/-}$ fetal livers available were $Ly9.2^{+}$, $H2^{d}$. Therefore I had to use BALB/c mice as hosts, which were $Ly9.1^{+}$, $H2^{d}$. Chimeras were sacrificed 8-10

weeks after transfer, a time point sufficient to ensure long-term reconstitution and donor-derived B lineage cells were analysed in organs of these mice.

4.2.2.1 Bone marrow

First I examined the bone marrow of the chimeras. In chimeras reconstituted with wt*Zap70 around 10% of all donor-derived bone marrow cells (Ly5.2$^+$) were infected (Fig.4.9a). B cells were present in both infected and uninfected donor-derived cells and in both cases, developed into mature B cells (Fig.4.9a). Similarly, in chimeras reconstituted with $Syk^{-/-}$*Zap70, around 5% of donor-derived cells (Ly5.2$^+$) were huCD2$^+$. As observed in section 4.2.1, infected $Syk^{-/-}$ B cells were able to develop into mature IgM$^+$IgD$^+$ cells, because they expressed Zap70, whereas uninfected B cells were completely arrested at the immature B cell stage (Fig.4.9b). In chimeras reconstituted with $Syk^{-/-}Zap70^{-/-}$*Zap70 more than half of all donor-derived cells (Ly9.1$^-$) were huCD2$^+$. Once again, infected B cells were able to develop into mature B cells, whereas uninfected B cells were mostly IgM$^-$IgD$^-$ B cells (Fig.4.9c). Surprisingly, a few IgM$^+$IgD$^+$ B cells were observed in the uninfected $Syk^{-/-}Zap70^{-/-}$ B cells. One possible explanation was that these B cells were infected and thus expressed Zap70, but their huCD2 expression was below detection levels. To examine this, I looked at the Zap70 expression levels in B cell populations of these mice. I found that both huCD2$^+$ and huCD2$^-$ donor-derived B lineage cells expressed higher levels of Zap70 than found in host-derived B lineage cells (Fig.4.10). Thus at least some huCD2$^-$ donor-derived B

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lineage cells must have been infected, but the level of huCD2 expressed by them was below the level of detection.
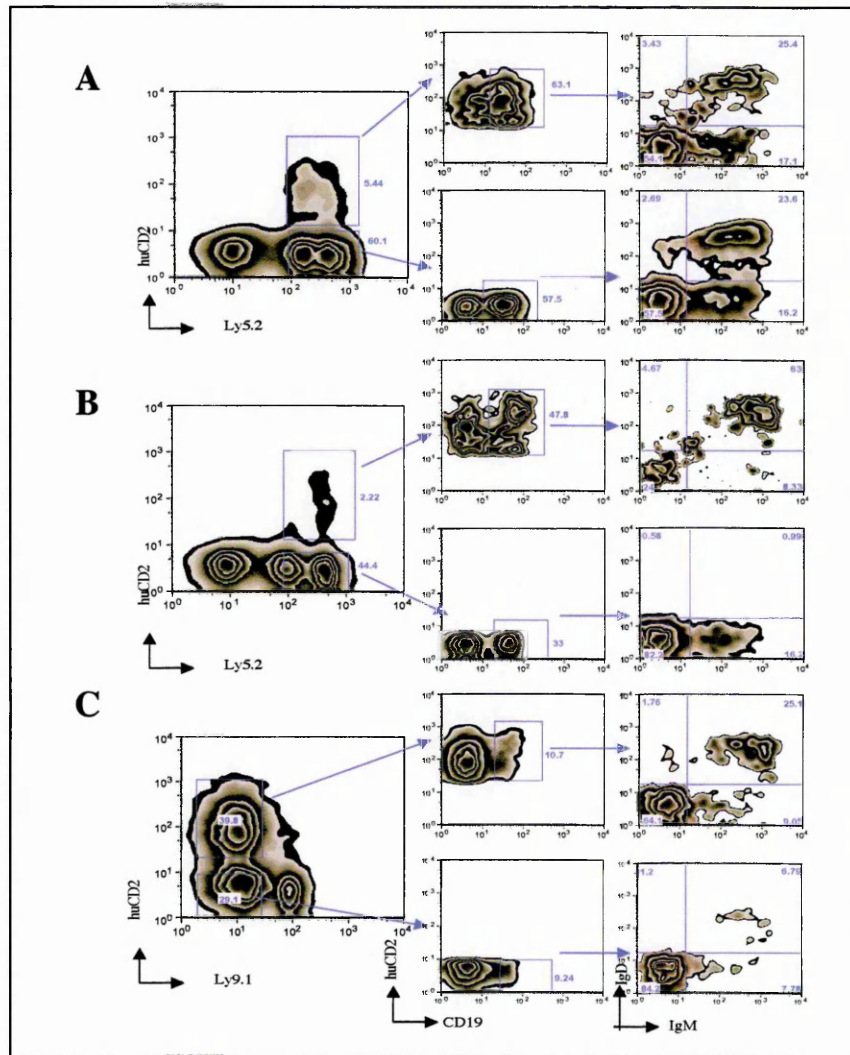
Figure 4.9. Bone marrow of radiation chimeras
Flow cytometric analysis of bone marrow cells from radiation chimeras. (A) C57BL/6.SJL-Ly5.1 mice reconstituted with wt*Zap70 fetal liver cells (Ly5.2⁺). (B) C57BL/6.SJL-Ly5.1 mice reconstituted with $Syk^{-/-}$*Zap70 fetal liver cells (Ly5.2⁺). (C) Balb/c mice (Ly9.1⁺) reconstituted with $Syk^{-/-}Zap70^{-/-}$*Zap70 fetal liver cells (Ly9.2⁺). Donor-derived cells (Ly5.2⁺ in (A) and (B) and Ly9.2⁺ in (C)) were separated into infected and uninfected cells on the basis of huCD2 expression. B lineage cells (CD19⁺) in these populations were analyses for expression of IgM and IgD. Numbers represent percentages falling into gates.

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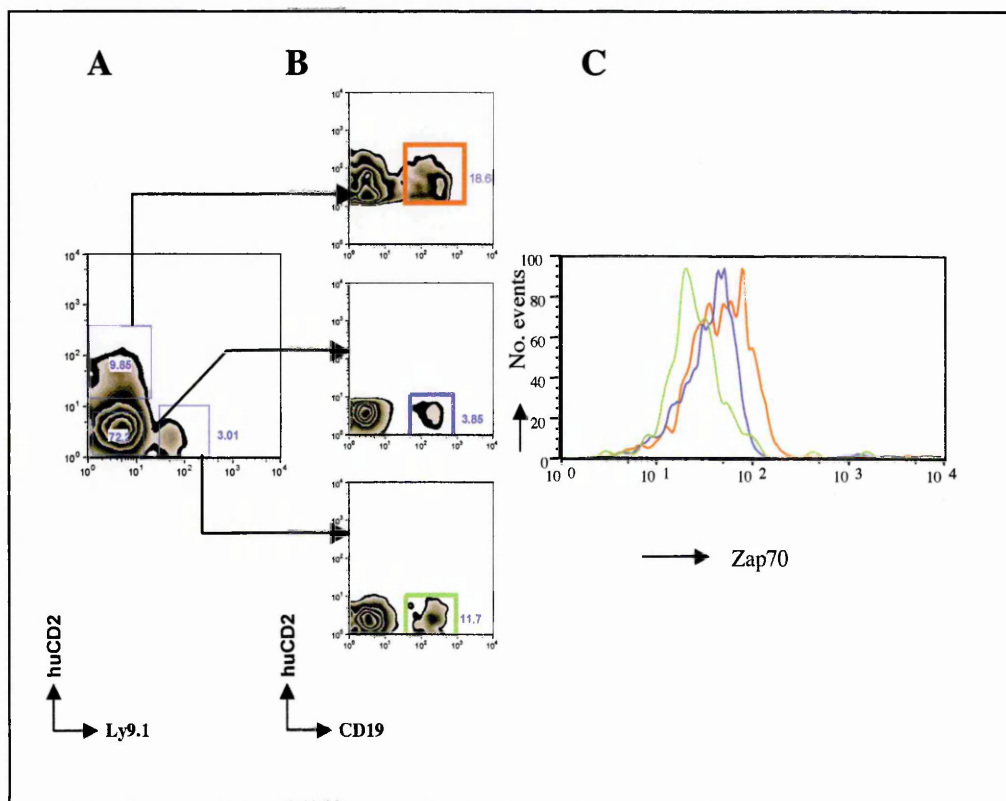
Figure 4.10. Zap70 expression on bone marrow B cells of radiation chimeras reconstituted with infected *Syk⁻/⁻Zap70⁻/⁻**Zap70 fetal liver cells.

Flow cytometric analysis of bone marrow cells from radiation chimeras made by reconstituting Balb/c (Ly9.1⁺) mice with *Syk⁻/⁻Zap70⁻/⁻**Zap70 fetal liver cells. (A) Expression of huCD2 and Ly9.1 distinguishes infected donor-derived cells (huCD2⁺Ly9.1⁻), uninfected donor-derived cells (huCD2⁻Ly9.1⁻) and host cells (huCD2⁻ Ly9.1⁺). (B) Each of the three populations in (A) was analysed for B lineage cells (CD19⁺). (C) Zap70 expression in huCD2⁺ donor-derived B cells (red), huCD2⁻ donor-derived B cells (blue) and host B cells (green), gates as in (A) and (B). Numbers represent percentages of cells falling into gates.

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4.2.2.2 Spleen

Next I examined the spleens of the chimeras. In chimeras reconstituted with wt*Zap70, B cells were present in both uninfected and infected donor-derived cells. As expected, both uninfected and infected B cells were able to develop into IgM$^+$IgD$^+$ mature B cells (Fig. 4.11a). huCD2$^+$ and huCD2$^-$ B cells were able to develop into B2 (or MRF) cells (CD23$^+$CD21^{low}) as well as marginal zone (MZ) B cells (CD23$^-$CD21^{high}) and newly formed (NF) B cells (CD23$^-$CD21$^-$) (Fig.4.12a).

As shown before, in chimeras reconstituted with $Syk^{-/-}$*Zap70 FL cells, only infected donor-derived cells gave rise to B cells, which developed into mature IgM$^+$IgD$^+$ B cells (Fig.4.11b). huCD2$^+$ B cells were mostly B2 cells (Fig.4.12b). In contrast, MZ B cells and NF B cells could not be clearly distinguished.

The same was observed in chimeras reconstituted with $Syk^{-/-}Zap70^{-/-}$*Zap70. IgM$^+$IgD$^+$ mature B cells were found in infected donor-derived cells, whereas no B cells could be seen in uninfected donor-derived cells (Fig.4.12c). huCD2$^+$ B cells developed into B2 cells, but, as in chimeras reconstituted with $Syk^{-/-}$*Zap70, MZ and NF B cells could not be clearly identified (Fig.4.12c). It may be that chimeras reconstituted with $Syk^{-/-}$*Zap70 or $Syk^{-/-}Zap70^{-/-}$*Zap70 do not express enough Zap70 to allow differentiation of MZ B cells, if the development of these cells is dependent on the strength of BCR signalling.

Figure 4.11. Spleen of radiation chimeras (I)

Flow cytometric analysis of spleen cells from radiation chimeras made by reconstituting C57BL/6.SJL-Ly5.1 mice with (A) wt*Zap70 (Ly5.2$^+$) or (B) $Syk^{-/-}$*Zap70 (Ly5.2$^+$) fetal liver cells, or (C) Balb/c (Ly9.1$^+$) mice with $Syk^{-/-}Zap70^{-/-}$*Zap70 (Ly9.2$^+$) fetal liver cells. Plots on the left show gates on infected (huCD2$^+$) or uninfected (huCD2$^-$) donor-derived cells ((A), (B) Ly5.2$^+$; (C) Ly9.2$^+$), which were then analysed for CD19 expression (middle plots) and CD19$^+$ cells further analysed for expression of IgM and IgD. Numbers represent percentages falling into gates or quadrants.

Figure 4.12. Spleen of radiation chimeras (II)

Flow cytometric analysis of spleen cells from radiation chimeras made by reconstituting C57BL/6.SJL-Ly5.1 mice with (A) wt*Zap70 (Ly5.2$^+$) or (B) $Syk^{-/-}$*Zap70 (Ly5.2$^+$) fetal liver cells, or (C) Balb/c (Ly9.1$^+$) mice with $Syk^{-/-}Zap70^{-/-}$*Zap70 (Ly9.2$^+$) fetal liver cells. Plots on the left show gates on infected (huCD2$^+$) or uninfected (huCD2$^-$) donor-derived cells ((A), (B) Ly5.2$^+$; (C) Ly9.2$^+$), which were then analysed for CD19 expression (middle plots) and CD19$^+$ cells further analysed for expression of CD21 and CD23. Gates identify newly formed (CD21$^-$CD23$^-$), mature recirculating follicular (CD21$^+$CD23$^+$) and marginal zone (CD21^{hi}CD23$^-$) B cells. Numbers represent percentages of cells falling into gates or quadrants.

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A similar picture was found in the lymph nodes of the chimeras (Fig.4.13). HuCD2$^+$ and huCD2$^-$ B cells from chimeras reconstituted with wt*Zap70 could develop into IgM$^+$IgD$^+$ B cells (Fig.4.13a). Whereas chimeras reconstituted with *Syk*$^{-/-}$*Zap70 or *Syk*$^{-/-}$*Zap70*$^{-/-}$*Zap70, only huCD2$^+$ donor-derived cells gave rise to IgM$^+$IgD$^+$ B cells (Fig.4.13b,c).

Figure 4.13. Lymph nodes of radiation chimeras
Flow cytometric analysis of lymph node cells from radiation chimeras made by reconstituting C57BL/6.SJL-Ly5.1 mice with (A) wt*Zap70 (Ly5.2$^+$) or (B) *Syk*$^{-/-}$*Zap70 (Ly5.2$^+$) fetal liver cells, or (C) Balb/c (Ly9.1$^+$) mice with *Syk*$^{-/-}$*Zap70*$^{-/-}$*Zap70 (Ly9.2$^+$) fetal liver cells. Plots on the left show gates on infected (huCD2$^+$) or uninfected (huCD2$^-$) donor-derived cells ((A), (B) Ly5.2$^+$; (C) Ly9.2$^+$), which were then analysed for CD19 expression (middle plots) and CD19$^+$ cells further analysed for expression of IgM and IgD. Numbers represent percentages falling into gates or quadrants.

4.2.2.4 Peritoneal cavity

Next, I analysed the peritoneal cavity of the chimeras. In chimeras reconstituted with wt*Zap70, huCD2$^+$ and huCD2$^-$ donor-derived cells gave rise to B cells. These B cells were mostly B2 cells (CD19$^+$Mac1$^-$), although a small number of B1 cells (CD19$^+$Mac1$^+$) could be seen (Fig.4.14a). However the number of B1 cells was much smaller than B2 cells. In radiation chimeras reconstituted with $Syk^{-/-}$*Zap70 or $Syk^{-/-}$ $Zap70^{-/-}$*Zap70 fetal liver cells, only huCD2$^+$ donor-derived B cells were able to develop into B cells, which were mostly B2 cells (Fig.4.14b,c).

