

The role of Syk protein tyrosine kinase in B cell development and function

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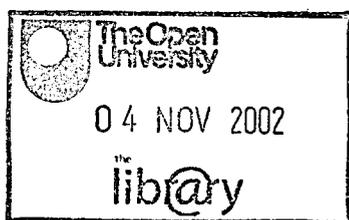
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Table of Contents

1	ABSTRACT	4
2	INTRODUCTION	5
2.1	EARLY B LYMPHOCYTE DEVELOPMENT IN THE MOUSE BONE MARROW	5
2.2	SIGNAL TRANSDUCTION IN B LYMPHOCYTES	21
2.3	SIGNAL TRANSDUCTION IN EARLY B LYMPHOCYTE DEVELOPMENT	28
2.4	SYK/ZAP FAMILY OF PROTEIN TYROSINE KINASES	31
2.5	GENE TARGETED MUTANT SYK ^o MICE	36
3	AIMS OF THE WORK IN THIS THESIS	39
4	RESULTS	2
4.1	RADIATION CHIMAERAS GENERATED FROM FROZEN FETAL LIVERS	40
4.2	SYK ^o LYN ^o DOUBLE MUTANT MICE.....	44
4.2.1	<i>B cell development in the absence of Syk and Lyn</i>	44
4.2.2	<i>Introduction of the 3-83 BCR transgene</i>	46
4.3	SYK AND ZAP-70 IN B CELL DEVELOPMENT	55
4.3.1	<i>B cell development in the absence of Syk and ZAP-70</i>	57
4.3.2	<i>Cell-autonomous effect of Syk and ZAP-70 deficiency</i>	64
4.3.3	<i>ZAP-70 protein expression in B lineage cells</i>	69
4.3.4	<i>Syk/ZAP-70 function in pro-BCR/pre-BCR signalling in vivo</i>	73
4.3.5	<i>Introduction of the 3-83 BCR transgene</i>	82
5	DISCUSSION	99
5.1	THE ROLE OF LYN TYROSINE KINASE IN EARLY B CELL DEVELOPMENT	100
5.2	THE ROLE OF ZAP-70 TYROSINE KINASE IN B CELL DEVELOPMENT	106
6	CONCLUSION	120
7	MATERIALS AND METHODS	122
7.1	SOLUTIONS AND MEDIA	122
7.2	MICE.....	123
7.3	ANTIBODIES AND CELL LINES:.....	123
7.3.1	<i>Purification of antibodies</i>	125
7.3.2	<i>Labelling of antibodies</i>	126
7.4	FACS ANALYSIS AND FACSORTING.....	127
7.4.1	<i>Intracellular staining</i>	127
7.5	GENOTYPING OF MICE	128
7.5.1	<i>FACS analysis</i>	128
7.5.2	<i>Southern Blot</i>	128
7.5.3	<i>Earpunch-PCR</i>	129
7.5.4	<i>Primer sets used for genotyping by PCR</i>	130
7.6	GENERATION OF RADIATION CHIMAERAS.....	130
7.7	FREEZING FETAL LIVERS	131
7.8	WESTERN BLOTTING.....	131
7.9	REARRANGEMENT ANALYSIS – BULK FETAL LIVER PCR	132
8	REFERENCES	135

1 Abstract

Signals reaching B cells through the B cell antigen receptor (BCR) expressed on the cell-surface can affect both the development, differentiation and activation status of B cells. One of the early mediators of signalling through the BCR is the protein tyrosine kinase (PTK) Syk. The exact mechanisms how Syk gets recruited to the BCR, how it becomes activated and what the downstream targets are in different responses still need to be clarified.

Gene targeted homozygous mutant (Syk^o) mice stress the importance of Syk in BCR signalling. B cell development is severely affected in these mice: very few cells reach the immature stage, and even these are prevented from further maturation, pointing to at least two steps in B cell differentiation where Syk is implicated.

To further explore the relative contribution of Syk and two other tyrosine kinases, Lyn and ZAP-70, to the early steps of B cell development, I conducted genetic experiments, generating double knockout (Syk^oLyn^o and Syk^oZAP-70^o) animals.

Analyses of these mice revealed a survival role for Syk and Lyn, a survival and differentiation role for Syk and ZAP-70. This latter was surprising, since ZAP-70 has not been assumed to function in B cell lineage cells. Results presented here demonstrate that ZAP-70 is indeed expressed and functional within B lineage cells, and can replace Syk in some, but not all aspects of pre/BCR signalling.

Importantly, pre-BCR signalling leading to differentiation (as defined by changes in surface protein expression), proliferation and allelic exclusion cannot proceed without the involvement of at least one of the Syk/ZAP-70 family protein kinases.

2 Introduction

2.1 Early B lymphocyte development in the mouse bone marrow

B-lymphocytes are key components of the immune system, as they can take up and present antigens to T cells and can differentiate into antibody-secreting plasma cells. The aim of B cell development is to generate B-lymphocytes that are capable of interacting with the widest possible variety of antigens and that can interact efficiently with T lymphocytes without endangering the integrity of the organism. B cells are generated in the bone marrow from their precursors through a series of developmental stages, passing through multiple checkpoints that can be followed by distinct patterns of surface protein expression and also by analysing the generation and expression of the B cell receptor (BCR).

The immunoglobulin inserted into the plasma membrane of B cells that serves as antigen receptor (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 unique receptors is served by the mechanism of gene rearrangement that during development brings together gene segments coding for different parts of the receptor proteins.

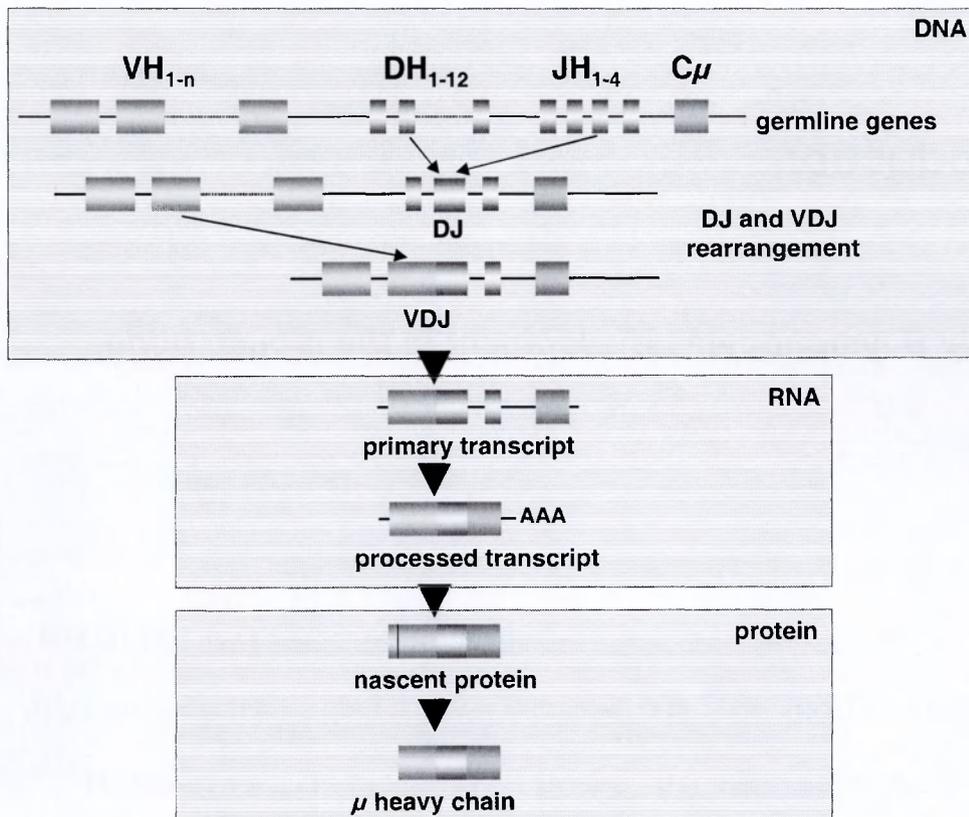


FIGURE 1. GENERATION OF μ HEAVY CHAIN PROTEIN – SCHEMATIC DRAWING (MODIFIED FROM [1]).

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 signals that are needed to start the process are unknown, but since the recombination machinery consists of multiple proteins that have to interact with the DNA, attention has been focused on locus accessibility (see later in this chapter).

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 (1 in 3), a heavy chain protein can be synthesised and tested (see below). Light chain generation follows that of the heavy chain, with a similar mechanism, but only joining V and J segments. 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 the 4 J_H segments. Light chain genes are grouped into κ and λ systems. The κ locus contains multiple V region families to be recombined with one of four J_κ segments, whereas the λ locus contains only 3 V genes with 3 J segments [1].

For a schematic drawing depicting the generation of a μ heavy chain protein from germline encoded gene segments see Figure 1.

Developing B cells in the bone marrow can be subdivided into different fractions from early pre-pro-B cells to mature recirculating B cells, as shown schematically with the two different nomenclatures in use in Figure 2 [2, 3]. Figure 3 shows the use of different antigens as markers of development using the flow cytometer.