4.2.2.5 Thymus

Finally, the thymi of the chimeras were analysed. In chimeras reconstituted with either wt*Zap70 or $Syk^{-/-}$*Zap70, both, huCD2$^+$ and huCD2$^-$ donor-derived cells showed normal T cell development. This was not surprising, as both types of chimeras expressed Zap70 (Fig.4.15 a,b). However, in radiation chimeras reconstituted with $Syk^{-/-}Zap70^{-/-}$ *Zap70 fetal liver cells, over 95% of donor-derived cells were huCD2$^+$ and these cells showed normal T cell development, whereas huCD2$^-$ thymocytes were completely blocked at the DN stage (Fig.4.15c, and data not shown) as previously described in mice deficient for Syk and Zap70 (Cheng et al., 1997).
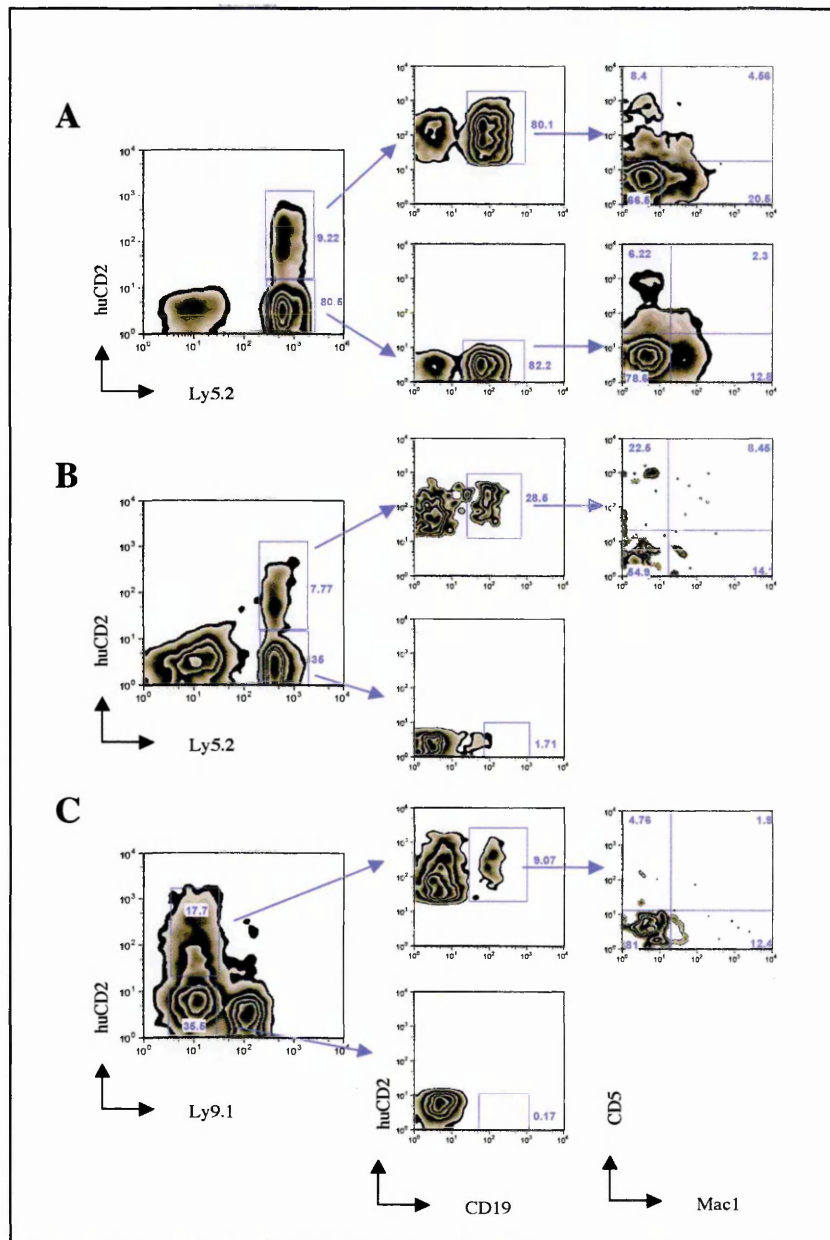
Figure 4.14. Peritoneal Cavity of radiation chimeras
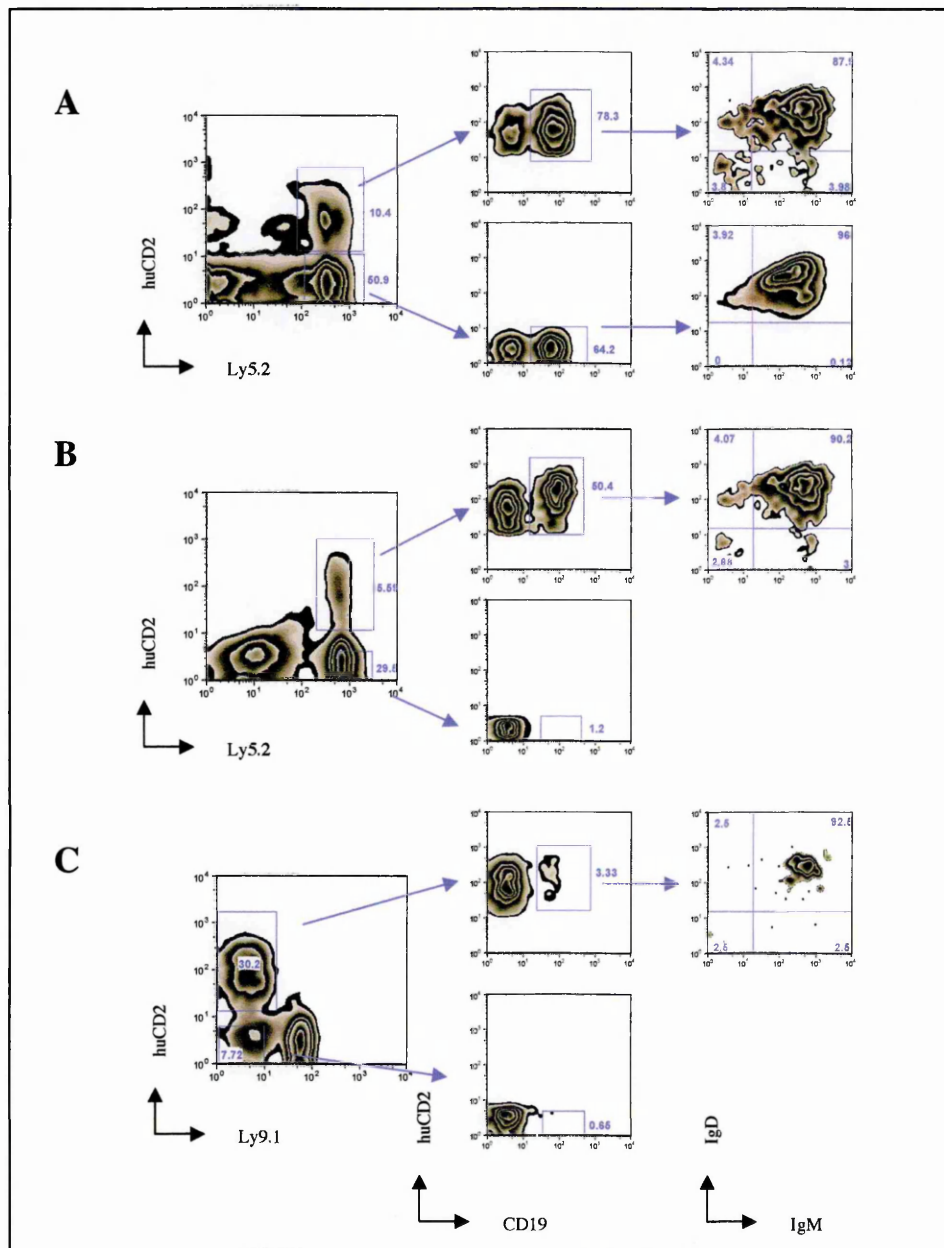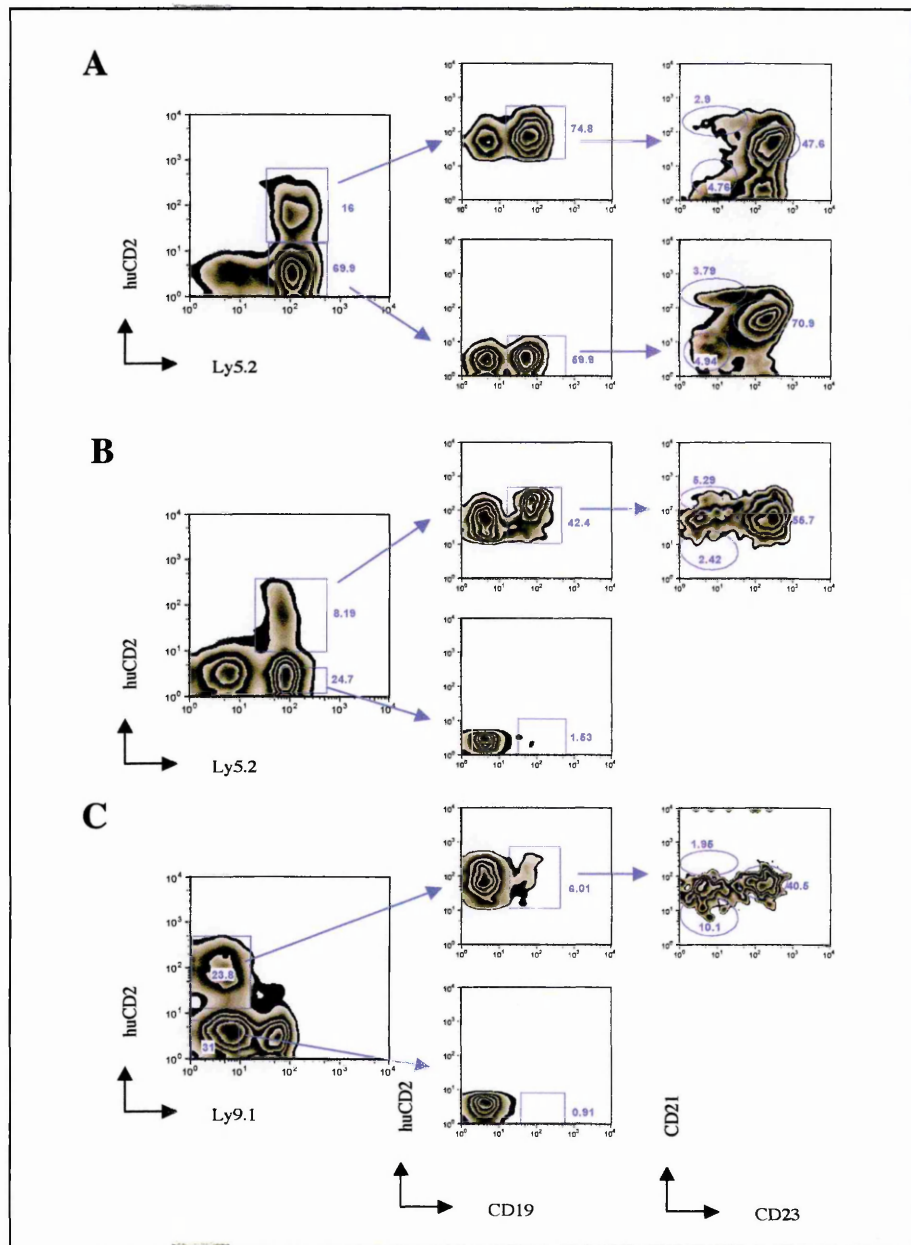
Flow cytometric analysis of lymph node cells from radiation chimeras made by reconstituting C57BL/6.SJL-Ly5.1 mice with (A) wt*Zap70 (Ly5.2$^+$) or (B) *Syk$^{-/-}$*Zap70 (Ly5.2$^+$) fetal liver cells, or (C) Balb/c (Ly9.1$^+$) mice with *Syk$^{-/-}$Zap70$^{-/-}$*Zap70 (Ly9.2$^+$) fetal liver cells. Plots on the left show gates on infected (huCD2$^+$) or uninfected (huCD2$^-$) donor-derived cells ((A), (B) Ly5.2$^+$; (C) Ly9.2$^+$), which were then analysed for CD19 expression (middle plots), CD19$^+$ cells were further analysed for expression of CD5 and Mac1. Gates identify B1a (CD5$^+$Mac1$^+$), B1b (CD5$^-$Mac1$^+$) and B2 (CD5$^-$Mac1$^-$) B cells. Numbers represent percentages falling into gates or quadrants.
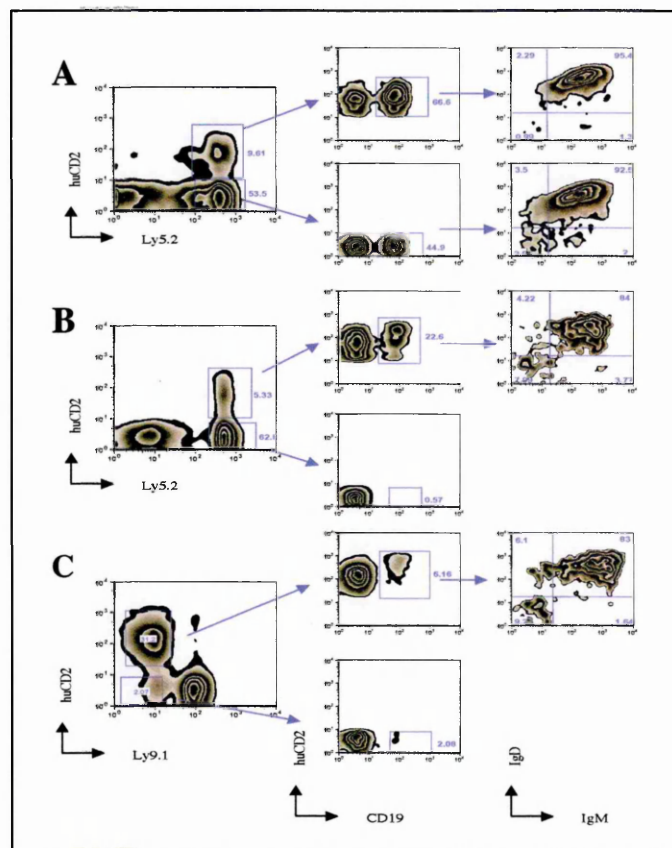
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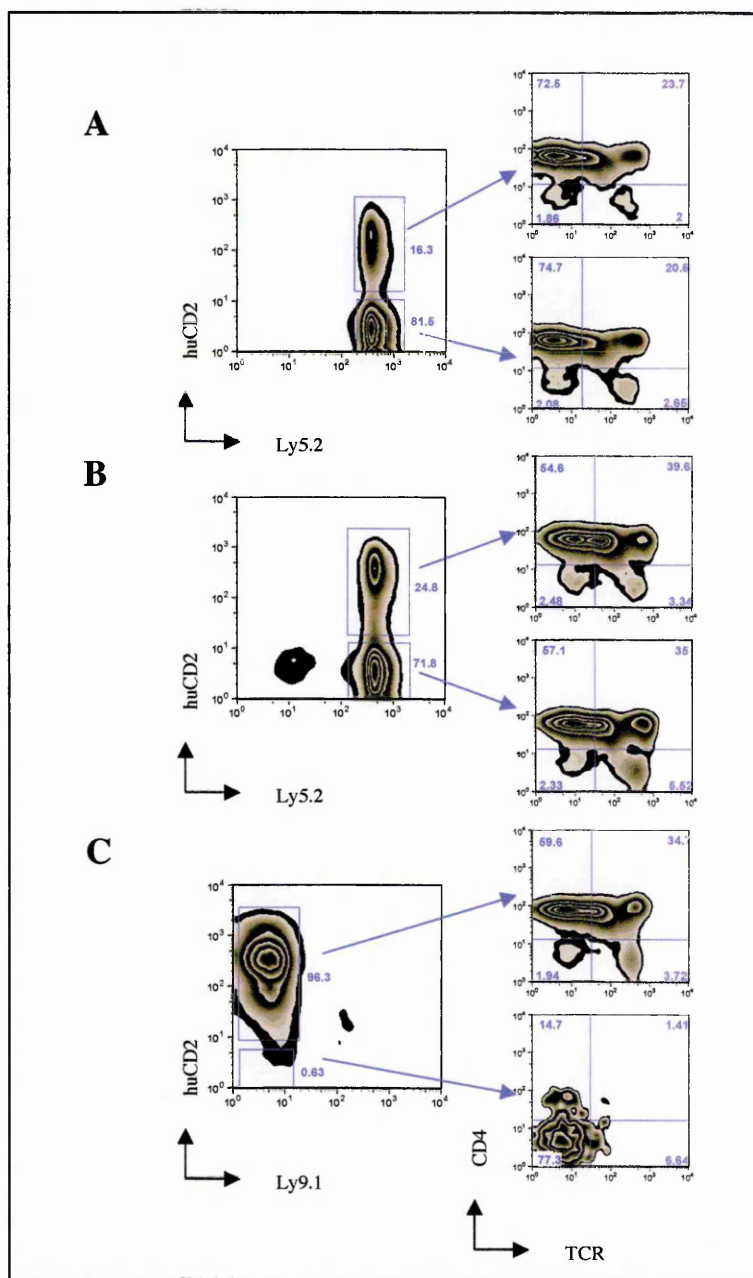
Figure 4.15. Thymus of radiation chimeras
Flow cytometric analysis of lymph node cells from radiation chimeras made by reconstituting C57BL/6.SJL-Ly5.1 mice with (A) wt*Zap70 (Ly5.2⁺) or (B) *Syk⁻/⁻*Zap70 (Ly5.2⁺) fetal liver cells, or (C) Balb/c (Ly9.1⁺) mice with *Syk⁻/⁻Zap70⁻/⁻*Zap70 (Ly9.2⁺) fetal liver cells. Plots on the left show gates on infected (huCD2⁺) or uninfected (huCD2⁻) donor-derived cells ((A), (B) Ly5.2⁺; (C) Ly9.2⁺), which were then analysed for the expression of CD4 and TCR. Numbers represent percentages falling into gates or quadrants.

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In summary, I have shown that overexpression of Zap70 in $Syk^{-/-}$ or $Syk^{-/-}Zap70^{-/-}$ fetal liver cells, which were used to reconstitute radiation chimeras could compensate for the lack of Syk and allow B cell development to proceed all the way to an $IgM^{+}IgD^{+}$ mature B cell stage. In all chimeras reconstituted with $Syk^{-/-}$*Zap70 fetal liver cells, overexpression of Zap70 overcame the complete block of B cell development at the immature to mature B cell transition in B cells in the bone marrow, and mature B cells were found in the spleen and lymph nodes. The same was found in chimeras reconstituted with $Syk^{-/-}Zap70^{-/-}$*Zap70 fetal liver cells. Overexpression of Zap70 could reconstitute B cell development from the pro-B to the mature B cell stage, alleviating the block at both the pre-BCR and the BCR checkpoints. Similarly, expression of Zap70 in thymi of these mice rescued T cell development.

4.2.3 Loss of CD21 expression on wt splenic B cells overexpressing Zap70

The analysis of splenic B cell populations in radiation chimeras reconstituted with wt FL infected with Zap70 revealed an unexpected phenotype. In three out of four mice a loss of CD21 expression on cell surface of follicular B cells was observed (Fig.4.16 and Fig 4.12a). Uninfected $huCD2^{-}$ B cells show a normal distribution of MZ (4.5-8%), MRF (70%) and NF (5.5-7.5%) B cells. Infected $huCD2^{+}$ B cells however, showed a loss of CD21 expression on follicular B cells ($CD23^{hi}$) and also a reduction in MZ B cells, which might be due to the loss of CD21 expression. $HuCD2^{+}$ and $huCD2^{-}$ B cells have normal CD19 expression (Fig.4.16) and showed normal IgM/IgD expression patterns (data not shown).
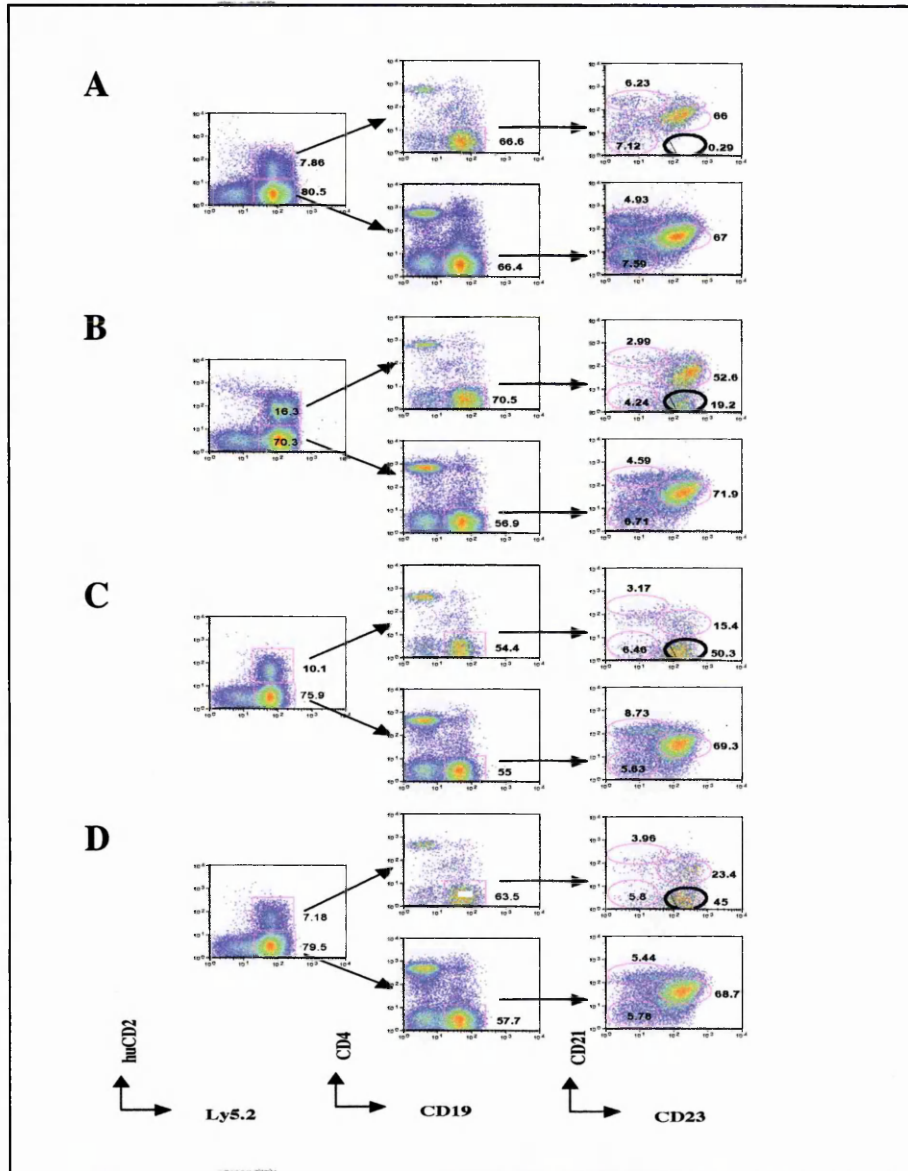
Figure 16 . Loss of CD21 expression on splenic wt follicular B cells overexpressing Zap70.
Flow cytometric analysis of spleen cells from radiation chimeras made by reconstituting C57BL/6.SJL-Ly5.1 mice with wt*Zap70 (Ly5.2+) fetal liver cells. (A)-(D) show results from four different chimeras analysed 8 weeks (A), 9 weeks (B) and 10 weeks (C, D) after reconstitution. Plots on the left show infected (huCD2+) and uninfected (huCD2-) donor-derived (Ly5.2+) cells. The plots in the central column show gates for CD19+ B cells, which were analysed in the plots on the right for expression of CD21 and CD23. Gates show newly formed (CD21-CD23-), mature recirculating follicular (CD21+CD23+) and marginal zone (CD21hiCD23-) B cells. The unusual CD21-CD23+ population is marked with a bold gate. Numbers represent percentages of cells falling into gates.

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5. Hormone-inducible Syk-ER fusion protein

Radiation chimeras reconstituted with $Syk^{-/-}$ fetal liver show a partial block in B cell development at the pro-B to pre-B cell transition. A few $Syk^{-/-}$ pre-B cells can develop as far as the immature B cell stage. However these cells can never complete their maturation into recirculating follicular B cells (Turner et al., 1995). Therefore there is a second, complete block in B cell development at this later stage.

Because there are no $Syk^{-/-}$ mature B cells, we are unable to address the role of Syk in BCR signalling at this stage of B cell development. It would be important to know if Syk was involved in mature B cell proliferation, in the differentiation into plasma cells, germinal centre cells or memory cells. Syk could play a role in somatic hypermutation or class switching. Furthermore, the BCR is required for survival of mature B cells, as ablation of the BCR leads to death of the B cell, suggesting that Syk also might play a role in survival (Lam et al., 1997).

One way of studying the role of Syk in mature B cell development, is to make Syk inducible, to be able to switch Syk on and off at different stages of B cell development, but maintaining its responsiveness to BCR signalling. The system I decided to use was to fuse the hormone-binding domain (HBD) of the estrogen receptor (ER) to Syk, thereby rendering Syk hormone-inducible.

5.1 Estrogen Receptor hormone binding domain / Hsp90 system

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The activities of many intracellular proteins, including the protein kinase Src, Abl and Raf1 (Jackson et al., 1993; Samuels et al., 1993), viral and cellular transcription factors HIV Rev (Hope et al., 1990), c-Myc (Eilers et al., 1989) v-Myb (Burk and Klempnauer, 1991), p53 (Roemer and Friedmann, 1993) and STAT6 (Kamogawa et al., 1998) have been rendered hormonally-regulated upon fusion to the HBD of different steroid receptors (Mattioni et al., 1994; Picard, 1993). The proposed mechanism of action is hormone-reversible protein-inactivation. In the absence of hormone, the inactive state is maintained by an Hsp90-containing complex that causes steric hindrance by blocking access of other proteins or DNA/RNA targets to the HBD-fused heterologous protein. Addition of hormone causes the HBD-fusion protein to release Hsp90 and thus now become active (Fig.5.1) (Schwenk et al., 1998).
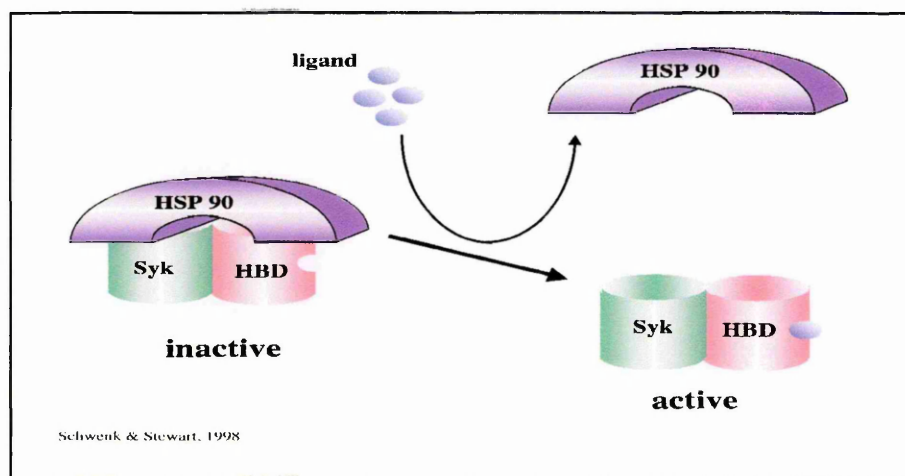
Figure 5.1. Hormone-inducible Syk-ER fusion protein
Syk-HBD fusion protein (Syk, green cylinder; HBD, pink cylinder) is kept in an inactive state in the presence of Hsp90 (violet). If ligand (blue circles) is present in the cell, it binds to the fusion protein, which will then undergo conformational changes and is now no longer accessible to Hsp90. This is the active form of the fusion protein (Schwenk et al., 1998).

The advantages of using this system are reversibility and rapid induction. Unlike systems employing inducible promoters it does not require transcription or translation for induction of activity, or apparently the decay of protein or mRNA for repression of activity. Furthermore, the system should work in all cells where the components of the Hsp90 complex are present; it has been demonstrated to function in mammalian, avian, amphibian and yeast cells.

Since estrogens are found in the serum added to tissue culture medium and are abundant *in vivo* in the mouse, they are likely to interfere with controlled induction. Therefore I chose to use mutant forms of ER-HBD (ER^{TM} or ER^{T2}), which are unable to bind estrogens, yet retain normal affinity for the synthetic ligand, 4-hydroxy-tamoxifen (4-OHT) (Danielian et al., 1993; Feil et al., 1996; Littlewood et al., 1995).