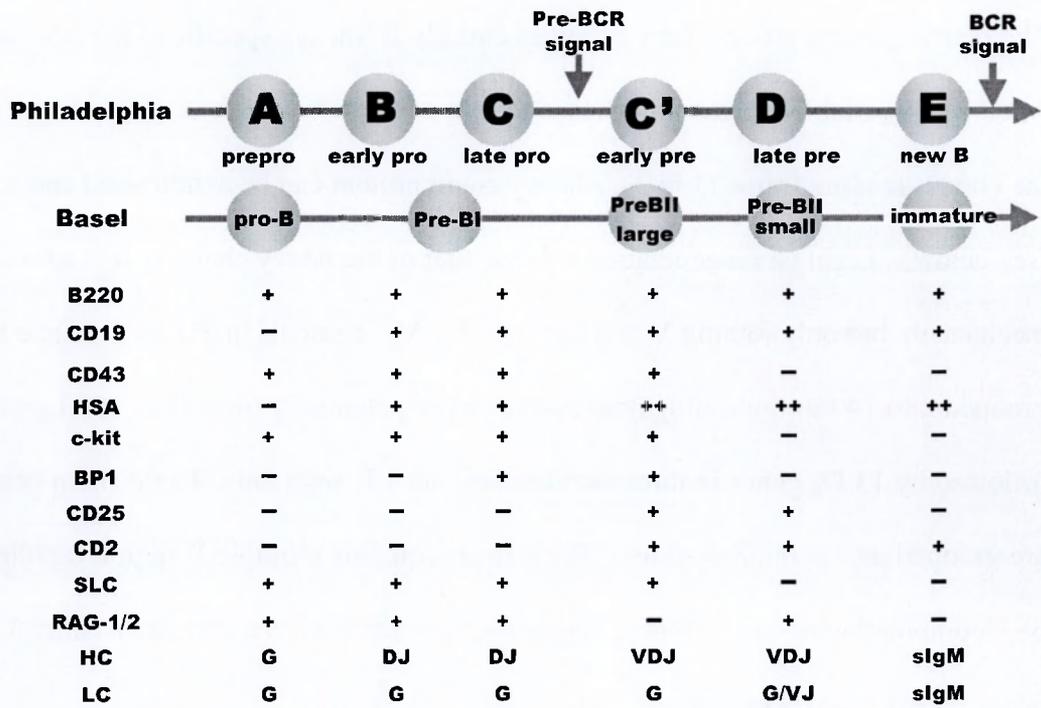


FIGURE 2. SCHEMATIC DEPICTION OF DISTINCT STEPS IN B CELL MATURATION

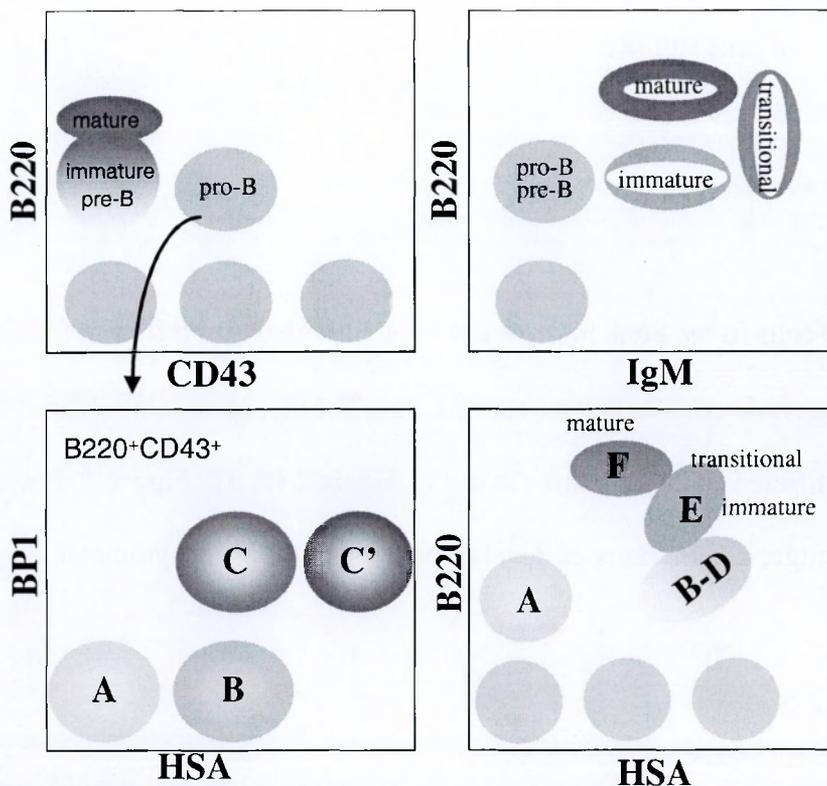


FIGURE 3. SURFACE ANTIGEN EXPRESSION USED TO DISTINGUISH DISTINCT MATURATIONAL STEPS IN B LYMPHOCYTE DEVELOPMENT

Further subdivision of the pre-pro-B cell population identified the earliest B lineage precursors in murine bone marrow as B220⁺ CD43⁺ HSA⁻ AA4.1⁺ CD4^{low} BP-1⁻ CD19⁻ cells [4] – see fraction (Fr.) A₁ in Table 1. While Fr.A₀ cells were shown to give rise to macrophages, granulocytes, erythroid colonies, T cells or B cells *in vitro*, Fr. A₁ appears to be fully B cell committed [5].

marker	A ₀	A ₁	A ₂	B
AA4.1	+	+	+	+
CD43	+	+	+	+
CD4 ^{low}	+	+	-	-
B220	-	+	+	+
HSA	-	-	-	+
CD19	-	-	-	+
μ_0	-	+++	+++	-
RAG-1/2	-	+/-	+	+++
Ig β	-	+	++	+++
Ig α	-	-	+	+++
λ_5	-	-	+	+++

TABLE 1. SURFACE ANTIGEN EXPRESSION IN FRACTIONS A AND B. (MODIFIED FROM: [4])

Other studies have identified a B220⁺ CD19⁻ DX5⁻ Ly-6C⁻ subset of bone marrow cells as the earliest B precursors, that do not appear to overlap with the above mentioned Fr. A cells [6].

Transcription from the unrearranged heavy chain locus (denoted as μ_0) is assumed to be a marker of locus accessibility, although whether it is only a marker, or a prerequisite for recombination is not clear. Detection of μ_0 already in Fr. A₁ suggests locus opening before the rearrangement machinery becomes active.

Among the proteins shown to be indispensable for efficient rearrangement (RAG-1/2, DNA-dependent-Protein Kinase, XRCC4, DNA ligase IV, Ku70 and Ku80) most widely studied have been the two RAG (recombinase activating genes) proteins. As shown in Table 1, RAG-1/RAG-2 activity can be first detected in Fr. A₂ and B. Accordingly, analysis of the immunoglobulin heavy chain locus shows minimal (~4%) D_H to J_H rearrangement in Fr. A₂, that increases to ~50% as cells reach the Fr. B stage [5].

Generation of gene targeted mutant mice have helped to elucidate mechanisms and molecules needed for distinct developmental steps. Different 'knockout' mice show impairment of B cell development at different stages, suggesting the involvement of the removed gene-product in the process.

RAG-1^o and RAG-2^o mice show complete lack of immunoglobulin heavy and light chain rearrangement, proving that both of these proteins are essential for the recombination machinery [7-9]. D_HJ_H, but not V_H-DJ_H rearrangement was found in the Igβ^o mice, suggesting that Igβ - that is already expressed in Fr. A₂ and later on acts as the signal transducing component of the pre/BCR – performs essential functions even before a pre-BCR or a BCR could assemble [10]. Based on this and other experiments revealing Igβ expression on the surface of pro-B cell lines, that have not undergone heavy chain rearrangement [11], the existence of a pro-BCR was proposed. This receptor could be responsible e.g. for initiating/allowing heavy chain V-DJ recombination.

The α chain of the IL-7 receptor was also shown to play a role in allowing V_H to DJ_H rearrangements, since in IL-7Rα^o animals only D-proximal, but not D-distal V_H gene

families can take part in recombination [12]. No germline transcripts were detected from the unrearranged 5' V_H gene families in these mice, pointing to a defect in locus accessibility. The expression level of the BSAP (B cell specific activation protein) transcription factor is also decreased in the absence of IL-7R α . BSAP (encoded by the *pax-5* gene) has been shown to bind an LCR (locus control region) at the 3' end of the IgH locus, and is essential for V-DJ_H joining early in B lymphopoiesis.

Consistent with this, pro-B cells in BSAP^o mice contain D_H-to-J_H rearrangements of the Ig HC locus at normal frequency, however V_H-to-DJ_H rearrangements are 50-fold reduced [13-15].

B cell development in RAG-1^o or RAG-2^o mice can be rescued by the introduction of a pre-rearranged BCR transgene [7], demonstrating that the maturational block was indeed due to the lack of immunoglobulin gene rearrangements. A BCR transgene, however, does not relieve the developmental block in BSAP^o mice, revealing that lack of recombination is only partly responsible for the defect [15].

Successful V_H-DJ_H rearrangement enables a developing B cell to synthesise a heavy chain (μ) protein, and transport a small portion of it to the membrane [16]. Pre-BCR complex is formed containing in addition to the μ chain the so-called surrogate light chain (SLC) components, VpreB and λ 5 [17-20] and also the signalling chains Ig α / β . Since the proper triplet codon reading frame must be maintained upon joining the different gene segments, productive V_H-DJ_H rearrangement is expected in about one-third of the cases ('in-frame' joints). In addition, only about 50% of μ H chains are capable of associating with SLC to form a pre-BCR, that is assumed to be a prerequisite for further developmental progression [21, 22]. Single *ex vivo* pro-B

cells proliferate to different extent, suggesting, that the 'fitness' of μ H-SLC pairing might have a role in determining the expansion of cells carrying distinct heavy chain proteins [23].

Phenotypic analyses of gene-targeted mutant mice missing different components of the pre-BCR provide us with information about the possible roles of this receptor:

- RAG-1^o and RAG-2^o mice: since no recombination can take place, these mutations effectively remove the heavy chain component of the pre-BCR. These mice show complete developmental arrest at B220⁺CD43⁺ pro-B cell stage (Fr. C, Figure 2) [7-9].
- μ MT mice: generated by targeted disruption of the μ chain membrane exon. The heavy chain locus undergoes rearrangement, but the protein product cannot be inserted into the membrane, the result of which is a developmental block at pro-B cell level. Heavy chain allelic exclusion - a process which stops recombination on the second allele if the first allele produces a productive VDJ_H joint, thereby ensuring that each B cell carries only one unique receptor - does not occur, resulting in some cells expressing a heavy chain protein from each of the two alleles. Light chain rearrangement is dramatically decreased, but not absent [24, 25].
- J_HT mice: deleting the whole J_H cluster by gene targeting prevents any heavy chain rearrangements in these mice, accordingly, no development is detected beyond the B220⁺CD43⁺ pro-B cell Fr. C [26, 27].

- $\lambda 5^{\circ}$ mice: in the absence of $\lambda 5$ no proper SLC complex can be formed. B cell maturation in these mice is incompletely blocked; the number of pre-B cells in the bone marrow is dramatically reduced [28]. The ‘leakiness’ of the mutation could be explained by assembly of a μ heavy chain with VpreB in the absence of $\lambda 5$, which appears to be possible in a portion of μ heavy chains that are normally capable of pairing with SLC [29]. Alternatively, early light chain rearrangements can also occur [30], providing the cells with a receptor, that cannot signal proliferation but can promote allelic exclusion, since heavy chain allelic exclusion is maintained in the absence of $\lambda 5$. In support of this latter possibility, combined $\lambda 5$ and Rag-1 deficiency in the presence of a heavy chain only transgene does not allow any progression into the pre-B cell compartment [31].
- Vpre-B1 $^{\circ}$ mice: the two Vpre-B genes in the mouse genome (Vpre-B1 and Vpre-B2) both encode proteins that can form a pre-BCR with a μ heavy chain and $\lambda 5$. Removal of Vpre-B1 protein lead to a partial block at the pre-BCR checkpoint, resulting in mildly decreased numbers of pre-B (pre-BII in the Basel nomenclature) cells, suggesting that Vpre-B2 can efficiently, though not completely substitute for Vpre-B1 [32].
- Vpre-B1 $^{\circ}$ Vpre-B2 $^{\circ}$ mice: upon removal of both VpreB gene products, no surface expression of any of the SLC components can be detected. Pre-B cells cannot expand resulting in severely reduced (~40-fold) pre-B cell numbers in the bone marrow showing the importance of a proper pre-BCR in inducing proliferative expansion. Surprisingly, allelic exclusion at the heavy chain locus is maintained. This finding could be explained by the assembly of yet to

be identified proteins with the μ heavy chain into an alternative pre-BCR, or light chain rearrangements happening earlier than normal, possibly helped by lack of proliferation [33].

D_H segments can be read in all three reading frames (RF), however, the frequency of the three RFs found in mature B cells are not equal. About 70% of mature B cells carry D regions using RF1, 30% uses RF3, and only 3% use RF2. The dominance of RF1 over RF3 is mainly due to STOP codons in RF3 in 8 of 12 D_H regions. RF2 is unique, in that it allows synthesis of the so-called $D\mu$ protein without the addition of any V_H regions. This $D\mu$ protein can form a complex with VpreB and $\lambda 5$ and assemble into a pre-BCR-like receptor with $Ig\alpha$ and $Ig\beta$. RF2 underrepresentation, which is already evident in pre-B cells, requires the membrane form of the protein, and also needs $\lambda 5$. Signalling through this receptor complex results in allelic exclusion, that is no further rearrangements can occur at the heavy chain locus [34-36]. Underrepresentation of RF2 suggests that this receptor cannot induce all the signalling events that a proper pre-BCR is capable of [37].