There are two different mutant versions of the ER-HBD, called ER^{TM} or ER^{T2} (Fig.5.2). Both HBD are tamoxifen-dependent. However, due their different mutations, ER^{T2} was shown to be 10x more sensitive to tamoxifen *in vivo* than the earlier version ER^{TM} (Indra et al., 1999).
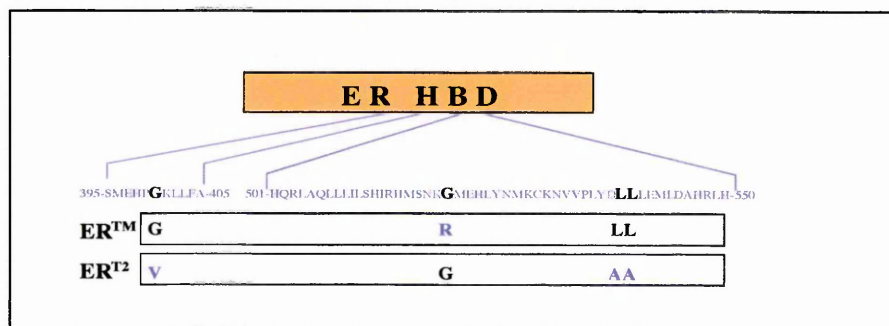
Figure 5.2. Tamoxifen-inducible estrogen hormone-binding domain (HBD)
The ER^{TM} is mutant form of the mouse ER bearing a G525R mutation, which makes it tamoxifen-dependent, but not estrogen-dependent. In contrast, the ER^{T2} is a mutant form of the human ER bearing G400V/L539A/L540A mutation, which makes it unable to bind estrogens, but 10x more sensitive to tamoxifen than ER^{TM}.

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5.2 Syk-ER in DT40 cells

To test the function of Syk-ER fusion proteins, I decided to use an *in vitro* test system, the chicken B lymphoma cell line DT40. Using DT40 cells has several advantages. First, crosslinking of the BCR expressed on the surface of DT40 cells induces a calcium flux, which is absolutely dependent on Syk, providing a rapid assay (Fig.5.3). Second, DT40 is a rapidly proliferating transformed cell line. Third, DT40 cells are very easy to transfect by electroporation. Finally, *Syk*$^{-/-}$ DT40 cells are available (Kurosaki et al., 1995).
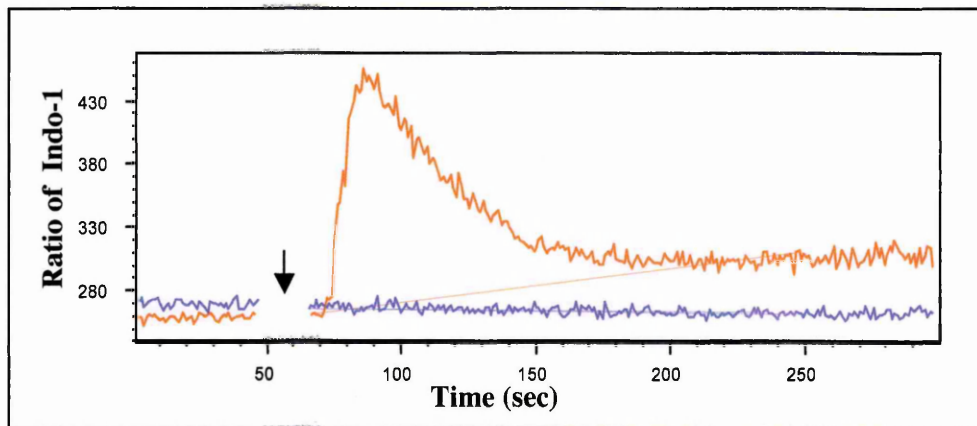
Figure 5.3. BCR-induced Calcium flux in DT40 cells is Syk-dependent
Wild type (red line) and *Syk*$^{-/-}$ (blue line) DT40 cells were loaded with Indo-1-AM. The graph shows the mean ratio of Indo-1 fluorescence at 405nm / 485nm as a measure of intracellular calcium. Cells were stimulated with anti-chicken IgM (M4) at the time indicated (black arrow).

Three different Syk fusion proteins were constructed previously in the lab by Geoff

Parsons, containing ER^{TM}-HBD at the N-terminus, hinge region or C-terminus of Syk

(Syk-NER^{TM}, Syk-HER^{TM}, Syk-CER^{TM}) and were cloned into the pApuro vector

(Fig.5.4). This vector has been shown to confer high levels of protein expression in

DT40 cells, from a chicken actin promoter (Takata et al., 1994).

To test the fusion proteins for their ability to give tamoxifen-dependent activity, I

decided to express them in $Syk^{-/-}$ DT40 cells and then analyse BCR-induced calcium

fluxes in the presence and absence of 4-OHT.
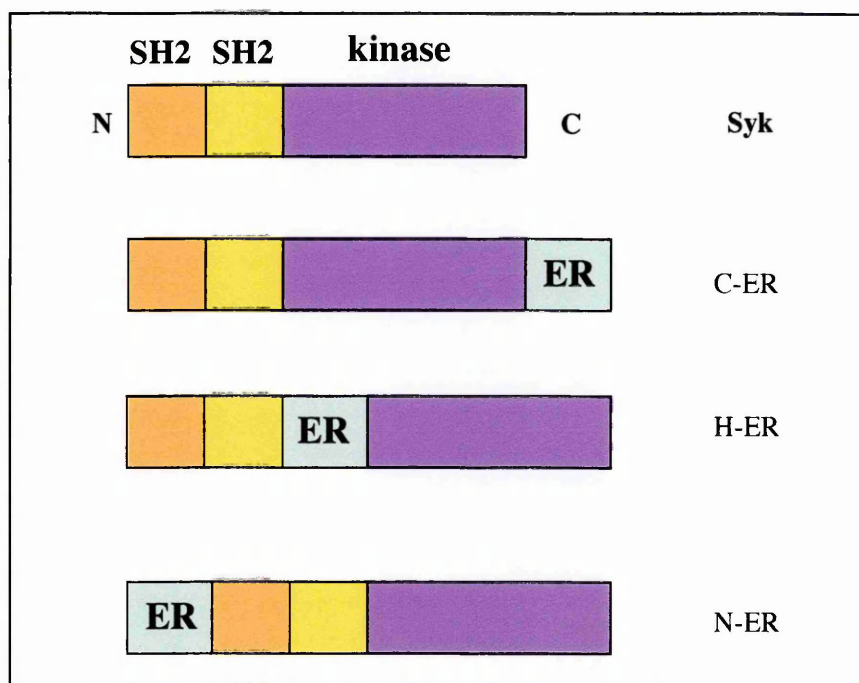
Figure 5.4. Syk-ER fusion proteins
The figure shows the three different fusion proteins containing the ER-HBD at the C-terminus, C-ER; hinge region, H-ER or N-terminus of Syk, N-ER. Structure of Syk is indicated on the top, with two N-terminal SH2 domains (orange and yellow) and a C-terminal kinase domain (purple); ER-HBD is marked in blue.

I electroporated $Syk^{-/-}$ DT40 cells with vectors expressing the three different Syk-ER™ fusion constructs and selected clones stably expressing the protein. I obtained more than 20 clones of $Syk^{-/-}$ DT40 cells transfected with vectors encoding Syk-NER™, Syk-HER™ or Syk-CER™ (Table 5.1). All clones were checked for protein expression, which showed that only a subset expressed the Syk protein. A typical example of expression is shown in Fig.5.5. Syk-CER™ was expressed in high levels in all clones, compared to the expression levels obtained for Syk-NER™ or Syk-HER™. The available antibodies against mouse Syk do not recognise chicken Syk (see also Fig.5.8), therefore the expression levels obtained with the clones could not be compared to endogenous Syk levels.

	KO-S	Ko-SNER™	Ko-SCER™	Ko-SHER™	Ko-SNER^T2
Number of puromycin resistant clones	28	32	25	31	17
Number of clones expressing Syk-ER	10	11	5	4	12

Table 5.1. Clones containing Syk-ER fusion constructs
Table shows the number of puromycin-resistant clones obtained and the number of these that expressed Syk or Syk-ER. The table lists DT40 clones stably transfected with murine Syk (KO-S), with Syk-NER (KO-SNER™ or KO-SNER^T2), with Syk-HER™ (KO-SHER™), or with Syk-CER™ (Ko-SCER™)
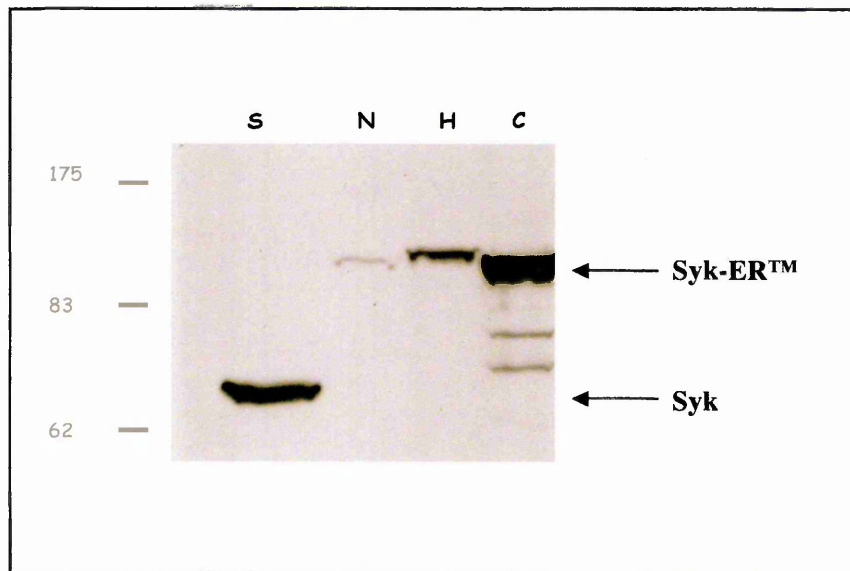
Figure 5.5. Protein expression in stable clones transfected with Syk-ER™ fusion constructs.
Western blot of total cell lysates of *Syk*$^{-/-}$ DT40 cells stably transfected with vector expressing wildtype murine Syk (clone Ko-S-62, S), Syk-NER™ (clone Ko-S-NER™ 64, N), Syk-HER™ (clone Ko-S-HER™ 63, H) or Syk-CER™ (clone Ko-S-CER™ 515, C). Total cell lysates of 3 million cells were loaded per lane. Blot was probed with rabbit anti-mouse Syk antiserum. Molecular weight marker (in kDa) is indicated in grey.

All expressing clones were used for calcium flux experiments, to test whether the introduced Syk-ER™ fusion proteins acted in a hormone-dependent manner. A typical example is shown in Fig.5.6. As shown in Fig. 5.6a calcium flux was induced in *Syk*$^{-/-}$ DT40 cells expressing murine Syk in response to IgM stimulation, but not in *Syk*$^{-/-}$ cells transfected with empty vector (Fig. 5.6a). This confirms that the defective BCR-induced

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calcium flux in Syk$^{-/-}$ DT40 chicken cells can be rescued by expression of murine Syk.

To test whether in *Syk$^{-/-}$* DT40 cells stably transfected with vectors expressing one of the three Syk-ER™ fusion constructs, calcium flux was induced only in the presence, but not in the absence of 4-OHT, the stably transfected clones were pre-incubated for 20 min with 4-OHT before starting the analysis.

In *Syk$^{-/-}$* DT40 cells expressing Syk-HER™ (Fig.5.6c) or Syk-NER™ (Fig.5.6d), calcium flux was induced in response to anti-IgM stimulation only when cells had been pre-incubated with 4-OHT. No calcium flux was seen in the absence of 4-OHT. However, *Syk$^{-/-}$* DT40 cells expressing Syk-CER™ were hormone-independent. Calcium flux was induced in response to BCR stimulation in the presence, but also in the absence of 4-OHT (Fig. 5.6b). This might be due to the fact that cells expressing Syk-CER™ expressed much higher levels of the fusion protein than cells expressing Syk-H-ER™ or Syk-N-ER™ (Fig. 5.5) and there might not be enough Hsp90 in the cell to keep the fusion protein in an inactive state. Pre-incubation of these cells with 4-OHT (Fig. 5.6b), lead to an elevated intracellular calcium concentration, which could not be further increased by BCR crosslinking, suggesting 4-OHT-induced activation of Syk.
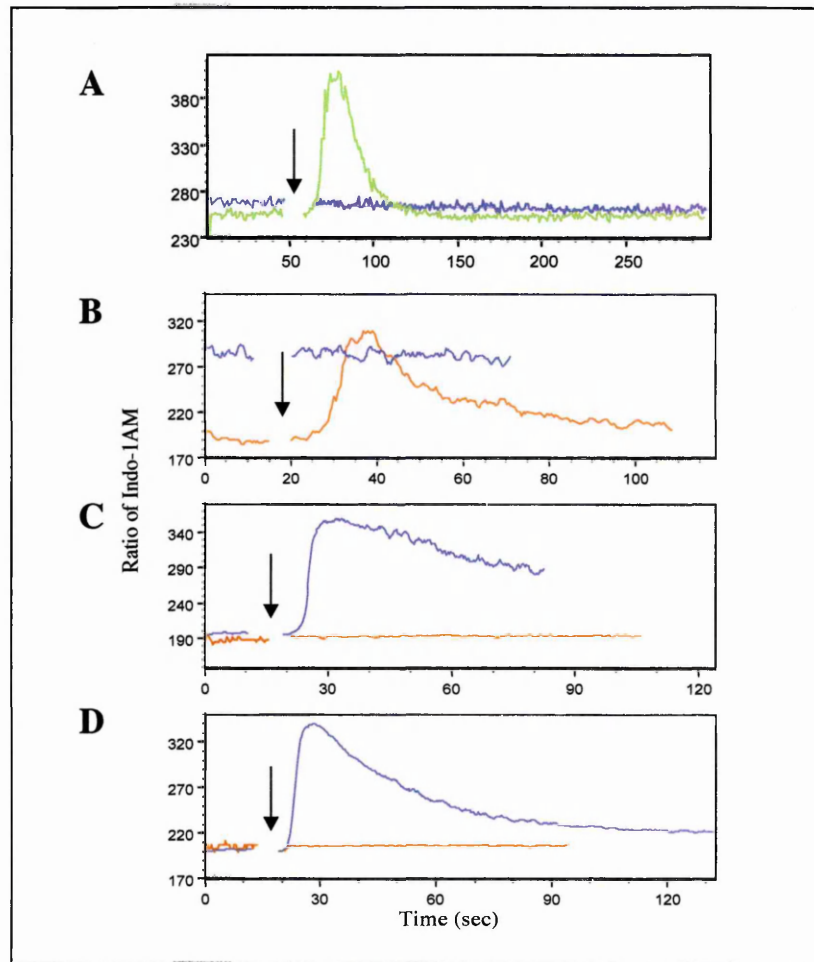
Figure 5.6. **[Ca²⁺] flux experiment on DT40 clones, stably transfected with Syk-ER™**

DT40 cells were loaded with Indo-1-AM. Graph shows the mean ratio of Indo-1 fluorescence at 405 nm/ 485 nm as a measure of intracellular [Ca²⁺]. Cells were stimulated with anti-chicken IgM (M4) at the indicated time (black arrow).

(A) *Syk⁻/⁻* DT40 cells stably transfected with an empty pApuro vector (clone Ko-pA62, red) or with a pApuro vector expressing wild type Syk (clone Ko-S62, green).

(B) *Syk⁻/⁻* DT40 cells stably transfected with a pApuro vector containing Syk-CER™ (clone Ko-C515). Cells were pre-incubated with 4-OHT for 20 min (blue) or were given no 4-OHT (red).

(C) *Syk⁻/⁻* DT40 cells stably transfected with a pApuro vector containing Syk-HER™ (clone Ko-H62). Cells were pre-incubated with 4-OHT for 20 min (blue) or were given no 4-OHT (red).

(D) *Syk⁻/⁻* DT40 cells stably transfected with a pApuro vector containing Syk-NER™ (clone Ko-N64). Cells were pre-incubated with 4-OHT for 20 min (blue) or were given no 4-OHT (red).

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A different approach to test the functionality of the Syk-ER™ fusion protein was to examine their tyrosine phosphorylation. As illustrated in Figure 5.7a, $Syk^{-/-}$ DT40 clones stably expressing murine Syk, showed tyrosine phosphorylation after BCR crosslinking. Clones expressing either Syk-NER™ or Syk-HER™ showed tyrosine phosphorylation after BCR stimulation only when pre-incubated with 4-OHT. No tyrosine phosphorylation was seen in the absence of the ligand (Fig.5.7b,c), confirming the N-terminal and H-regional HBDs were indeed able to repress Syk in the absence of hormone. Clones expressing Syk-CER™ however, showed higher basal levels of tyrosine phosphorylation. The Syk-CER™ protein was tyrosine phosphorylated after BCR crosslinking in the absence of 4-OHT. Once cells were pre-incubated with 4-OHT, high levels of tyrosine phosphorylation were observed even without BCR stimulation (Fig. 5.7d), similar to the calcium flux (Fig.5.6).

In summary, two of the three fusion constructs, the Syk-NER™ and Syk-HER™, were activated in a ligand-dependent manner, however the overall results from Syk-NER™ were more promising than from the Syk-HER™, since the return to basal calcium levels was closer to wild type in Syk-NER™ compared to Syk-HER™ (see Fig. 5.6 c,d).

The newer version of the ER, ER^{T2}, was shown to need ten times less tamoxifen for its activation that the earlier version ER™ (Indra et al., 1999). As for later purposes in a mouse model, I want to use the lowest amount of tamoxifen as possible, I decided to make a Syk-NER^{T2}. I electroporated $Syk^{-/-}$ DT40 cells with the vector expressing Syk-NER^{T2}, selected clones and screened them for expression of the fusion protein. A typical example is shown in Fig. 5.8.
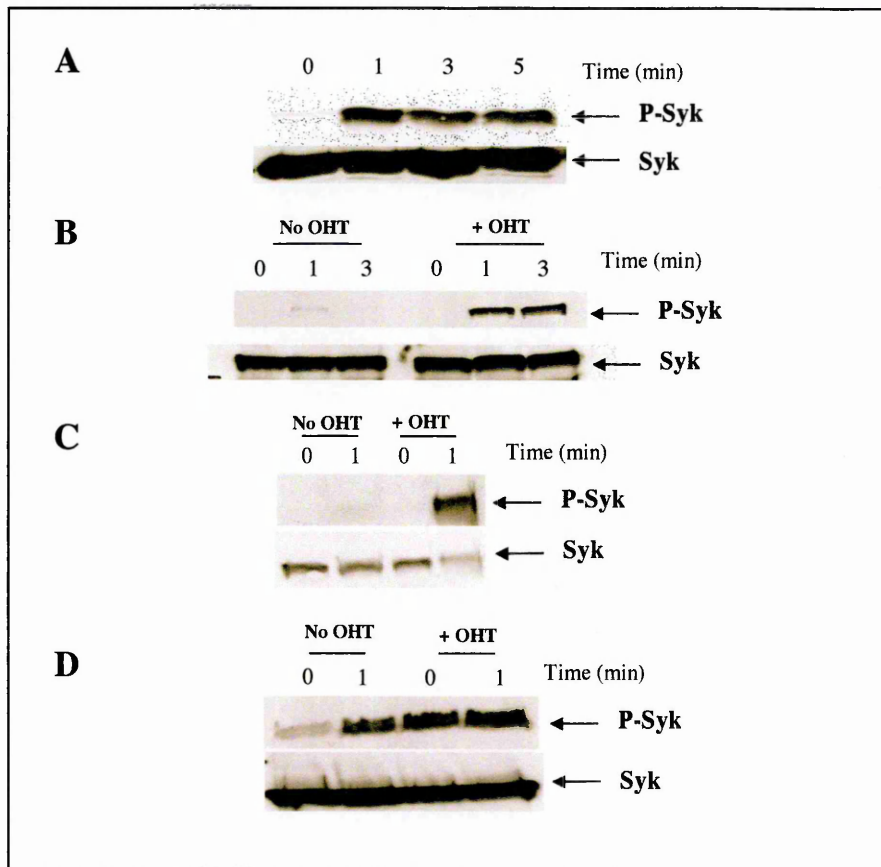
Figure 5.7. Tyrosine-Phosphorylation of Syk-ER protein after anti-IgM stimulation

Immunoblot of total cell lystaes from DT40 clones stimulated crosslinking of the BCR for the indicated times. Cells were either not treated with 4-OHT, or were pre-incubated with 4-OHT for 10 min prior to stimulation. Blots were probed with rabbit anti-mouse phospho-Syk (P-Syk). Blots were then stripped and re-probed with rabbit anti-mouse Syk antiserum (Syk). Analysis of clones expressing Syk (A), Syk-NER™ (B), Syk-HER™ (C) and Syk-CER™ (D) are shown.
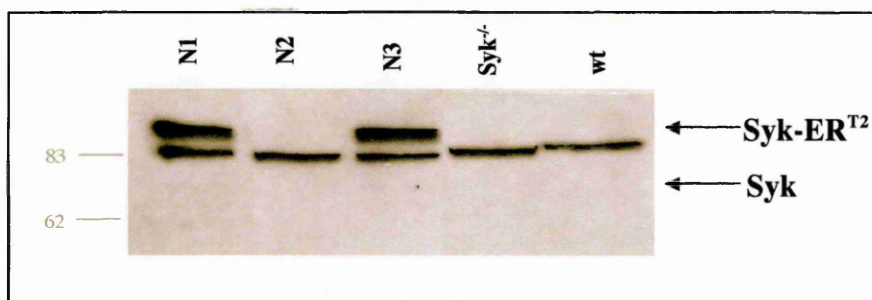
Figure 5.8. Protein expression in clones stably expressing Syk-NER^{T2}

Immunoblot of total cell lysates of $Syk^{-/-}$ DT40 clones transfected with vector containing Syk-NER^{T2}. 3 stably tranfected Syk-NER^{T2} clones are shown here (N1, N2, N3). N1 and N3, but not N2, express the Syk-ER fusion protein. Lysates from wt DT40 cells (wt) and $Syk^{-/-}$ DT40 cells ($Syk^{-/-}$) are also shown. Blot was probed with rabbit anti-mouse Syk antiserum, which only recognises murine Syk and not endogenous chicken Syk (see wt-lane). Top arrow points to Syk-ER fusion protein band (109 kDa); bottom arrow indicates position of Syk (72kDa). The 83 kDa band visible in all lysates is an non-specific band seen with this antibody.

All clones expressing Syk-NER^{T2} were used for calcium flux analysis, to test whether it would function in a hormone-dependent manner, as did the Syk-NER^{TM} fusion protein (Fig.5.9). Interestingly, in all clones stably expressing Syk-NER^{T2} calcium flux was induced in response to anti-IgM in the presence, but also in the absence of 4-OHT (Fig.5.9b). Although a greater calcium flux was observed when cells were pre-incubated with 4-OHT, calcium flux was not entirely hormone-dependent, in contrast to clones stably expressing Syk-NER^{TM}, where cells showed a BCR-induced calcium flux only when the hormone was present in the cell (Fig. 5.9a).

Because the Syk-NER^{T2} did not appear to be fully hormone-dependent, I decided to use

clones expressing the Syk-NER^{TM} for further experiments.
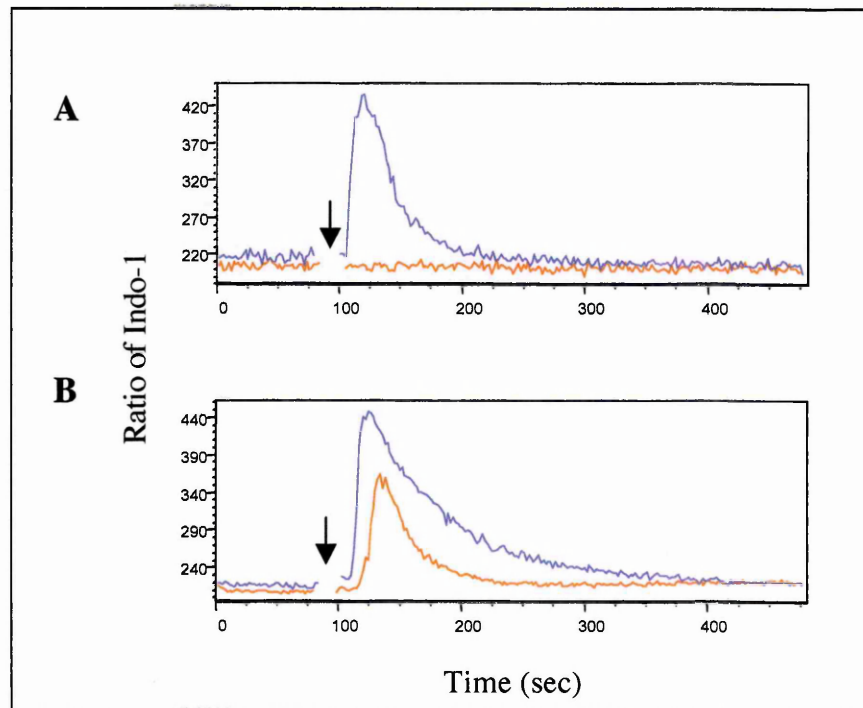
Figure 5.9.   Calcium flux in DT40 clones expressing Syk-NER^{TM} and Syk-NER^{T2}
DT40 cells were loaded with Indo-1-AM. Graphs show the mean ratio of Indo-1 fluorescence at 405 nm/ 485 nm as a measure of intracellular Calcium. Cells were stimulated with anti-chicken IgM (M4) at times indicated (black arrow).
(A) *Syk$^{-/-}$* DT40 cells stably transfected with pApuro vector containing Syk-NER^{TM} (clone Ko-N5.1). Cells were either pre-incubated with 4-OHT for 20 min (blue), or were given no 4-OHT (red).
(B) *Syk$^{-/-}$* DT40 cells stably transfected with pApuro vector containing Syk-NER^{T2} (clone Ko-N5.8). Cells were either pre-incubated with 4-OHT for 20 min (blue), or were given no 4-OHT (red).

One of the potential advantages of the ER system over other inducible systems (e.g.

tetracycline-inducible system) is its quick response time. Therefore, I wanted to

determine the shortest pre-incubation time needed to observe a BCR-induced calcium

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flux in clones expressing Syk-NER™. To do this, cells were pre-incubated with 4-OHT for 5, 10, 15 or 20 min (Fig. 5.10a). Cells showed a BCR-induced calcium flux after only 10min of pre-incubation with 4-OHT. However, cells pre-incubated for 15 min or 20 min showed a greater BCR-induced calcium flux. In contrast, 5 min pre-incubation time seemed to be too short to achieve a visible BCR-induced calcium flux.

Next, I investigated the lowest concentration of 4-OHT needed to allow efficient BCR-induced calcium flux. Clones expressing Syk-NER™ were pre-incubated for 20 min with 400nM (standard concentration used in all previous experiments), 200nM, 100nM, 50nM and 25nM 4-OHT. As shown in Fig.5.10b, 100nM of 4-OHT is the lowest concentration needed to permit a BCR-mediated calcium flux. A greater calcium flux was visible after pre-incubation with either 200nM or 400nM 4-OHT. However no calcium flux was obtained when pre-incubating cells with 50nM or 25nM 4-OHT.
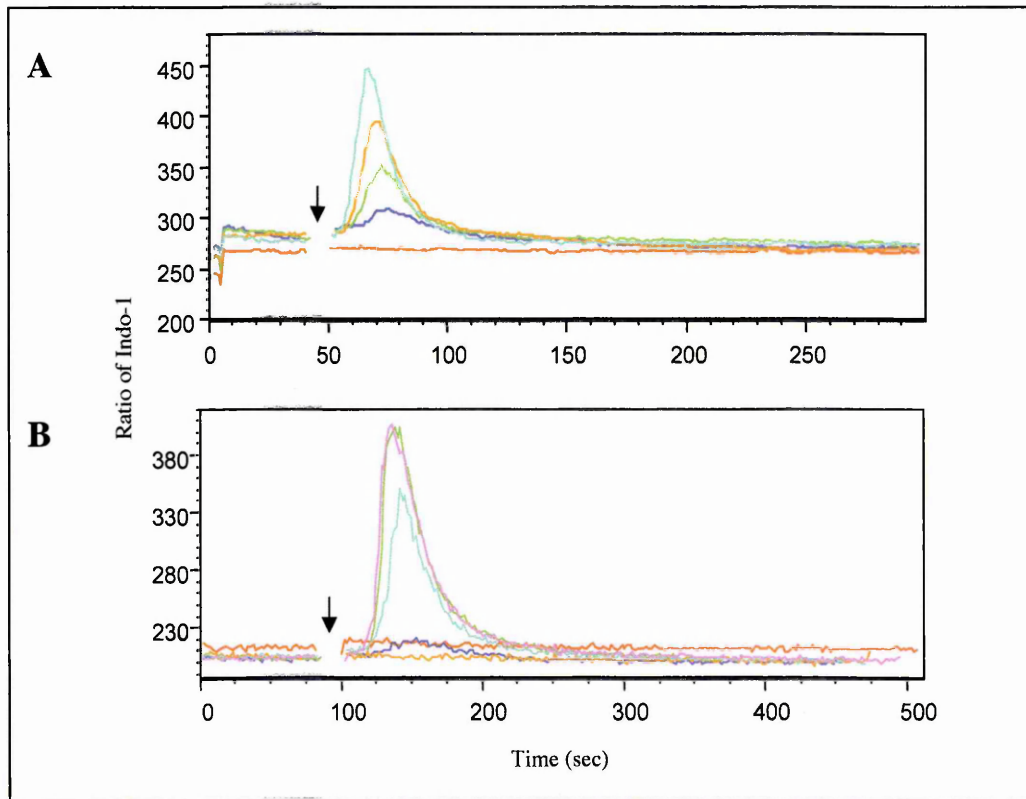
Figure 5.10. Time and concentration dependent activation of Syk-NER™

Syk⁻ᐟ⁻ DT40 cells were stably transfected with a pApuro vector containing Syk-NER™. DT40 cells were loaded with Indo-1-AM. Graphs show the mean ratio of Indo-1 fluorescence at 405 nm/ 485 nm as a measure of intracellular Calcium. Cells were stimulated with anti-chicken IgM (M4) at times indicated (black arrow).