Pre-BCR signalling has been shown to cause down-regulation of Rag1/2 activity and the synthesis of SLC components –VpreB and $\lambda 5$ – is also turned off, which allows allelic exclusion to occur and also leads to the cessation of the pre-BCR signal [38].

At the next stage of development the SLC will be exchanged for conventional light chains (see below). Whether the pre-BCR induces light chain rearrangement or not is a matter of debate. Light chain rearrangements have been shown to occur even in the absence of any heavy chain recombination [30, 39]. On the other hand, induced pre-BCR expression in previously pre-BCR⁻ cells lead to germline κ_0 transcription and rearrangement [40, 41]. These conflicting data can be reconciled by suggesting that

pre-BCR is not strictly required for light chain rearrangement, but the frequency/efficiency of light chain recombination is increased by signals emanating from the pre-BCR.

As mentioned above, IL-7R α is critical for complete V_H-DJ_H rearrangement.. At a later stage, at the pro-B→pre-B transition, pre-BCR assembly and expression was needed for increased IL-7 responsiveness, suggesting that the interplay between the pre-BCR and IL-7 allows proliferation at this maturational step [42-44].

In summary, the role of the pre-BCR is to

- promote differentiation of pro-B cells into pre-B cells
- selectively amplify pre-B cells that have a productively rearranged μ heavy chain gene coding for a protein capable of associating with the SLC
- ensure allelic exclusion at the heavy chain locus
- arrest D μ producing cells
- promote light chain rearrangement
- downregulate SLC expression
- increase IL-7 responsiveness.

The next stage of B cell development leads to the maturation of pre-B cells into IgM expressing immature B cells. The hallmark of this step is the replacement of the pre-BCR with a conventional BCR that necessitates the complex formation between the tested μ heavy chain and a newly synthesised light chain. After the proliferative phase – induced by pre-BCR signalling – pre-B cells have to enter a resting phase, so that they can re-induce RAG protein expression that is down-regulated in dividing cells [45].

As mentioned above, light chain genes are organised into κ and λ loci. Analyses of light chain rearrangements [29, 46-49] have shown that:

- light chain rearrangements are completely absent in Fr. C' (large pre-BII in the Basel nomenclature) cells, and become prominent in the Fr. D (small pre-BII) population;
- most κ^+ B cells have their λ locus in germline configuration, whereas most λ expressing cells have non-productive rearrangements on both of their κ alleles, the majority of these using the most 3' J κ segments, suggesting that κ rearrangements precede that of the λ locus;
- κ and λ LC loci can rearrange independently of each other, since prior recombination of a κ allele is not prerequisite for λ rearrangement as demonstrated using mutant mice incapable of rearranging κ
- rearrangements at the κ locus are induced 5-10-fold more frequently than at the λ locus, they also start earlier, which leads to the ~10:1 ratio of κ : λ rearrangements that is established as soon as light chain rearrangements start
- cells can attempt multiple rearrangements on the same κ allele – probably to replace the original rearrangement that was either out-of-frame, produced a light chain protein that was incapable of pairing with the heavy chain expressed in the cell, or formed an autoreactive BCR with the μ chain (see 'receptor editing' below).

A second major checkpoint in B cell development (the first being the pre-BCR step) is the testing of the newly formed BCR. Since B cells carrying a BCR specific for

an autoantigen could be deleterious for the organism, the process of negative selection is needed to ensure that only self-innocuous B cells leave the bone marrow.

A number of different mechanisms of tolerance/negative selection have been described for B lymphocytes acting at different stages of maturation:

- receptor editing: preserves the cell, but eliminates the original receptor that proved to be autoreactive. This can be achieved by exchanging the heavy or light chain of the receptor by secondary rearrangements [50-54]. The original κ light chain rearrangement leaves upstream $V\kappa$ and downstream $J\kappa$ segments intact and available for subsequent recombination. Heavy chain replacement, however, is mechanistically more complicated, since V_HDJ_H rearrangement eliminates the sequences between the segments used (see Figure 1); the cryptic recombination signal sequences (RSS) that can be used for this type of secondary heavy chain recombination are embedded at the 3' end of most V_H genes [55-57].

This method of negative selection appears to be ontogenically the first possibility : emerging B lymphocytes expressing low levels of IgM respond to their cognate ligand by increased RAG expression, making continued immunoglobulin rearrangements possible [58];

- clonal deletion eliminates autoreactive B cells from the repertoire by apoptosis [59-62]. The likely target cell population for deletion is the so-called transitional cells. These cells are in transition from the immature (HSA^{bright} , $B220^{dull}$) to the mature (HSA^{dull} , $B220^{bright}$) stage. They also express high levels of IgM, Fas antigen, but no anti-apoptotic Bcl-2, which makes them rather

sensitive to this form of negative selection [58, 63]. Cells at this transitional stage are believed to be leaving the bone marrow for the periphery, which might also contribute to their increased sensitivity to deletion rather than receptor editing : cellular elements of the bone marrow were shown to be protective against apoptosis [64];

- 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 [65], which – analysing normal, polyclonal populations – may make anergy almost indistinguishable from deletion [66-69]. Whether cells get deleted or anergized probably depends on the quality of receptor-ligand interaction, including avidity, receptor cross-linking [61] or the possibility of repeated antigen encounter [70];
- follicular exclusion: anergic cells – when competing within a normal repertoire of B cells - do not seem to be able to enter the long-lived follicular B cell population of the spleen. This exclusion leads to shorter life span (1-3 days) which is inversely proportional to the degree of self-reactivity. Being confined to the outer T cell zone, these anergic B cells are destined to die, unless a robust signal (e.g. a strongly cross-reactive foreign antigen) rescues them from death. Since these anergic cell require a more powerful signal for their activation, and because without this signal, they are short-lived, the risk of autoimmunity induction is probably smaller, than the advantage that can be

gained by the extension of the repertoire by granting them a 'second chance' [67, 71, 72];

- another interpretation of the above findings that led to the concepts of anergy and follicular exclusion is that these two phenomena are detectable in transgenic systems only, where the competition between cells is artificially kept at minimum. Under more physiological circumstances, in a competitive environment, no or very few anergic cells are seen. In the HEL system the T cells were shown to be tolerant, therefore there is no T cell help available. The B cells see their cognate antigen, move into the T zone and eventually die. This process is essentially peripheral tolerance effected by lack of T cell help.
- peripheral tolerance: in addition to central tolerance affecting developing B lymphocytes in the bone marrow, clonal deletion or anergy can affect mature B cells in the peripheral organs, as well, which is essential for autoantigens only expressed outside of the bone marrow [73-75].

Some autoreactive cells can also escape deletion and survive in the peritoneal cavity as B-1 (see below) B cells, expanding in the absence of antigen, and contributing to autoimmunity [76, 77].

B1 cells [78] are a special subset of B cells that in mouse is mainly found in the peritoneal cavity. Phenotypically these cells are B220^{low} IgM^{hi} IgD⁻ CD23⁻ Mac-1⁺, expressing (B1a) or not (B1b) CD5. B1 cells are self-renewing, they secrete IgM, IgG3 and IgA and are thought to be responsible for natural immunity.

There are interesting similarities between anergic B cells and cells of the B1(a) lineage: both express CD5, have received stimulation by antigen, show suboptimal response to B cell receptor crosslinking *in vitro*, have undergone extensive receptor

editing and both are excluded from germinal centres [79]. Whether there is more connection between anergic and B1 cell populations than mere phenotypic resemblance is currently a focus of interest.

In addition to negative selection, evidence is accumulating that a 'positive selection' mechanism also operates. The aim of this process would be to ensure that only cells equipped with a functional, well-fitting heavy-light chain pair could enter the long-lived, mature B cell pool.

First indication for the existence of positive selection in B cells came from comparison of HC and LC gene usage in the bone marrow and in the periphery [80-82]. Results from these studies show that the peripheral repertoire is more restricted than that of the bone marrow, suggesting that only cells with 'selected' receptors are allowed to complete maturation. Also, since mature B cells need continuous 'tickling' of their BCR for survival [83], it seems reasonable to assume that BCRs need to be selected before the cells reach full maturity.

Direct evidence for positive selection comes from a study of B1 cells showing naturally generated autoreactive cells accumulating only in the presence of autoantigen [84]. Experiments on another special B cell subset, the marginal zone B cells also suggest the existence of positive selection in the B lineage [85].

2.2 Signal transduction in B lymphocytes

The main aim of B cell development is to provide the organism with B-lymphocytes carrying unique receptors that enables them to bind and react to antigens. As mentioned above, during maturation the components of the B cell receptor are tested, which inevitably involves signal transduction through the different versions of the BCR, though whether or not an external ligand is also involved in the 'tests' is still a matter of debate.

Most of the studies analysing signal transduction through the BCR have used mature B lymphocytes, and whether the same mechanisms operate similarly in B cells at earlier stages of development has for most processes not been confirmed yet.

Signal transduction through the B cell receptor on mature B cells is initiated by ligand binding to the receptor complex, that is composed of a membrane-bound immunoglobulin noncovalently bound to an $Ig\alpha/\beta$ complex in 1:1 stoichiometry. Monomers of the BCR appear to form oligomeric complexes on the cell surface, which is probably crucial for efficient signal propagation [86].

Among the earliest events after receptor crosslinking is tyrosine phosphorylation of a group of cellular proteins (reviewed in [87-91]). Since the BCR does not have intrinsic tyrosine kinase activity, it has to recruit cytoplasmic protein tyrosine kinases (PTK) using its signalling components, $Ig\alpha$ and $Ig\beta$. The cytoplasmic tail of both of these coreceptors harbours an ITAM (Immunoreceptor Tyrosin-based Activation Motif) sequence: $(D/E)_x7(D/E)_{xx}Y_{xx}L/I_x6-8Y_{xx}L/I$ [92]. The tyrosines in these

ITAMs are rapidly phosphorylated upon receptor ligation probably by Src-family tyrosine kinases (Lyn, Fyn, Blk and/or Lck), since a small amount of these kinases is associated with the BCR before stimulation [93], and they are among the earliest kinases to be activated upon BCR stimulation [94]. If both ITAM tyrosines are phosphorylated (about 20% of the ITAMs), they become binding sites for the tandem SH2 domains of the Syk protein kinase (see Syk structure, below) of the Syk/Zap-70 family of tyrosine kinases. Activation of Syk ensues, either by auto-phosphorylation, cross-phosphorylation by neighbouring Syk molecules, and/or by Src-family kinases (See Figure 4, No.1.).

Active Syk is required for the recruitment/activation of the adapter protein BLNK (B cell LiNKer protein), that couples Syk to multiple downstream signalling cascades (Figure 4, No.2) [95-98].

One of the targets recruited by BLNK is PLC γ (phospholipase-C γ) that also binds to and becomes phosphorylated by Btk (Figure 5)and potentially Syk (Figure 4, No.3.). PLC γ cleaves the membrane lipid component PIP2 (phosphatidylinositol-4,5-bisphosphate) to IP3 (inositol-1,4,5-trisphosphate) and DAG (diacylglycerol). DAG leads to PKC (protein kinase C) activation, whereas IP3 is crucial in the regulation of intracellular Ca⁺⁺ concentration (see below and Figure 5). Elevated Ca⁺⁺ level leads to the dephosphorylation and nuclear translocation of NF-AT (nuclear factor of activated T cells) via the calcium-dependent phosphatase, calcineurin; NF- κ B proteins c-Rel and RelA also move to the nucleus as a result of increased Ca⁺⁺ concentration (Figure 4, No.4), where they regulate gene expression.

Another pathway starting from Syk and BLNK involves the tyrosine phosphorylation and activation of Vav, that acts as a GEF (guanine nucleotide exchange factor) for the Rho family of GTPases (Rac-1, RhoA and Cdc42). Their downstream targets include the ERK (extracellular signal regulated kinase), JNK (c-Jun N-terminal kinase) and p38 kinases and the cytoskeleton (Figure 4, No.5) [96, 99].

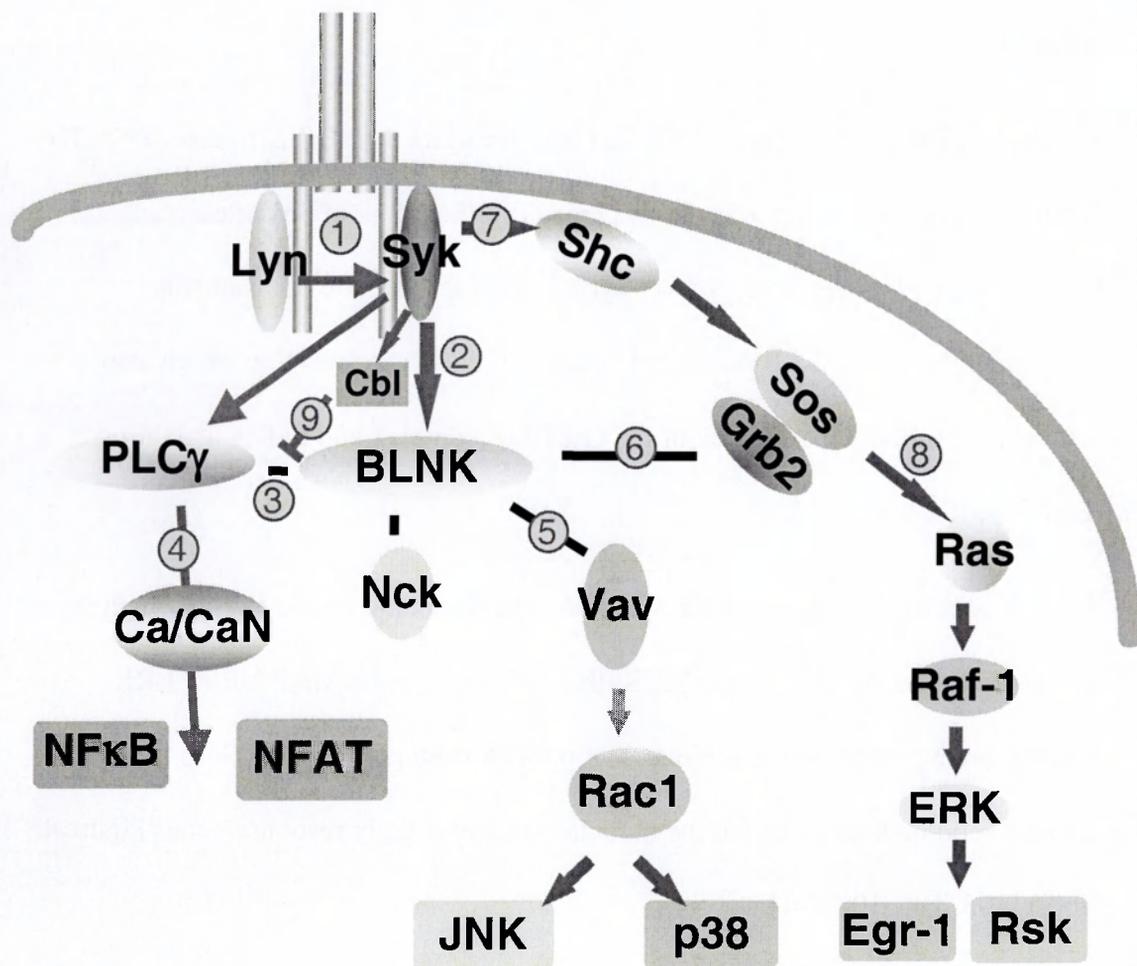


FIGURE 4. MAJOR SIGNALLING PATHWAYS IN B-LYMPHOCYTES UPON BCR STIMULATION.

See text for description. For additional molecules and pathways see also Figure 5.

Cbl (cellular homologue of the transforming gene of the Cas-NS1 murine retrovirus) has also been shown to bind to and be phosphorylated by Syk [100, 101]. More recently Cbl has been shown to be an E3 ubiquitin ligase [102], and as such appears to regulate Syk ubiquitination and degradation [103]. In DT40 chicken B lymphoma cells Cbl was able to interact with BLNK, obstruct BLNK-PLC γ 2 interactions, thereby preventing PLC γ 2 activation [104] (Figure 4, No.9.). Both of these mechanisms are probably part of a negative feedback loop leading to termination of B cell activation.

Following BCR crosslinking, BLNK can also associate with Grb2, translocate to the membrane and activate the Ras pathway via Grb2/Sos (Son of Sevenless; GEF for Ras) complex [95] (Figure 4, No.6). Syk can also activate the Ras pathway independently of BLNK: Syk interacts with and phosphorylates Shc which also results in membrane localisation of the Grb2/Sos complex and in Ras activation (Figure 4, No.7) [95, 99].

Ras activates its downstream effector, the serine-threonine kinase Raf-1, which in turn activates the MAPK kinase MEK, that will activate the MAP kinase ERK leading among others to the activation of p90Rsk (mitogen-induced S6 serine/threonine kinase) and to the induction of *Egr-1* early response gene (Figure 4, No.8) [105, 106, 107 504].

As mentioned above, Syk recruits and phosphorylates PLC γ 2 via BLNK (Figure 5, No.1.), leading to the production of IP $_3$ (Figure 5, No.2.). IP $_3$ allows Ca $^{++}$ release from IP $_3$ receptor-gated intracellular stores (Figure 5, No.3.). This can be detected as the initial rapid peak in Ca $^{++}$ flux measurements. Btk (Bruton's tyrosine kinase), a member of Btk/Tec family of protein tyrosine kinases, is also important in activating

PLC γ 2 (Figure 5, No.4), thereby enhancing peak IP $_3$ levels leading to a profound depletion of intracellular calcium stores. This store depletion results in the opening of plasma membrane Ca $^{++}$ -channels via store-operated calcium entry (SOC) mechanism (No.5.), making Btk responsible for the sustained increase in intracellular Ca $^{++}$ -concentration following BCR activation [108-110].

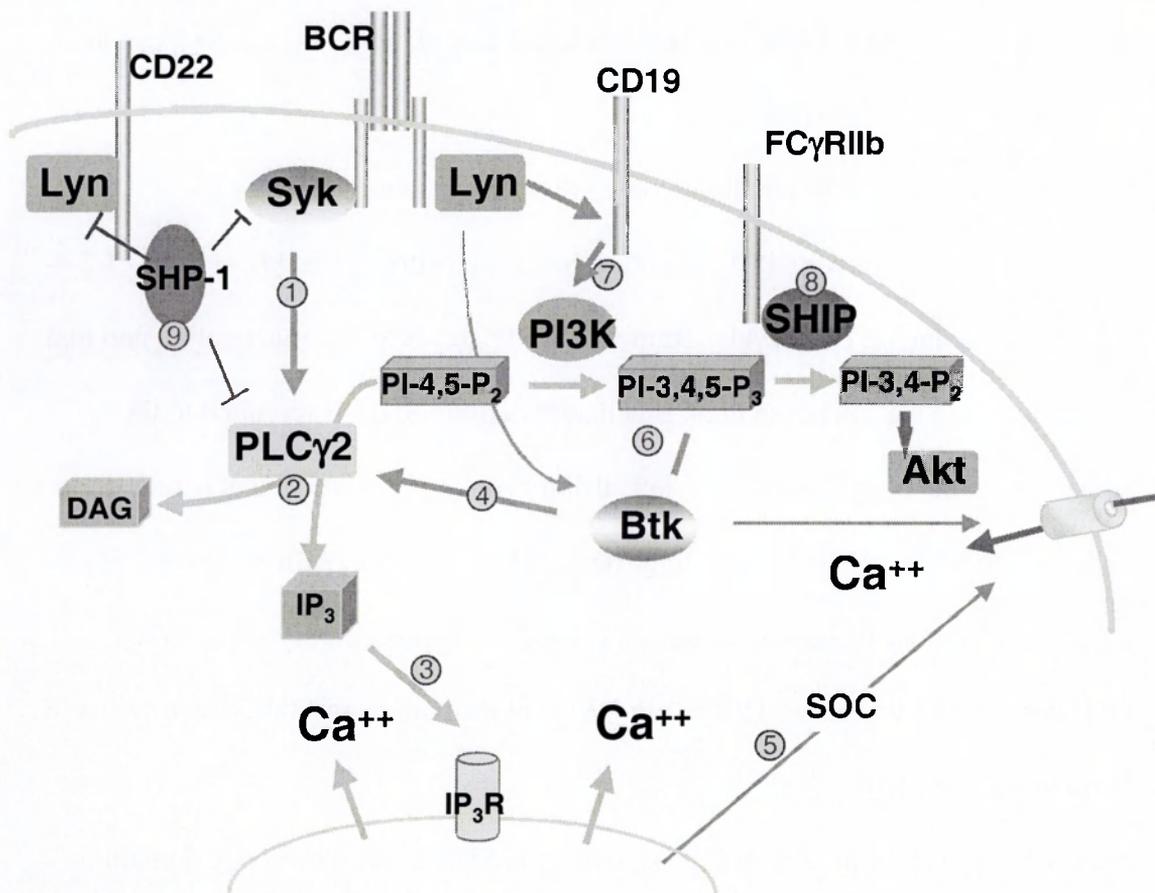


FIGURE 5. ADDITIONAL MOLECULES INVOLVED IN BCR-MEDIATED SIGNALLING THAT ARE ESPECIALLY IMPORTANT IN REGULATING INTRACELLULAR Ca $^{++}$ CONCENTRATION.

Btk activation requires phosphorylation by Lyn, binding to tyrosine phosphorylated BLNK and also membrane localisation, which is achieved by binding of PIP $_3$

(phosphatidylinositol-3,4,5-trisphosphate) to the PH (pleckstrin homology) domain of Btk (Figure 5, No.6) [111]. PIP₃ also binds to the PH domain of PLC γ , thereby contributing to the membrane localisation of this lipase [112].

PIP₃ is a product of PI3K (phosphatidyl-inositol-3-kinase), which in turn is subject to multiple potential regulatory pathways: (a) PI3K can associate with CD19, but only after BCR crosslinking that leads to lyn activation, which in turn can phosphorylate multiple tyrosine residues in the cytoplasmic tail of CD19 [113] (Figure 5, No.7); (b) Vav-3 can also activate PI3K, possibly via Rac-1 stimulation[114]; (c) Syk can also contribute to PI3K activation [115].