(A) Cells were pre-incubated with 4-OHT for 20 min (light blue), 15 min (orange), 10 min (green), 5 min (dark blue) before starting analysis or were analysed without the addition of 4-OHT (red).

(B) Cells were pre-incubated for 20 min with 400nM 4-OHT (pink), 200nM 4-OHT (green), 100nM 4-OHT (light blue), 50nM 4-OHT (dark blue), 25nM 4-OHT (orange) before starting analysis or were analysed without addition of 4-OHT (red).

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In addition to being quick in responding, the ER fusion protein should also be reversible. Therefore, I wanted to assess the rapidity of inactivation of Syk-ER™ in transformed cells upon removal of 4-OHT. *Syk⁻ᐟ⁻* DT40 cells stably expressing Syk-NER™ were pre-incubated with 4-OHT before measuring BCR-induced calcium flux (Fig.5.11). 4-OHT was then removed by extensive washing before allowing the cells to rest in ligand-free medium for 1 hour, 2 hours, 7 hours or 24 hours before measuring calcium flux (Fig.5.11). Calcium flux was reduced already 1 hour after removal of 4-OHT. This was further reduced after 2 and 7 hours in the absence of 4-OHT and had completely vanished 24 hours after removal of the hormone.
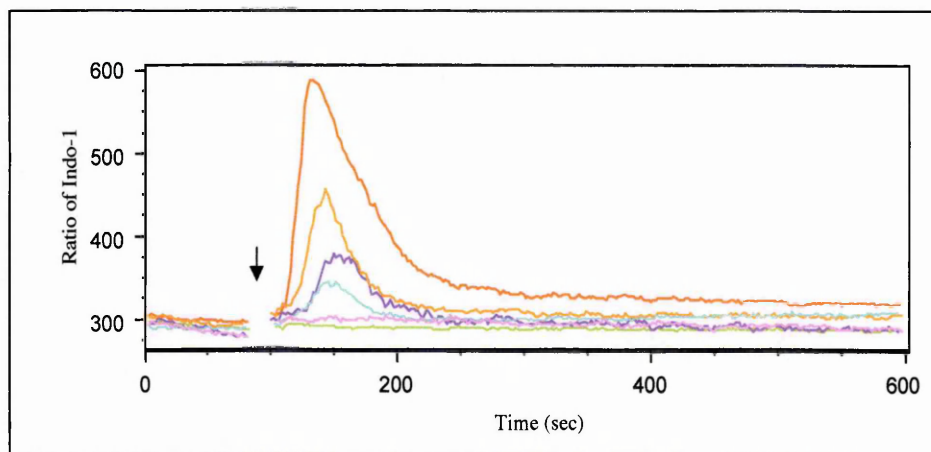
Figure 5.11. Time course of Syk-NER™ inactivation after removal of 4-OHT
Graph shows *Syk⁻ᐟ⁻* DT40 cells expressing Syk-NER™. DT40 cells were loaded with Indo-1-AM. Graphs show the mean ratio of Indo-1 fluorescence at 405 nm/ 485 nm as a measure of intracellular Calcium. Cells were stimulated with anti-chicken IgM (M4) at times indicated (black arrow).
 Cells were analysed in the absence of 4-OHT (green) or pre-incubated with 400nM 4-OHT for 20 min (red), then washed extensively before starting analysis 1 hour (orange), 2 hours (purple), 7 hours (light blue) or 24 hours (pink) after removal of 4-OHT.

In summary, the calcium flux analysis in DT40 cells provided me with an appropriate read-out for assessing the functionality of the Syk-ER fusion constructs. Analysis of stably transfected clones expressing individual Syk-ER^{TM} fusion constructs showed that clones expressing either Syk-NER^{TM} or Syk-HER^{TM} gave a BCR-induced calcium flux in the presence, but not in the absence of 4-OHT. Clones expressing Syk-CER^{TM}, however, were hormone-independent (Fig.5.6), which might be due to their high protein expression levels compared to the Syk-NER^{TM} or Syk-HER^{TM}, allowing some of the fusion protein to "escape" inhibition perhaps because of limiting amounts of Hsp90.

The best results were achieved using the Syk-NER^{TM} construct. Therefore I chose the N-terminus to test the ER^{T2} HBD. This version of the ER HBD is reported to be 10x more sensitive to 4-OHT, which might have been useful for later *in vivo* studies in the mouse. Calcium flux analysis in clones stably expressing Syk-NER^{T2} however revealed that this fusion construct was "leaky". Although greater BCR-induced calcium fluxes were observed in the presence of 4-OHT, calcium flux was also induced in the absence of 4-OHT (Fig.5.9). Returning to analysis of Syk-NER^{TM}, I established the lowest concentration of 4-OHT (100nM) and the shortest pre-incubation time needed (10 min) to fully activate the Syk-ER (Fig.5.10). Furthermore, I found that 24 hours after removal of 4-OHT, Syk-ER was back to its inactive state, confirming that the system was indeed reversible (Fig.5.11).

5.3 Future work: Syk-ER™ Knock-in mouse

The best tool to study the role of Syk in mature B cells using the hormone-inducible Syk *in vivo*, would be a knock-in mouse strain carrying Syk-ER in the endogenous *Syk* gene. I plan to introduce the ER™ domain at the appropriate position into the endogenous *Syk* gene via homologous recombination and thus to generate such a strain. The advantage of this approach is that the knock-in will result in correct temporal and tissue-specific expression of the fusion protein at the correct expression levels, since it will be under the control of endogenous regulatory elements. To circumvent the perinatal lethality of the Syk mutation, pregnant mice carrying the Syk-ER™ fusion gene could be treated with tamoxifen (which is metabolized into the active ligand 4-OHT in the liver) from mid-gestation onwards in order to induce Syk activity. This procedure should also rescue B cell development. Such mice would be kept on tamoxifen continuously in order to keep Syk active, and thus prevent bleeding disorders.

As the N-terminal Syk-ER™ fusion construct gave the most promising results in DT40 cells, I decided to knock-in the ER™ at this exact position in the *Syk* gene. More precisely, the ER™ will be introduced into exon 2 between amino acids 8 and 9 (Fig.5.12). That is the position where the ER™ was placed in the Syk-N-ER™ fusion construct.
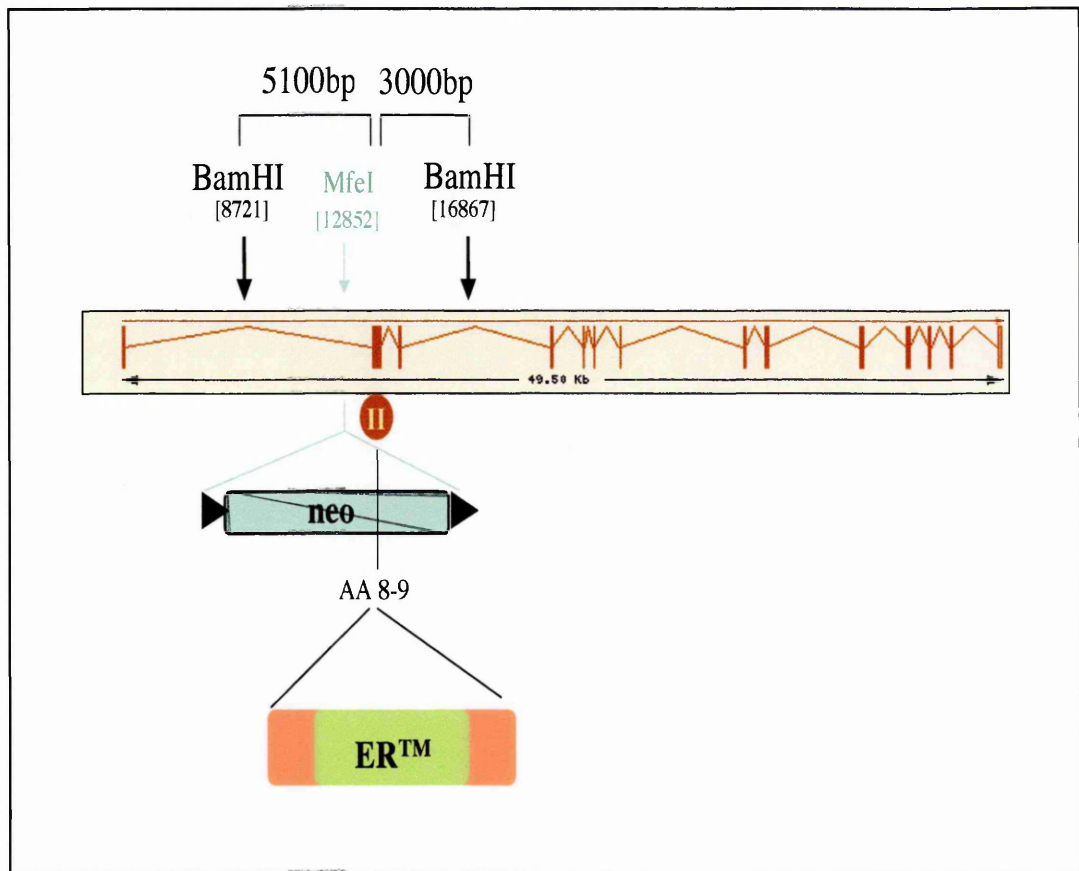
Figure 5.12. Knock-in strategy

Figure shows the knock-in strategy. The ER^{TM} will be knocked in at the N-terminus of the Syk gene.

Syk is indicated in the cream rectangle; exons, small red rectangle; introns, red lines.

The position where the ER^{TM} is going to be introduced is indicated in exon II between amino acids 8-9 (AA 8-9). As part of the targeting strategy, a neomycin resistance cassette (neo, blue rectangle) flanked by two loxP sites (black arrows) will be introduced into an MfeI site in intron 1, and will be subsequently removed by Cre-mediated excision either in the targeted ES cells or in the resultant mice.

I found two convenient BamHI sites in the genomic sequence of C57BL/6J mice, which flanked a region of 8.1kb containing exon 2 and which will be used as homologous regions (HR) for the targeting construct.

Since the available genomic sequence is from the C57BL/6J strain, and the ES cell line I am intending to use is from 129/Sv, the next step was to check whether the BamHI sites also exist in 129/Sv mice. A Southern blot was performed using a 457bp probe containing exon 2 and was found to hybridise to a 8.1kb BamHI fragment in 129/Sv DNA (Fig. 5.13a.)

Next I screened a mouse 129/Sv PAC library with the 457bp probe. I obtained 6 PAC clones that hybridised to my probe. An example is shown in Fig. 5.14b. DNA from the 6 clones was digested with BamHI and Southern blot analysis was performed using Syk cDNA as a probe to determine which one of the clones contained the 8.1kb fragment and which one of them, if any, contained the whole *Syk* gene, which might be useful for further targeting work. 3 of the 6 clones contained the whole *Syk* gene (one example is shown in Fig. 5.13 c, lane 1). One clone seemed to contain only the N-terminus of the *Syk* gene (Fig. 5.13 c, lane 2), spanning the regions from Exon 1 to 3, and one clone contained Exons 1 to 10 (Fig. 5.13 c, lane 3).
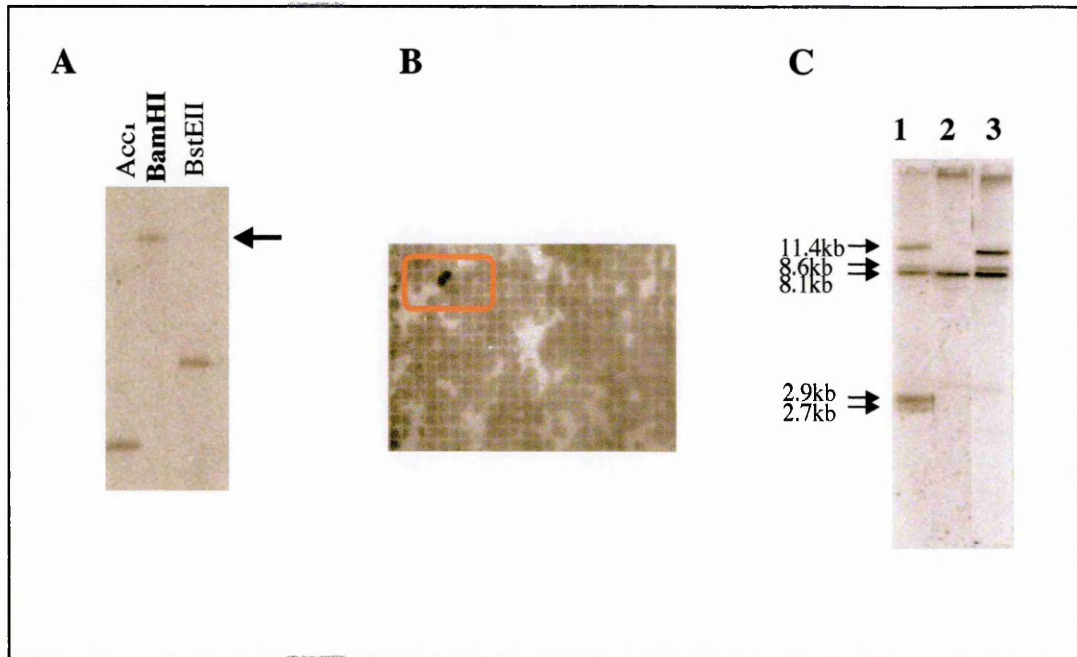
Figure 5.13. Screening PAC library for homologous region
(A) Southern blot of 129/Sv genomic DNA digested with AccI, BamHI and BstEII and probed with 457bp probe. Arrow points to the 8.1kb BamHI fragment.
(B) Part of one filter carrying the PAC mouse genomic library screened for *Syk* using the 457bp probe. One positive clone is indicated in the red box.
(C) Southern blot shows three different PAC clones (1,2,3) digested with BamHI and probed with *Syk* cDNA. Black arrow points to expected fragments (fragment sizes: 11.4kb, 8.6kb, 8.1kb, 2.9kb and 2.1kb). All three PAC clones contained the 8.1kb fragment.

The next step will be to clone the ER^{TM} into the N-terminus of *Syk*. Homologous recombination will be used in ES cells to knock-in the Syk-ER^{TM} fusion construct into the endogenous *Syk* gene using standard techniques. Correctly targeted ES cells will be injected into mouse blastocysts to generate chimaeric mice and male chimaeras will be bred to wild type females to transmit the mutant allele carrying the N-ER^{TM} to the next generation. Mice heterozygous for this mutant allele will be intercrossed and pregnant females will be treated with tamoxifen to prevent the perinatal lethality as described

before. If tamoxifen is unable to rescue this lethality, perhaps because it is not compatible with maintenance of pregnancy, Syk-ER™ fetal liver will be transplanted into irradiated mice. These radiation chimeras in turn will be treated with tamoxifen in order to rescue B cell development.

The question of whether Syk was needed for maintenance / survival of mature peripheral B cells could then be addressed. Tamoxifen treatment of knock-in mice should allow the development of mature B cells. Upon removal of tamoxifen one could measure the persistence of these B cells. If removing the ligand fully from the host proves to be impossible, these cells could be adoptively transferred into a new ligand-free host.

Furthermore, to study the ability of B cells to transduce BCR signals in the absence of functional Syk, the mice will be taken off tamoxifen. First the disappearance of Syk activity following removal of tamoxifen could be monitored by stimulating B cells through the BCR and looking at phosphorylation of Syk. Once the Syk-ER™ is fully sequestered by Hsp90 it will no longer be recruited to the BCR and will not be phosphorylated.

To examine the role of Syk in B cell signalling, cells could be stimulated through the BCR in the presence and absence of 4-OHT and their ability to proliferate could be measured. The effect of a lack of Syk on intracellular signalling pathways could be studied. In particular the BCR-induced tyrosine phosphorylation of the BCR subunits, of Src-family kinases and of other intracellular proteins will be studied, as well as the ability of the cells to induce a rise of intracellular calcium.

The Syk-ER™ knock-in mice will also be used to study the role of Syk in BCR-mediated responses *in vivo*. B cell function in these mice will be assessed by taking the

mice off tamoxifen, immunising them with T-dependent or T-independent antigens, and measuring antibody responses, germinal center formation, class switching and somatic hypermutaion, and the generation and persistence of B cell memory.

B cells coming from these knock-in mice will allow a direct study of the role of Syk in the function of mature B cells, which is not possible using $Syk^{-/-}$ mice, due to the complete lack of mature B cells.

6. Discussion

The Syk-family of PTKs plays an important role in lymphocyte development and signalling. Gene targeted mutant mice revealed that Syk plays a significant role in B cell development, whereas Zap70 is important for T cell development. $Syk^{-/-}$ mice show a partial block in B cell development at the pro-B to pre-B cell transition and a complete block at the immature to mature B cell transition (Cheng et al., 1995; Turner et al., 1995). Mice deficient for Zap70 have normal numbers of DP thymocytes, but no CD4$^+$ and CD8$^+$ SP T cells (Negishi et al., 1995).

Unexpectedly, mice deficient for both Syk and Zap70 show a complete block in B cell development at the pro-B to pre-B cell transition, indicating that Zap70 plays a role in B cells, as well (Schweighoffer et al., 2003). Furthermore, Zap70 is expressed in a B-CLL cells from a subset of patients (Orchard et al., 2004; Wiestner et al., 2003). Interestingly, Zap70 expression correlates very strongly with poor prognostic outcome.

I set out to further explore the role Zap70 plays in B cell development and function. Furthermore, I wanted to find out whether Syk and Zap70 play similar or differential roles and whether Zap70 was able to compensate for the lack of Syk in B cell development. I also wanted to investigate the role of Syk in mature B cells. In order to do this, I made a hormone-inducible Syk protein and tested it in a chicken B lymphoma (DT40) cell line.

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6.1 Zap70 expression in different B cell subsets

First, I wanted to determine which subset of mature B cells expressed Zap70 and whether there was a particular subset that expressed more or less Zap70 than other B cell populations. I attempted to use flow cytometry to measure intracellular Zap70 expression levels in B lineage cells. I optimised the conditions for the detection of intracellular Zap70 using thymocytes, which express high levels of this kinase (Fig.3.1). With the optimised staining method I was able to clearly detect Zap70 in thymocytes. The antibody used only detected Zap70, and did not detect the related Syk kinase. However, using this method, I was unable to detect Zap70 expression in B lineage cells from the bone marrow, spleen or peritoneal cavity (Fig.3.2 and data not shown), suggesting that if B lineage cells do express Zap70 kinase, they do so at a level well below that seen in thymocytes or mature T cells.

Next, I examined Zap70 expression in B lineage cells using immunoblotting. Using this technique, I was indeed able to detect Zap70 expression in all developing and mature B cell populations, including B1 and marginal zone B cells. I also wanted to quantify Zap70 expression during B cell development from the bone marrow to the spleen and compare it to levels in thymocytes. Normalised to the expression of ERK2, pro-B cells expressed 30-fold less Zap70 than $TCR\alpha^{-/-}$ thymocytes, whereas pre-B, immature and mature B cells expressed between 60- to 100-fold less (Fig.3.4). When normalised to the number of cells lysed, pro-B cells expressed 8-fold less while pre-B, immature and mature B cells expressed 30-fold less Zap70 than $TCR\alpha^{-/-}$ thymocytes. Thus pro-B cells appear to express 2 to 3 times more Zap70 than all other B cell subsets. This correlates

with the fact that in *Syk$^{-/-}$* mice B cell development is only partially blocked at the pro-B to pre-B cell transition, but completely arrested at the immature B cell stage (Cheng et al., 1995; Turner et al., 1995). Pro-B cells seem to have sufficient amounts of Zap70 to drive B cell development through the pre-BCR checkpoint in the absence of Syk. However, because Zap70 expression levels are lower in immature B cells, these cells may not express enough Zap70 to allow B cell development to progress further in the absence of Syk, thus leading to a complete block of B cell development at the immature B cell stage in *Syk$^{-/-}$* mice.

As an extension of this study, it would be interesting to explore the expression pattern of Zap70 in plasma cells, memory B cells and germinal centre B cells. This would be difficult to achieve in naïve mice, because the numbers of cells in the B cell populations mentioned above are too low to be able to detect Zap70 using immunoblotting. Therefore mice could be immunised to boost cell numbers, before purifying the above B cell populations for immunoblotting. Furthermore, I could look at expression of Zap70 in activated B cells. This could be done by activating naïve B cells *in vitro* with for example, anti-IgM or LPS, and comparing Zap70 expression levels in activated and naïve B cells.

6.2 The role of Zap70 in B cell development

I wanted to explore whether Zap70 plays a role at distinct stages of B cell development, other than the pre-BCR checkpoint. Therefore, I examined B cell populations from bone marrow, spleen, lymph nodes and peritoneal cavity of *Zap70$^{-/-}$* mice compared to wild-

type mice. I also compared $Zap70^{-/-}$ B cell subsets to those obtained from $TCR\alpha^{-/-}$ mice that, similarly to $Zap70^{-/-}$ mice, lack mature $\alpha\beta$ T cells. This allowed me to assess whether differences seen in $Zap70^{-/-}$ B cell populations were due to the lack of Zap70 in these B cells or to the absence of $\alpha\beta$ T cells, and cytokines produced by $\alpha\beta$ T cells, that might differentially affect the development of specific B cell subsets.

A significant increase was found in MZ B cells in the spleen and B1b cells in the peritoneal cavity of $Zap70^{-/-}$ mice. In contrast, $TCR\alpha^{-/-}$ mice had no change in numbers of MZ B cells in the spleen and showed a decrease of B1b cells in the peritoneal cavity. These findings suggest that the increase in MZ B cells and B1b cells in $Zap70^{-/-}$ mice was due to the lack of Zap70 in the B cells themselves, and not due to the lack of $\alpha\beta$ T cells. However, these results were only partly confirmed in radiation chimeras reconstituted with $Zap70^{-/-}/\mu$MT bone marrow, where a significant increase in percentage of B1b cells and in MZ B cell numbers. However, because cell numbers vary a lot from mouse to mouse, more radiation chimeras need to be analysed.

Although MZ and B1 B cells are distinct subsets, they share many phenotypic and functional characteristics (Berland and Wortis, 2002; Martin and Kearney, 2000a; Martin et al., 2001). Cells of both subsets are long-lived and participate in antibody responses to TI antigens. Both B1 and MZ B cells maintain normal numbers throughout life after arrest of B lymphopoiesis, suggesting that they are self-renewing (Hao and Rajewsky, 2001). It is clear that BCR signalling is involved in the enrichment of certain B1 and MZ B cell clones, since this process is impaired in xid, $CD19^{-/-}$ and $CD45^{-/-}$ mice (Martin and Kearney, 2000b; Martin and Kearney, 2001). B1 cells, enriched in the

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peritoneal and pleural cavities, are generated and maintained through continuous IgM signalling (Arnold et al., 1994; Hardy et al., 1994). Selection into the B1 compartment might require higher strength BCR signalling than selection into MRF and MZ B cell population (Casola et al., 2004). It has been reported that B cells assume the MZ phenotype as a consequence of receiving lower levels of BCR signalling than are required for entry into the MRF B cell pool (Cariappa et al., 2001). This conclusion was based on the fact that mice lacking the transcription factor Aiolos have B cells that are hyperresponsive to BCR signalling and essentially lack MZ B cells. In contrast *xid* mice, which have defective BCR signalling, have normal numbers of MZ B cells. Interestingly, however, mice lacking Aiolos, despite their hyperresponsive BCR signalling have reduced numbers of B1 cells (Wang et al., 1998). Inconsistent with weaker BCR signalling being necessary for MZ B cell development is the fact that both MZ B and B1 cells are absent or significantly decreased in CD19 knock-out mice, which should have a weakened BCR signal (Martin and Kearney, 2000b; Rickert et al., 1995; Sato et al., 1996). Furthermore, it has been reported recently using a monoclonal BCR mouse line, specific for the self-Thy-1/CD90 glycoprotein that an increase in BCR signal strength, induced by low-dose self-antigen, directed naïve immature B cells to mature, not into the MRF B cell population, but instead into the MZ B cells subset (Wen et al., 2005).

Furthermore, Notch2$^{+/-}$ mice show a reduction in frequency and absolute number of both B1 and MZ B cells, suggesting that Notch2 plays a role in determining the generation of MZ and B1 B cells. This also supports the notion that these two subsets share some common elements in their developmental pathways (Witt et al., 2003).

In addition, p110δ-mutant mice have reduced numbers of MZ B cells and B1 cells (Clayton et al., 2002; Okkenhaug and Vanhaesebroeck, 2003). The same phenotype is also observed in $CD19^{-/-}$ mice, which is not surprising as CD19 is one of the main regulators of PI3-K in B cells (Tuveson et al., 1993) and mice expressing a tyrosine-to-phenylalanine mutant of CD19 that cannot bind PI3-K also lack B1 and MZ B cells (Wang et al., 2002). These results suggest that a PI3-K-transmitted signal from CD19 is required for the differentiation of B1 and MZ B cells.

These findings together suggest that B1 and MZ B cells might need similar factors and signal transduction pathways for their development and differentiation.

$Zap70^{-/-}$ B cells may have altered BCR signal strength favouring the development of B1 and MZ B cells rather than MRF B cells. To test this hypothesis, a future set of experiments could involve transducing wt, $Syk^{-/-}$, $Zap70^{-/-}$ or $Syk^{-/-}Zap70^{-/-}$ hematopoietic stem cells with Zap70 or Syk and reconstitute lethally irradiated hosts with these infected cells. In the resulting chimeric mice one could monitor the development of B cells expressing different amounts of Syk or Zap70 kinases and ask whether changes in signal-strength may cause skewing towards particular B cell subsets.

Alternatively, different BCR repertoires could favour the development of either MZ B and B1 cells or MRF B cells. A potential role of Zap70 and also Syk may be to facilitate positive selection into the MRF B cell pool, by enhanced proliferation and/or survival of immature B cells with BCR repertoires suited for MRF B cells. In the absence of Zap70 immature B cells with BCR repertoires in favour of MZ and B1 B cells may be preferentially outgrown.

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As CD19, Btk and PI3-K have been shown to be involved in development of MZ and B1 B cells, because mice deficient in these proteins lack or have reduced numbers of B1 and MZ B cells. It is possible that other molecules, for example Zap70, are involved in the preferential development and maintenance of MRF B cells rather than MZ B and B1 cells.

Differences in B cell subsets in *Zap70$^{-/-}$* and wt mice could be more apparent in a competitive environment where *Zap70$^{-/-}$* B cells have to compete with wild-type B cells for a specific 'niche'. Therefore the generation of competitive chimeras, which are made with different ratios of *Zap70$^{-/-}$* and wild type fetal liver, is going to be my next approach. Fetal liver cells will be used rather than bone marrow cells for these specific chimeras, because the development of B1 cells is of particular interest, which appear to be generated to their full potential only from fetal liver stem cells.

Also, if in *Zap70$^{-/-}$* mice BCR repertoires and/or signalling is altered due to decreased expression levels of the Syk family of PTKs, a similar increase in MZ and B1b cells could be observed in mice heterozygous for Syk.

6.3 The role of Zap70 in B cell function

I first examined the role of Zap70 in B cell function by analysing immune responses to TD (T cell dependent) and TI (T cell independent) antigens. I made radiation chimeras using sublethally irradiated *Rag1$^{-/-}$* recipient mice reconstituted with a mixture of wt/μMT or *Zap70$^{-/-}$*/μMT bone marrow. All B cells in these chimeras come from wt or *Zap70$^{-/-}$* bone marrow whereas T cells come from wt or μMT bone marrow and have no

signalling mutations. I chose to immunize these mice using TD and TI antigens dissolved in PBS to generate weak responses that are more likely to reveal differences caused by Zap70 deficiency. Immunisation with TD antigen TNP-KLH and TI-antigens TNP-Ficoll or TNP-LPS showed no significant difference in responses of $Zap70^{-/-}$ and wt chimeras. However, the TNP-Ficoll and TNP-KLH responses were very weak and need to be repeated and extended, perhaps using more antigen, or antigen with adjuvant.

Next, I examined the role of Zap70 in mobilisation of calcium flux upon BCR stimulation in B cell subsets in the spleen and peritoneal cavity. No difference in mobilising calcium fluxes could be observed in $Zap70^{-/-}$ and wt mice.

In conclusion, Zap70 does not appear to play a role in the B cell responses examined, which suggest that Syk, the other member of the Syk/Zap kinase family is the dominant kinase during these processes.

However, the study concerning the role of Zap70 in B cell function is still very preliminary and needs to be extended. Future experiments will include exploring the turnover rate of developing and mature B cells by Bromodeoxyuridine (BrdU) labelling. Furthermore, I am going to examine germinal centre formation in the spleen in response to TD immunisation using immunohistochemistry. These TD immunisations need to be done as above in radiation chimeras reconstituted with $Zap70^{-/-}/\mu$MT bone marrow compared to radiation chimeras reconstituted with wt/μMT bone marrow as controls. In addition, I could look at migration of $Zap70^{+/+}$ and $Zap70^{-/-}$ B cells. It would be interesting to find out whether Zap70 is involved in integrin signalling of B cells. Furthermore, I could explore whether Zap70 is involved in class switching, by culturing

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naïve B cells and activating them with LPS or anti-CD40 or CD40L. Then cells could be monitored for proliferation and for class switch recombination.