PIP₃ is substrate for SHIP (src homology 2 domain-containing inositol 5'-phosphatase), that converts PIP₃ into phosphatidyl-inositol-3,4-bisphosphate (PI-3,4-P₂), thereby reducing PIP₃ levels. Removal of PIP₃ leads to Btk inactivation and loss of the sustained Ca⁺⁺ increase upon B cell stimulation. SHIP is recruited to the signalling complex by Fc γ RIIb, the low affinity receptor for IgG that acts as a negative modulator of BCR signalling (No.8.) [116-118]. Fc γ RIIb, as several other negative regulatory receptors, carries an ITIM (immunoreceptor tyrosine-based inhibitory motif) sequence - (I/V)x(p)YXXL - in its cytoplasmic tail, that serves as a binding site for SHIP.

Another negative modulator of Ca⁺⁺ signalling is SHP-1 (Src-homology domain 2 containing tyrosine phosphatase-1). Upon BCR ligation activated Lyn phosphorylates and recruits CD22 to the receptor complex, whose phosphorylated ITIM in turn binds SHP-1. SHP-1 seems to influence multiple early and late events in B cell activation: it inhibits Syk, Lyn and PLC γ 2 activation, leading to decreased Ca⁺⁺ release from intracellular stores and also blocks ERK activation [119, 120].

Moreover, since SHP-1 is weakly associated with the resting BCR, and has been

shown to dephosphorylate $Ig\alpha/\beta$ *in vitro*, SHP-1 might be responsible for keeping the BCR complex in an unphosphorylated, quiescent state [121].

2.3 Signal transduction in early B lymphocyte development

The earliest B cell specific receptor appears to be the pro-BCR, which was found on the surface of pro-B cells before any heavy or light chain rearrangement occurs [11]. In the absence of any clonotypic component, this receptor contains CD79a/Ig α and CD79b/Ig β chains in complex with calnexin and other as yet unidentified proteins [11]. In RAG $^{\circ}$ bone marrow cells this receptor has been shown to be capable of signalling molecules become tyrosine phosphorylated (Syk, Vav, p110 PI3K, ERK1; Ig α) [122]. Also, as mentioned in the previous chapter, Ig β° mice show D-J_H, but not V-DJ_H recombination, suggesting a role for an Ig β -containing receptor prior to any μ HC expression [10]. A similar complex has been also found on the surface of pro-T cells (CIC: clonotype-independent complex) that is assumed to influence T cell maturation before the start of TCR β expression [123, 124]. Identification and removal of the other – as yet unknown – components of the putative pro-BCR should help elucidating the role and importance of this receptor in early B cell development. *In vivo* crosslinking of Ig β on the surface of RAG $^{\circ}$ pro-B cells was shown to induce differentiation as measured by loss of CD43, λ 5 and c-kit expression and upregulation of CD25, BP-1 and CD2 [122]. This response is thought to mimic a pre-BCR signal in the absence of μ heavy chains in these recombinase-deficient pro-B cells.

The signal transduction machinery involved in the pre-BCR checkpoint has also been described with the help of gene targeted mutant mice:

- Ig β ^o mice: as mentioned above, no V_H-DJ_H rearrangement takes place in these mice, therefore the role of Ig β as a component of the pre-BCR cannot be assessed [10].
- Ig α ^{Δ c/ Δ c} mice: while no complete knockouts of the Ig α gene have been reported, truncation of the intracellular portion of this pre-BCR component leads to the accumulation of 70% more than normal pro-B cells, suggesting a partial block at the pre-BCR step [125].
- Ig α ^{FF/FF} / Ig β ^{Δ c Δ c} mice: combination of Ig α cytoplasmic tail tyrosine to phenylalanine mutations with truncation of the Ig β protein cytoplasmic tail leads to a complete arrest of B cell development at pro-B cell stage, demonstrating the importance of Ig α / β as pre-BCR signal transducing components [126];
- Syk^o mice: in the absence of Syk protein tyrosine kinase B cell development is blocked incompletely at the pro-B \rightarrow pre-B, and completely at the immature \rightarrow mature transition (see below in detail) [127, 128];
- BLNK^o mice: removal of BLNK adapter protein leads to incomplete block at the pre-BCR step resulting in increased numbers of pro-B cells, while reduced numbers in subsequent maturational stages. IgM expressing cells do develop and with time accumulate in the periphery, but are less responsive to BCR stimulation than wild type B cells [129, 130].
- xid (X-linked immunodeficiency) mice: a point mutation in the PH domain (R28C) of the *btk* gene causes XLA (X-linked agammaglobulinemia) in humans and a milder immunodeficiency in mice. Xid mice show somewhat impaired pro-B to pre-B transition [131] and defective λ light chain

rearrangement [132]. Anti-Ig β treatment of RAG-2 $^{\circ}$ pro-B cells cannot induce differentiation into pre-B cells in the absence of Btk, also suggesting a role for Btk in pro-BCR and/or pre-BCR signalling [133].

- BLNK $^{\circ}$ xid mice: combined mutations resulting in the absence of both BLNK and functional Btk leads to a much more severe phenotype than the single mutations: B cell development is blocked, albeit incompletely at the B220 $^{+}$ CD43 $^{+}$ CD25 $^{+}$ pre-BCR $^{+}$ Fr. C' (large pre-BII) stage [134].
- CD19 transgenic mice: while removing CD19 does not lead to any obvious perturbation of B cell development in the bone marrow, overexpression of CD19 in the form of a transgene causes early B cell maturation defect, with normal B220 $^{+}$ IgM $^{-}$ pro- and pre-B cell numbers, but significant reduction in the number of B220 $^{+}$ IgM $^{+}$ bone marrow cells [135].

The phenotype of the above mutant mice suggests that at least some of the key molecules are shared between pre-BCR and BCR signal transduction. There are, however, several knockout mouse models showing significant defects at the immature \rightarrow mature B cell transition, that show no, or only mildly impaired B cell maturation at the early steps in the bone marrow, e.g. lyn $^{\circ}$ [136, 137], CD45 $^{\circ}$ [138], xid [139, 140], PKC β° [141], p85 α (PI3K) $^{\circ}$ [142], vav-1 $^{\circ}$ vav-2 $^{\circ}$ [143], PLC γ -2 $^{\circ}$ [144], or lyn $^{\circ}$ xid [145] mice.

The lack of earlier B cell developmental impairment in these mutant mice might suggest different 'wiring' from the pre-BCR and from the BCR, or might reveal higher level of redundancy at the early steps.

2.4 Syk/ZAP family of protein tyrosine kinases

Protein tyrosine kinase Syk has been shown to be important in a variety of cell types of the haemopoietic system: B and T lymphocytes, mast cells, NK cells, macrophages and platelets [146, 147]. The protein sequence of Syk (and Zap-70) predicts a structure of two SH2 domains and a kinase domain (Figure 6).

Both SH2 domains have been shown to be necessary for Syk binding to tyrosine-phosphorylated Ig α and Ig β , and this binding is essential for phosphorylation at Y519 and Y520 after BCR ligation [148]. Tyrosines 518 and 519 were found to be the major autophosphorylation sites on Syk, and *in vivo* they are indispensable for signal propagation [149]. Since Syk with mutated tyrosines at 518 and 519 is still almost fully kinase-active, these phosphotyrosines are probably more important in allowing protein-protein interactions (e.g. Src-family kinase binding to Syk) than directly influencing Syk enzymatic activity [150].

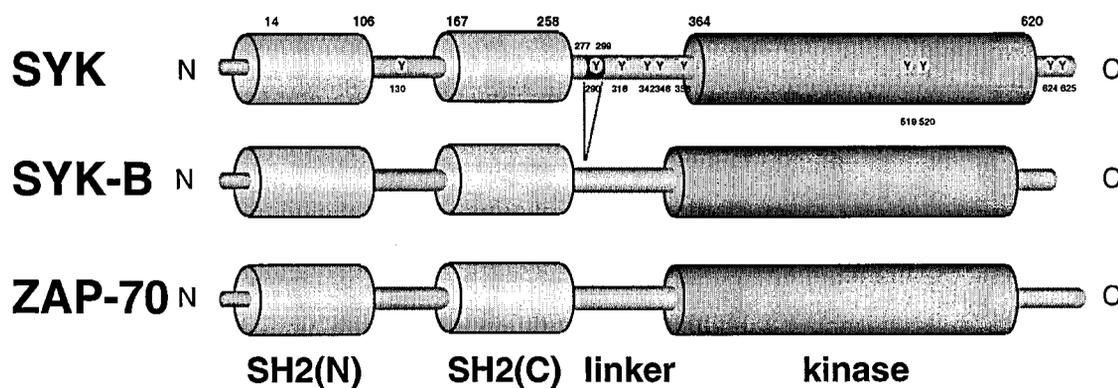


FIGURE 6. SCHEMATIC STRUCTURE OF SYK-FAMILY PROTEIN TYROSINE KINASES.

The tandem SH2 domains are shown as light cylinders, the kinase domain as dark cylinder, the tube depicts the linker region connecting them. The dark section refers to the linker insert (Syk only) that differentiates Syk from SykB. Also shown are the tyrosines mapped in Syk as potentially important phosphorylation sites. Some of these - but not all - have been shown to map to homologous regions in ZAP-70.

Several other tyrosines were shown to be important in interacting with known downstream effectors:

- Y342 and to a lesser degree Y346 within the linker region of Syk are required for interacting with Vav [99];
- Y316 is a putative Cbl binding site [151];
- tyrosines in the linker region (e.g. Y341) are necessary for interaction with the C-terminal SH2 domain of PLC γ [152].

In addition, phosphorylated Y130 causes conformational changes in the inter-SH2 domain, altering both the SH2-SH2 interface interactions and the interactions between the inter-SH2 region and the kinase active site. It has been proposed that phosphorylation of Y130 negatively influences the binding of Syk to the antigen

receptor and at the same time allows increased access of the catalytic site to protein substrates [153].

The linker region is highly susceptible to proteolysis, and is thus likely to be exposed *in vivo*, suggesting participation in protein-protein interactions; it probably functions as a hinge - regulating intramolecular interactions between the N- and C-terminal portions of Syk [154]. The 23 amino-acid linker-insert found in Syk, but not in the alternatively splice isoform SykB, appears to facilitate ITAM binding [155].

K396 in the catalytic, kinase domain is located within the ATP-binding site, whereas the above mentioned Y518/519 lie in the conserved activation loop [146].

Figure 6 shows very similar domain structures for Syk and ZAP-70, the only two known members of the Syk/ZAP family of protein tyrosine kinases. There are, however, important differences between the two kinases:

- expression pattern: Syk is known to be expressed in almost all haemopoietic cell lineage, in vascular endothelium and mammary epithelium, whereas ZAP-70 appears to be restricted to T and NK lineage cells [156];
- the catalytic activity of Syk is ~100-fold greater than that of ZAP-70, which seems to be an intrinsic characteristics of the respective catalytic domains [157]
- comparison of the crystal structures suggests that the two SH2 domains in Syk are more flexible and more independent from each other, than is the case for ZAP-70, offering an explanation why Syk might be able to interact with a

wider variety of proteins, which might also explain its ubiquitous expression pattern [158];

- Syk can be activated in the absence of Src family tyrosine kinases (SFK), whereas ZAP-70 depends on SFKs; a possible explanation for this difference may come from tyrosines 519,520 in the activating loop, that are substrates for autophosphorylation in Syk, but not in ZAP -70 [146];

Other receptors that can bind Syk upon ITAM phosphorylation

A number of receptors, known as immunoreceptors, found on the surface of haemopoietic cells show strong structural similarities: they all have separate subunits for antigen recognition and signal transduction (see Figure 7.). They include the TCR, FcεRI, FcγRI and FcγRIII. Similarly to the B cell signalling described above, ligation of these receptors induces ITAM phosphorylation and Syk recruitment.