6.4 Overexpression of Zap70 can rescue the Syk⁻/⁻ phenotype

Radiation chimeras reconstituted with $Syk^{-/-}$ fetal liver cells show a partial block at the pro-B to pre-B cell transition and a complete block at the immature B cell stage (Cheng et al., 1995; Turner et al., 1995). The partial block is due to the fact that Zap70 can compensate for the lack of Syk at the pre-BCR checkpoint (Schweighoffer et al., 2003). $Zap70^{-/-}$ mice have no block in B cell development, but are blocked at the DP to SP transition in the thymus during T cell development. Mice deficient for both Syk and Zap70 are arrested at the DN to DP stage in T cell development, indicating a role of Syk at the pre-TCR checkpoint.

It has been shown that expression of a Syk transgene in $Zap70^{-/-}$ mice can rescue T cell development and therefore overexpression of Syk can compensate for the lack of Zap70 in these mice (Gong et al., 1997). Furthermore, expression of Zap70 in $Syk^{-/-}$ DT40 cells rescues BCR signalling, suggesting that Zap70 is functionally equivalent to Syk in BCR signalling at least in chicken B cells (Kong et al., 1995).

I wanted to investigate whether the same was true for pre-BCR and BCR signals during murine B cell development and whether the complete block at the BCR checkpoint is due to insufficient levels of Zap70 in immature B cells. Alternatively, Syk and Zap70 could have distinct functions and Zap70 may not be able to transduce the signals through the BCR in quite the same way as Syk and may thus be unable to support the selection

of immature B cells into the peripheral B cell pool. In support of this suggestion, it has been shown that the specific *in vitro* kinase activity of Syk is 100-fold higher than that of Zap70 (Latour et al., 1996), which might lead to the phosphorylation of a different set of downstream targets, and that Syk, but not Zap70 can be activated in an Lck-independent fashion (Latour et al., 1997).

I used retroviral gene transfer technology to overexpress Zap70 in $Syk^{-/-}$ B lineage cells. $Syk^{-/-}$ embryos die around birth, so as a source of HSC I used fetal liver cells that were infected with retroviruses *in vitro* and then used to reconstitute lethally irradiated recipient mice. This technology is faster than making a transgenic mouse and in a chimeric mouse there are donor-derived cells that are infected with the retrovirus expressing the gene of interest, but also donor-derived uninfected cells, which can be used as internal controls.

I have also tried bone marrow cells as a source of HSC. These bone marrow cells were obtained from 5-FU (5-fluorouracil) treated $Syk^{-/-}$ chimeras to enrich for HSC and were then retrovirally infected as described for fetal liver cells (data not shown) and secondary chimeras were made. However, $Syk^{-/-}$ chimeras have $Syk^{-/-}$ as well as wild type HSC. Unfortunately $Syk^{-/-}$ cells were outcompeted by wt cells in the secondary chimeras and therefore could not be used to analyse the overexpression of Zap70 in $Syk^{-/-}$ B cells.

After optimising transfection of the appropriate packaging cell line (PlatE) and transfection reagent (Genejuice), I infected $Syk^{-/-}$ fetal liver cells with retrovirus expressing Zap70 (pMSCV-IRES-huCD2-Zap70) and reconstituted lethally irradiated recipients. This system had two main advantages: first, donor-derived cells could be

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easily distinguished from host cells, by their expression of the cell surface marker Ly5.2, whereas the host was Ly5.1$^+$. Second, infected donor-derived cells were identified by the expression of huCD2.

In the first set of experiments, I infected $Syk^{-/-}$ fetal liver cells with a retrovirus expressing Zap70 or an "empty" retrovirus as a control. Radiation chimeras were sacrificed 8-10 weeks after reconstitution, a period that is sufficient to ensure long-term reconstitution. Survival of the mice 8-10 weeks after reconstitution also confirmed that the *ex vivo* culture protocol did not compromise the ability of cultured HSC to mediate long-term radioprotection. Infected, huCD2$^+$ donor-derived B cells from radiation chimeras reconstituted with $Syk^{-/-}$*Zap70 fetal liver cells were able to overcome the block at the BCR checkpoint, which is a feature of $Syk^{-/-}$ B cells, and develop into IgM$^+$IgD$^+$ mature B cells in the bone marrow, spleen and lymph nodes. In contrast, infected, huCD2$^+$ donor-derived B cells from radiation chimeras reconstituted with $Syk^{-/-}$ *empty vector were still arrested at the immature B cell stage in the bone marrow, and no donor-derived B cells were found in the spleen and lymph nodes.

Next, I extended the experiment and infected wt, $Syk^{-/-}$ and $Syk^{-/-}Zap70^{-/-}$ fetal livers cells with a retrovirus expressing Zap70 and reconstituted lethally irradiated hosts. Fetal livers deficient in both Syk and Zap70, have no member of the Syk-family of PTKs, and are therefore a 'cleaner' model to observe whether overexpression of Zap70 alone drives B cells through both pre-BCR and BCR checkpoints. Donor-derived infected B cells of radiation chimeras were observed 8-10 weeks after reconstitution and the presence of mature B cells in the bone marrow, B2 cells in the spleen, lymph nodes and peritoneal cavity confirmed that in radiation chimeras reconstituted with $Syk^{-/-}$*Zap70 and $Syk^{-/-}$

*Zap70$^{-/-}$*Zap70 fetal liver cells, overexpression of Zap70 can drive cells through both developmental checkpoints. However, B lineage cells in the spleen of radiation chimeras reconstituted with *Syk$^{-/-}$*Zap70 and *Syk$^{-/-}$Zap70$^{-/-}$*Zap70 fetal liver, were mainly B2 cells; MZ B cells could not be clearly distinguished. Furthermore, in the peritoneal cavity of these radiation chimeras, donor-derived B cells were again mainly B2 cells, whereas B1 cells could not be seen. Surprisingly, this was also true for radiation chimeras reconstituted with wt*Zap70 fetal liver cells. Although it is believed that fetal liver cells give rise to a normal compartment of B1 cells in the peritoneal cavity and adoptive transfer studies have confirmed this (Herzenberg, 2000; Kantor and Herzenberg, 1993), it is unknown whether the *in vitro* culture of the fetal liver cells skews them towards the development of B2 cells rather than B1 and MZ B cells.

Zap70 expression determined by intracellular flow cytometry revealed that according to mean fluorescence intensities, infected B cells in the lymph nodes expressed at least 5 times more Zap70 than uninfected B cells and around 4 times less than CD19$^-$ lymph node cells, which are mainly T cells (Fig.4.8). This finding shows that Zap70 expression levels in these *Syk$^{-/-}$* B cells do not need to be as high as in T cells to be able to overcome the *Syk$^{-/-}$* B cell developmental block.

In conclusion my results from this part of the study showed that Zap70 is able to transduce signals from the pre-BCR and BCR that are required to allow B cell development to proceed to a mature B cell stage. My results also suggest that the reason why Zap70 is unable to compensate for the lack of Syk in developmentally arrested *Syk$^{-/-}$* immature B cells is probably its very low expression level. Based on the above findings

I cannot exclude, however, that even if present in sufficient quantities, Zap70 may not be able to perform all the necessary tasks as efficiently as Syk.

6.5 Loss of CD21 expression on wt splenic B cells expressing Zap70

An interesting observation was made while analysing radiation chimeras reconstituted with wt*Zap70 fetal liver cells. Splenic donor-derived infected B cells (huCD2$^+$CD19$^+$CD23$^+$) have little or no CD21 expression, whereas donor-derived uninfected B cells had normal expression of CD21. This observation was made in 3 out of 4 radiation chimeras analysed. The expression pattern of other surface markers, e.g. CD19, IgM and IgD were unchanged.

It is known that the surface expression of CD21 in follicular B cells decreases in autoimmune MRL/lpr mice, and it has been speculated that this decrease might reflect activation by antigen (Mandik-Nayak et al., 1999; Takahashi et al., 1997). A similar decrease in CD21 expression has been seen in patients with SLE (Wehr et al., 2004; Wilson et al., 1986). Anti-IgM crosslinking *in vitro* of normal human B cells has been shown to contribute to a decrease in CD21 expression (Boyd et al., 1985). Follicular B cells that lack Aiolos have markedly reduced expression levels of CD21, possibly because these cells receive constitutive BCR-derived signals (Cariappa et al., 2001). It is therefore possible that BCR signalling can cause a reduction in CD21 expression.

B cells that express normal amounts of endogenous Syk-family of protein tyrosine kinases and in addition express Zap70 from the retroviral vector, might receive enhanced

or even constitutive BCR signals. This activation might result in a reduction or loss of CD21 expression on follicular B cells.

To address this further I will examine other cell surface markers on wild-type B cells overexpressing Zap70, e.g. activation markers like CD86 or MHC class II.

To take these experiments further, I plan to create a series of radiation chimeras expressing different levels of one or both of the Syk-family kinases. To achieve this I will use wt, $Zap70^{-/-}$, $Syk^{-/-}$ or $Syk^{-/-}Zap70^{-/-}$ fetal liver cells and transduce them with retrovirus carrying Syk, Zap70 or neither of these molecules.

If BCR strength does indeed influence cell fate decisions during B cell development, and if altering the levels of these kinases changes the strength of signalling, these chimeric mice could reveal the correlation between the presence or absence of different B cell populations and BCR signal strength.

6.6. Hormone-inducible Syk-ER fusion protein

As B cell development is completely blocked at the immature B cell stage in the absence of Syk, it is not possible to study the role of Syk in mature B cell development in conventional gene-targeted animals. However, by making Syk inducible, such that it could be switched on and off at different stages of B cell development, it may be possible to study the role of Syk in mature B cells.

There are many different techniques of generating animals with inducible expression of genes. One system is the Cre-loxP system. Two loxP site could be introduced within the

Syk gene so that after Cre-mediated recombination Syk would be inactivated. This choice would allow B cell development to proceed to the mature B cell stage. And one could explore the role of Syk in mature B cells. However, the disadvantage of this system is that mice with introduced loxP site in the gene of interest need to be crossed to mouse lines expressing Cre recombinase in order to obtain Cre-mediated recombination, which is not a quick process.

The tetracycline-based inducible system (tet-system) is one option of making an inducible and reversible Syk protein. This system uses the reverse transactivator, rtTA, which binds to the tetracycline operator *tetO*, in the presence of the tetracycline derivate doxycycline, and induces transcription of the gene of interest, which is placed under the control of *tetO*. In the absence of doxycycline rtTA is not able to bind to *tetO*, thus the gene of interest is not transcribed. The disadvantage of this system is that is slow, needing around four days for the protein to be fully switched off and around 24 hours for the system to be switched on (Legname et al., 2000).

The system I decided to use was to fuse the ER-HBD (estrogen receptor hormone-binding domain) to Syk, thereby rendering Syk hormone-inducible. The advantages of this system are reversibility and rapid induction. Unlike the tet-system, it neither requires transcription nor translation for induction of activity. To test the function of the Syk-ER fusion proteins, I used DT40 cells as an *in vitro* test system, where the BCR-induced calcium flux is completely dependent on Syk. Three different fusion proteins (Syk-NER™, Syk-HER™ and Syk-CER™) were expressed in *Syk⁻/⁻* DT40 cells, and BCR-induced calcium flux in the presence and absence of 4-OHT was analysed. This experiments revealed that both the Syk-NER™ and Syk-HER™ were activated in a

hormone-dependent manner. Clones expressing the Syk-CER™ fusion protein were constitutively active. This might be due to the high expression levels of the Syk-CER™, resulting in insufficient amounts of Hsp90 in the cell to keep the fusion protein in an inactive state.

Two versions of the ER HBD are available, the ER™ and the newer version ER^{T2}. The ER^{T2} is believed to be 10x more sensitive to tamoxifen than the ER™, which might be useful for later studies of Syk-ER in an *in vivo* mouse model. I therefore made Syk-NER^{T2} and tested its homone-inducibility in *Syk$^{-/-}$* DT40 cells. Clones expressing Syk-NER^{T2}, however, were not entirely hormone-dependent, in contrast to clones stably expressing Syk-NER™, therefore I decided to proceed with the Syk-ER™ fusion protein. Returning to analysis of Syk-NER™, I established the lowest concentration of 4-OHT (100nM) and the shortest incubation time (10min) needed to fully activate Syk-ER. Furthermore, I found that Syk-ER was indeed reversible; 24 hours after removal of 4-OHT Syk-ER was inactive.

The exact mechanism of 4-OHT action on these fusion proteins is unknown. One possibility is that once 4-OHT binds to the HBD it cannot dissociate anymore. The hormone needs to be degraded first, before HBD is accessible for Hsp90 binding. As the half-life of 4-OHT is 6 hours (Danielian et al., 1998), it might take 24 hours before the cell is hormone-free and the fusion-protein therefore inactivated. Another possibility is that the conformational changes caused by hormone-binding are not reversible. Thus to eliminate active Syk, the fusion protein would need to be degraded, new fusion protein would need to be synthesised, which would now be complexed with Hsp90 and hence inactive.

A knock-in mouse expressing the Syk-ER would enable me to study the role of Syk in mature B cells. One could study the role of Syk in TD- and TI-independent immune responses, in plasma cell generation and germinal centre formation. One could monitor B cell proliferation after BCR-stimulation as well as B cell persistence in the presence and absence of Syk. Finally, one could address the role of Syk in class switch recombination and somatic hypermutation, and memory B cell formation and peristance.

6.7 Overall conclusions

In these studies I have investigated the role of Zap70 in B cell development and function. I have shown that Zap70 is expressed in all developing and mature B cell subsets. However the highest expression levels were observed in pro-B cells, in which Zap70 seems to be able to drive cells through the pre-BCR checkpoint. In terms of the involvement of Zap70 in B cell development, I have also shown an increase in MZ and B1 B cells in *Zap70$^{-/-}$* mice, which seems to be due to the lack of Zap70 in the B cell lineage itself. In terms of B cell function, I have explored the role of Zap70 in TD and TI immune responses and BCR-induced calcium release. However, *Zap70$^{-/-}$* B cells were able to mount responses to both, TD and TI antigens. Furthermore, Zap70-deficient B cells had no defect in BCR-induce calcium release.

Using retroviral gene transfer technology I was able to show that Zap70 can compensate for the lack of Syk at the pre-BCR and BCR checkpoints and therefore, the complete block of *Syk$^{-/-}$* B cells at the BCR checkpoint likely due to insufficient amounts of Zap70.

Finally, I have also generated an inducible Syk kinase. I confirmed that the Syk-N-ER™ works in a hormone-inducible manner and made the first step toward the Syk-ER knock-in mouse.

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