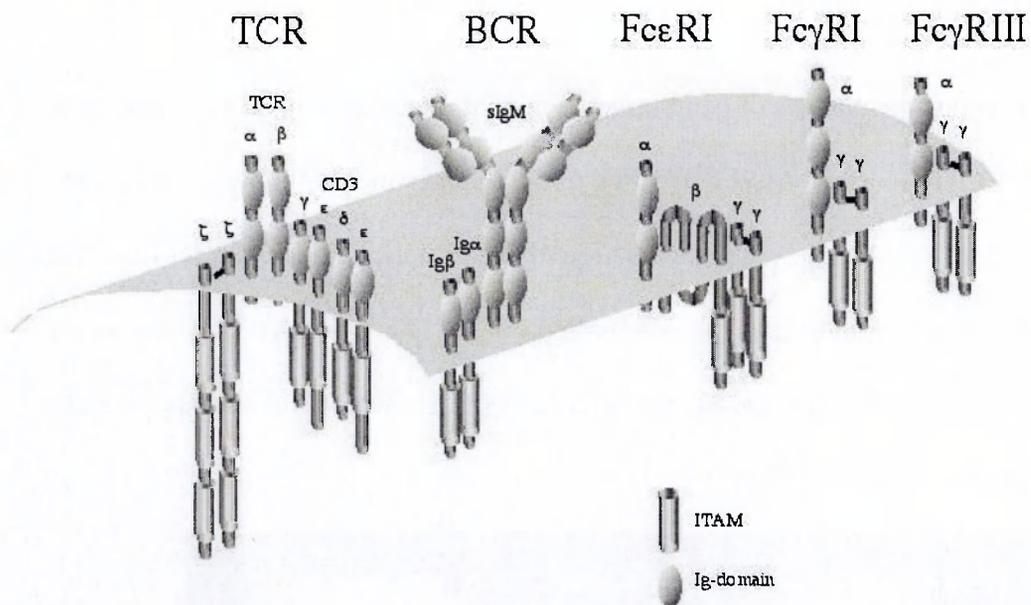


FIGURE 7. FAMILY OF HEMATOPOIETIC RECEPTORS WITH ITAM-CONTAINING SUBUNITS

The majority of T cells recognise antigen via the TCR (T cell receptor) $\alpha\beta$ dimer expressed on their surface in complex with the signalling CD3 subunits γ , δ , ϵ and ζ . In a smaller population of T cells TCR γ and δ substitute for the $\alpha\beta$ dimer (not shown). Similarly, B cells use the membrane-bound IgM tetramer (2 heavy + 2 light chains) for antigen recognition, whereas the signalling function of the B cell receptor (BCR) comes from the Ig α /Ig β heterodimer (CD79a/b). The other three receptors shown bind the Fc portion of IgE (Fc ϵ RI) or IgG (Fc γ RI, Fc γ RIII) antibodies and also consist of recognition and signalling chains. Fc ϵ RI is found on mast cells and basophils, while the Fc γ R are expressed on macrophages, neutrophils and NK cells. Fc γ RIIA (not shown) combines the Ig-like subdomain and the ITAM in one chain. Some Fc γ RIIIs contain ζ chains instead of Fc γ R; conversely, some TCR complexes use Fc γ R instead of ζ .

The balls in the recognition subunits symbolise the Ig-like subdomains, while the ITAM (Immunoreceptor Tyrosine-based Activation Motif) sequences are shown as cylinders.

2.5 Gene targeted mutant *Syk*^o mice

Generation and analysis of gene-targeted mutant mice carrying no functional *syk* alleles lead to further dissection of the role *Syk* plays in B cell development and function. [127, 128]. *Syk*^o mice die around birth for as yet unknown reasons, though extensive petechial haemorrhaging throughout the fetal body that is visible around day 16 of gestation (Figure 8), points to a defect in vascular endothelium, platelet function or wound healing.

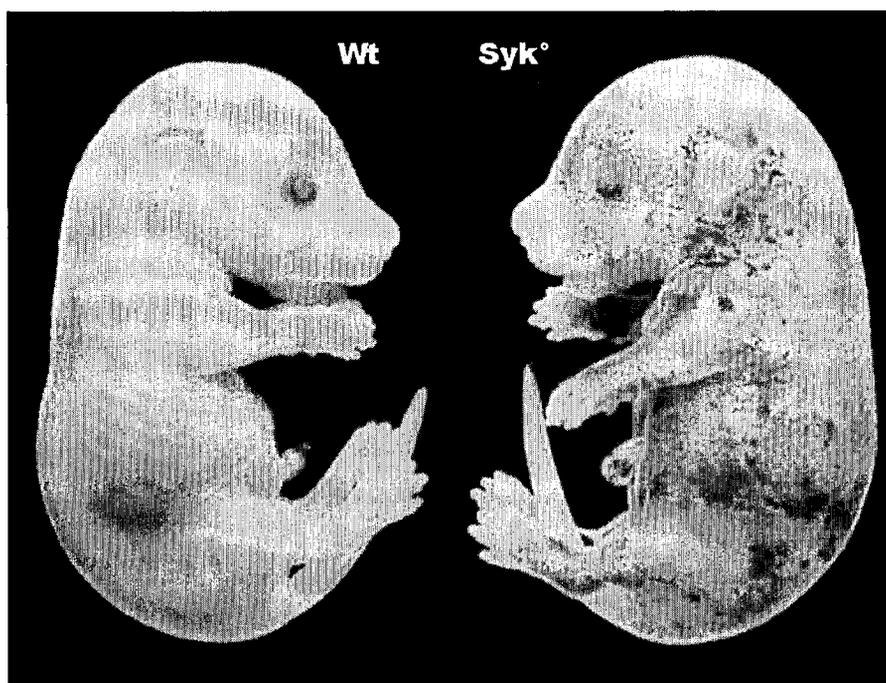


FIGURE 8. HAEMORRHAGES IN *SYK*^o EMBRYOS

16.5 day old embryos are shown of the indicated genotypes. The *Syk*^o embryo shows characteristic petechiae and haemorrhaging throughout the body, accompanied by oedema.

B cell development is severely affected in these mice: irradiated mice reconstituted with Syk-deficient fetal liver showed a partial block in B-cell development at the pro-B to pre-B cell transition, consistent with a key role for Syk in pre-B-cell receptor signalling. This block is not complete, as immature cells are generated albeit in reduced numbers. These cells, however, cannot complete their maturation and become recirculating mature IgM⁺IgD⁺ cells, suggesting a second developmental block in the absence of Syk, a complete block at the positive selection step (Figure 9) [128, 159] .

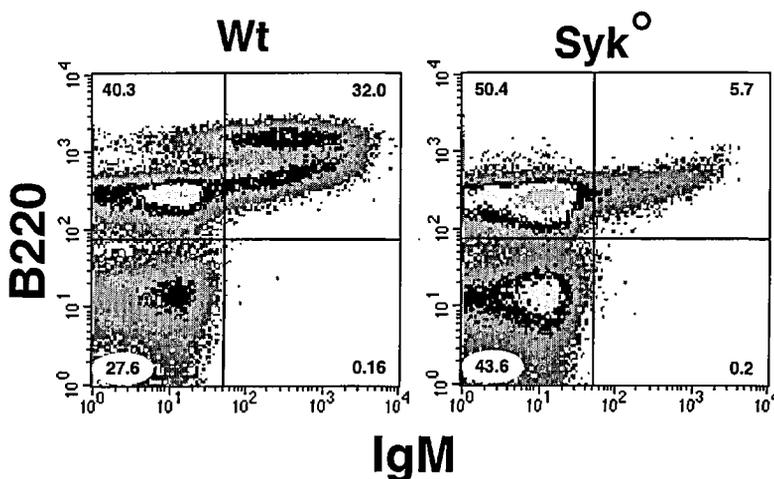


FIGURE 9. B CELL DEVELOPMENT BLOCK IN THE ABSENCE OF SYK

Donor-derived bone marrow cells from chimaeric mice are shown. As donors day 16.5 fetal liver cells from embryos of the indicated genotype were used.

A few immature syk⁰ B cells can travel from the bone marrow to the spleen, but they are found lined up at the edge of the T cell zone, and they do not enter the follicles. A possible explanation for this could be impaired chemokine-signalling, based on the similar phenotype seen in the BLR-1⁰ mice [159, 160].

Introducing a HEL (hen egg lysozyme) – specific BCR into Syk^o animals together with soluble HEL as cognate antigen at levels that in Syk⁺ animals do not affect B cell maturation markers, leads to IgM downregulation, potentially suggesting a negative role for Syk in this model of B cell negative selection [161].

3 Aims of the work in this thesis

B lymphocyte development progresses through multiple stages, that are defined by distinct gene expression patterns. One of the major checkpoints a developing B cell has to pass through is the pre-BCR signalling step, allowing differentiation of pro-B cells into pre-B cells. The signalling molecules taking part in this process and the mechanism of their interactions are the focus of this work. In particular I have been interested in the role of Syk/ZAP-70 family protein tyrosine kinases. Removal of Syk in gene targeted mutant mice has a major effect on B cell development, resulting in a severe, but incomplete block at the pre-BCR step. This partial block suggests that other pathways can also operate that can mediate pro-B → pre-B transition or that other molecules can replace Syk in this process. Using combination mutant (double knockout) and transgenic animals I attempted to define alternative routes that can lead from the pre-BCR to changes in gene expression.

4 Results

4.1 *Radiation chimaeras generated from frozen fetal livers*

Mice carrying no functional alleles of the *syk* gene (*Syk*^o mice) die perinatally [127], therefore all analysis of B cell development in mice carrying this mutation must be carried out in radiation chimaeras. We have been using fetal liver cells to reconstitute the radiosensitive haemopoietic system of lethally irradiated (1000 rads) recipient mice. In these experiments donor-derived cells must be identified using an appropriate marker, since the depletion of host cells is never entirely complete. Furthermore, if the donor fetal liver cells also carry transgene(s), we have to make sure that the transgene is only present on one of the alleles, so as not to disrupt potentially important sequences on both alleles at the unknown integration site. Finally, we wanted to inject fetal liver cells of multiple different genetic compositions on the same day into the same cohort of irradiated recipients. To generate chimaeric mice from fetal livers of the same embryonic age and to make sure of having a complete set of all required genotypes, we have to know the genetic makeup of the livers prior to reconstitution. All these requirements make the use of freshly harvested fetal livers as donors difficult, if not impossible.

As an alternative, we decided to try to generate a 'library' of frozen (-70°C) fetal livers, that would enable us to collect livers of all the desired genotypes over several weeks, which can then be used for reconstitution at the same time at a later timepoint.

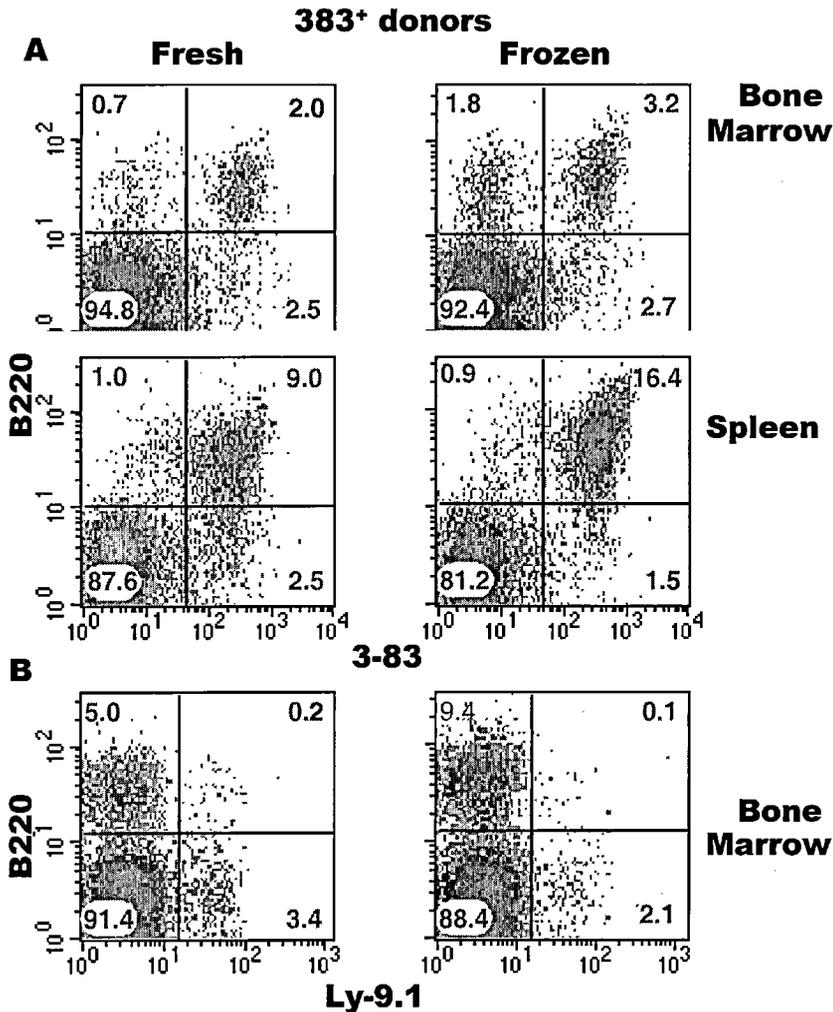


FIGURE 10. PREVIOUSLY FROZEN FETAL LIVER CELLS CAN RECONSTITUTE THE HAEMOPOIETIC SYSTEM OF LETHALLY IRRADIATED RECIPIENTS.

Freshly harvested and previously frozen (-70°C) fetal liver cells were used to reconstitute irradiated recipients. Donors: 3-83⁺H2^{d/d} Ly-9.2/9.2, recipients: BALB/c-(H2^{d/d}Ly-9.1/9.1).

A) B220⁺3-83⁺ cells are donor-derived transgenic B lymphocytes

B) B220⁺ Ly-9.1⁻ cells are donor-derived B cells, showing that the vast majority of B-lineage cells are donor-derived.

As shown in Figure 10, using fetal livers cells carrying the 3-83 B cell receptor (BCR) transgene, frozen/thawed fetal livers are capable of reconstituting the lymphoid lineages of irradiated recipients as well as fresh fetal livers. We typically find that $\geq 90\%$ of B lineage cells in both bone marrow and spleen are derived from the injected donor fetal livers.

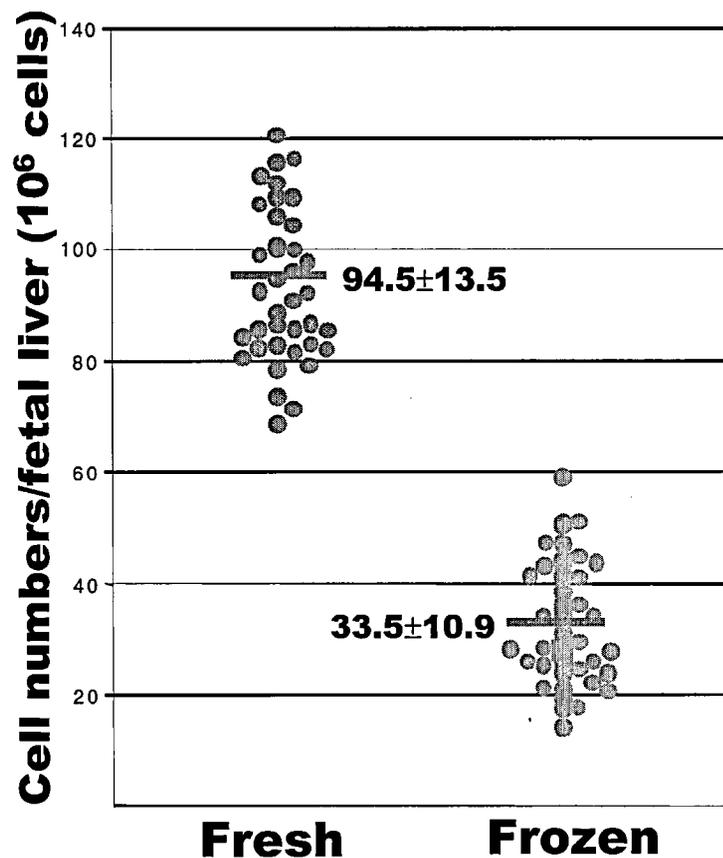


FIGURE 11. COMPARISON OF FETAL LIVER CELL NUMBERS BEFORE FREEZING AT -70°C AND AFTER THAWING.

Day 16.5 fetal livers were harvested, and cell numbers were determined on the same day ('fresh'). Alternatively, fetal liver cell suspensions were frozen at -70°C (in 90% FCS + 10% DMSO) and kept up to 4 months before thawing ('frozen'). Before freezing and after thawing cell numbers were determined using Casyton cell counter (using $4.8\mu\text{m} - 15\mu\text{m}$ diameter setting). Each symbol represents one fetal liver. Numbers shown are mean \pm SD ($n=40$) for both sets of samples.

Freezing and thawing of fetal liver cell suspensions leads to a reduction in the number of viable cells, as determined by trypan blue exclusion or by Casyton cell counter.

We can recover on average 33.5 ± 10.9 million cells from each previously frozen liver compared to 94.5 ± 13.5 million cells harvested from fresh livers (Figure 11.). We have not observed any significant differences in cell recovery from fetal livers of different genetic make-ups (Syk^o, Syk^oZAP-70^o, Syk^oLyn^o, etc.).

4.2 *Syk*[°] *Lyn*[°] double mutant mice

One of the important group of tyrosine kinases shown to be involved in early signalling from the BCR is the Src family, with Lyn being its most prominent representative in the B lineage. Lyn has been implicated both as positive and negative regulator of B cell signalling (see Introduction). Gene targeted mutant *Lyn*[°] mice show reduced numbers of recirculating mature B cells, but development does not appear to be impaired at the pre-BCR checkpoint [137].

In order to examine the relative roles of Syk and Lyn in early B cell development, *Syk*[°] *Lyn*[°] double mutant mice were generated and analysed.

Cornall et al. suggested that Lyn is not necessary for the initiation of BCR signalling, but may rather have an important negative effect [120], based on which one could have expected that the two mutations, Syk (positive regulator) and Lyn (negative regulator) neutralize each other, resulting in normal development. As discussed below, this does not appear to be the case.

4.2.1 B cell development in the absence of Syk and Lyn

The first question I wanted to address using *Syk*[°]*Lyn*[°] double knockout (DKO) mice was whether the incomplete block at the pro-B→pre-B cell transition becomes more or less severe by removing Lyn in addition to Syk.

Similarly to the Syk° animals, $Syk^{\circ}Lyn^{\circ}$ mice also die around birth, therefore all the experiments used fetal liver radiation chimeras. Figure 12 shows the analysis of bone marrow B cell development in chimaeras in the presence and absence of Syk and Lyn .

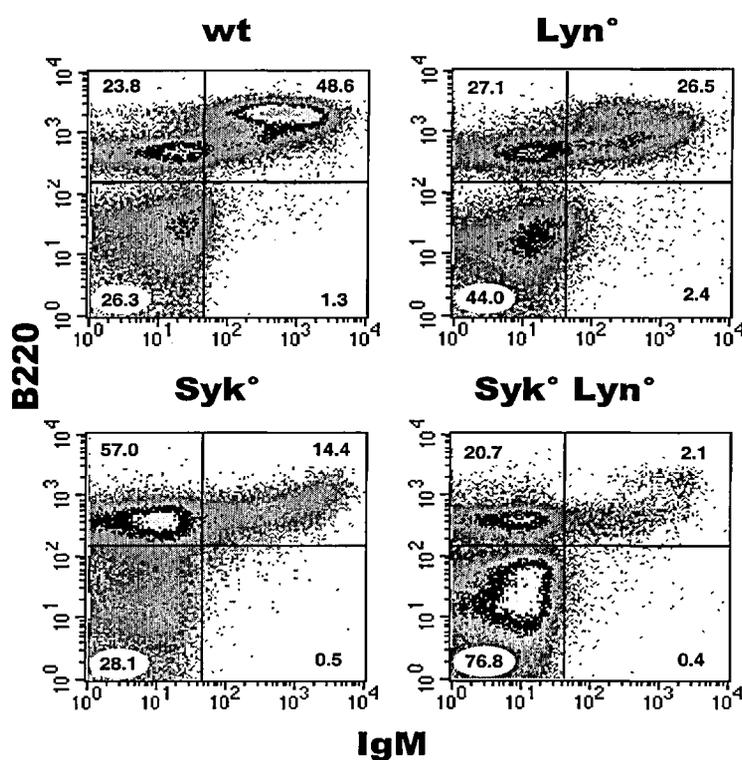


FIGURE 12. SEVERE BLOCK IN B CELL DEVELOPMENT IN THE ABSENCE OF SYK AND LYN

Chimaeric mice were generated using wild type, Lyn° , Syk° or $Syk^{\circ}Lyn^{\circ}$ fetal liver cells as labelled. Ly-9.1- (donor-derived) bone marrow cells are shown. Numbers refer to percentages of Ly-9.1- cells falling into each quadrant.

Lyn° mice show lower percentages of recirculating mature $B220^{hi}$ cells as described [137], whereas Syk° B cells do not develop beyond the $B220^{low}IgM^{+}$ stage as shown in Figure 9 (in the *Introduction*).

In the absence of both Syk and Lyn the ratio of B cells expressing IgM is further decreased, showing that without Syk and Lyn the pro-B→pre-B block is more severe, but by no means complete.

The more severe phenotype of the Syk^oLyn^o double-knockout compared to the single mutant animals suggests that Syk and Lyn play partially non-overlapping roles in B cell development, i.e. Lyn not only contributes to the activation of Syk, but also has Syk-independent functions.

4.2.2 Introduction of the 3-83 BCR transgene

Adding a pre-rearranged BCR transgene to single and double knockout animals by crossing them to mice carrying the 3-83 BCR transgene allows us to address the following questions:

- Does a BCR transgene relieve the B cell developmental block seen in the single and double mutant non-transgenic mice?
- Since this transgene was shown to effectively shut down endogenous immunoglobulin gene rearrangement [53]: is allelic exclusion functioning in mice deficient in Syk and/or Lyn?
- Is negative selection of autoreactive B cells intact in the absence of Syk and/or Lyn? The cognate ligand for the 3-83 BCR is known (MHC class I H-2K^{k,b}), and can be carried by the chimera hosts.

4.2.2.1 Effect of a rearranged transgene on B cell development

It has been shown for Syk^o 3-83⁺ chimaeric mice [159] that B cell lineage cells in the bone marrow express IgM^a (the transgenic heavy chain), but only low levels of B220 (see also Figure 13). No IgD expression is detected (not shown). Lyn^o mice bearing the same 3-83 Tg express higher levels of B220 than their Syk^o counterparts, but remain below that of the wild type mice. Few transitional cells (B220^{hi} IgMa^{hi} IgD^{lo}) are also detected (not shown). Syk^oLyn^o double knockout mice contain very few IgM^a-expressing cells in their bone marrow, with very low B220-levels (Figure 13).

Addition of the anti-apoptotic regulator Bcl-2 as a transgene [162] increases the number of B220⁺ cells to reach same levels in all four genotypes, but B220 and IgM^a expression levels do not change significantly. The finding that in the presence of the Bcl-2 transgene the significant difference in B cell numbers between Syk^o and Syk^oLyn^o mice disappears, suggests that both Syk and Lyn have survival roles in early B cell development, and Lyn performs this role - at least to some extent - independently of Syk.

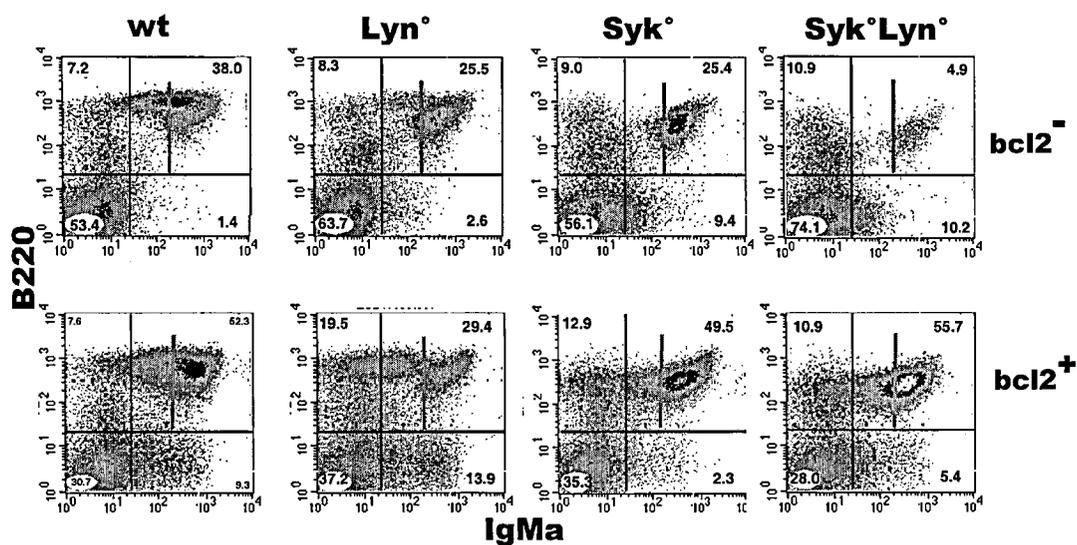


FIGURE 13. B CELL DEVELOPMENT IN THE BONE MARROW OF 3-83 IG TRANSGENIC MICE IN THE ABSENCE OF SYK AND/OR LYN.

Irradiated B10.D2 (H2^{d/d}) recipients were reconstituted with 3-83⁺ Ly-9.1/9.1 fetal liver cells of shown Syk/Lyn genotypes. 8 weeks later bone marrow cells were analysed. Cells were stained with anti-Ly-9.1-FITC, anti-IgMa-PE and B220-APC. Plots shown are gated on Ly-9.1⁺ donor-derived cells. Vertical lines divide IgMa^{hi} and IgMa^{lo} cells.

Survival, however, cannot be the only role for Syk and Lyn, given that addition of the Bcl-2 transgene does not lead to the appearance of fully mature B cells in these mutant mice.

4.2.2.2 Allelic exclusion

The 3-83 Ig transgene carries Ig κ light chain, therefore any Ig λ expression on the cell surface must be due to endogenous light chain rearrangement. Figure 14. shows mildly increased Ig λ expression in Lyn $^{\circ}$ 3-83 $^{+}$ Bcl-2 $^{+}$ mice, while in Syk $^{\circ}$ 3-83 $^{+}$ Bcl-2 $^{+}$ bone marrow 20% of donor-derived B220 $^{+}$ cells coexpress IgMa and Ig λ . (Similar results were also obtained by M. Turner - unpublished). Even higher proportion (25%) of lambda expressing cells are found in Syk $^{\circ}$ Lyn $^{\circ}$ double knockout chimaeras.

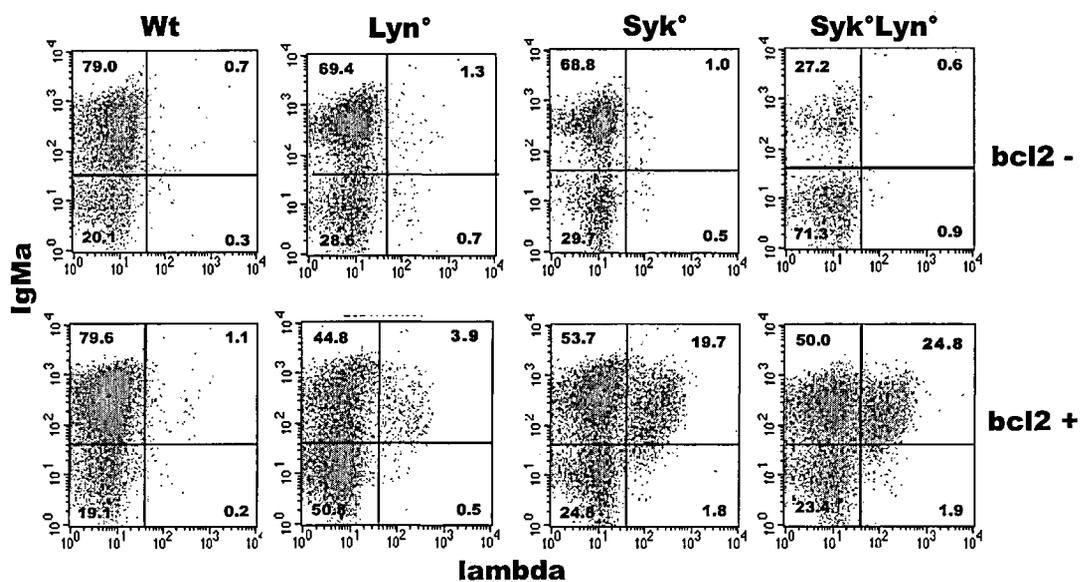


FIGURE 14. ENDOGENOUS LIGHT CHAIN REARRANGEMENT IN 3-83 TRANSGENIC MICE IN THE ABSENCE OF SYK.

Irradiated B10.D2 (H-2 $^{d/d}$ Ly-9.2/9.2) recipients were reconstituted with 3-83 $^{+}$ H-2 $^{d/d}$ Ly-9.1/9.1 fetal liver cells of shown Syk/Lyn genotypes. 8 weeks later bone marrow cells were analysed. Cells were stained with anti-Ly-9.1-FITC, anti-IgMa-PE, biotin-anti-Ig λ /Streptavidin-Red613 and anti-B220-APC. Plots shown are gated on B220 $^{+}$ Ly-9.1 $^{+}$ donor-derived B cells.

Likewise, 8% and 12% of donor-derived B cells also express endogenous heavy chain in Syk^o 3-83⁺ Bcl-2⁺ and Syk^oLyn^o 3-83⁺ Bcl-2⁺ mice, respectively (Figure 15).

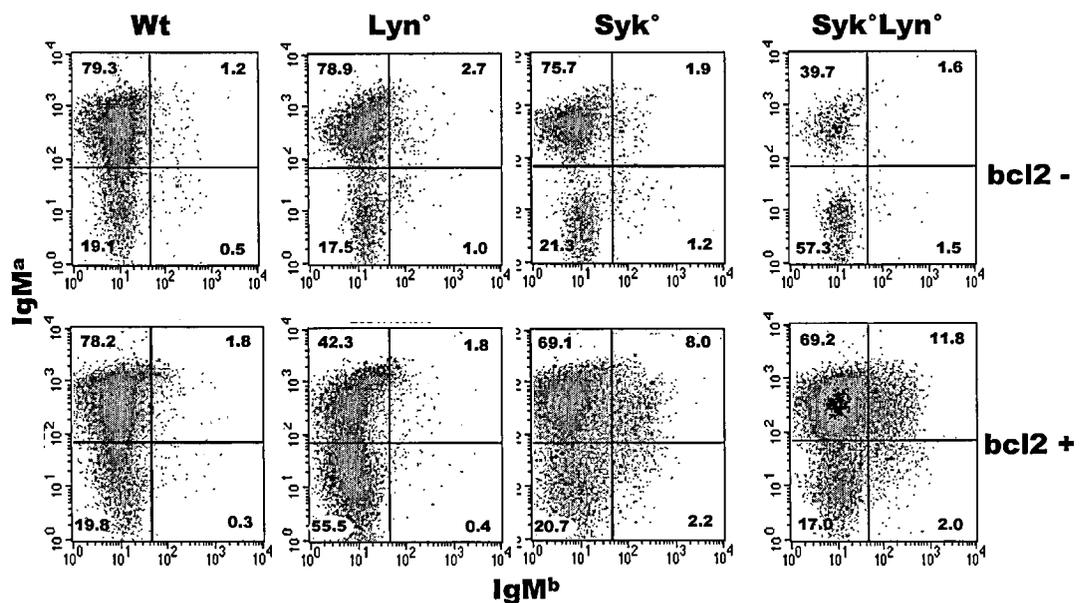


FIGURE 15. ENDOGENOUS HEAVY CHAIN REARRANGEMENT IN 3-83 TRANSGENIC MICE IN THE ABSENCE OF SYK.

Irradiated B10.D2 (H-2^{d/d} Ly-9.2/9.2) recipients were reconstituted with 3-83⁺ H-2^{d/d} Ly-9.1/9.1 fetal liver cells of shown Syk/Lyn genotypes. 8 weeks later bone marrow cells were analysed. Cells were stained with anti-IgM^b-FITC, anti-IgM^a-PE, biotin-anti-Ly-9.1/Streptavidin-Red613 and anti-B220-APC. Plots shown are gated on B220⁺ Ly-9.1⁺ donor-derived B cells.

Endogenous rearrangement resulting in cell surface expression of Igλ and IgM^b in these 3-83 Ig transgenic mice could be explained by lack of allelic exclusion, suggesting that signals through both the pre-BCR and BCR to stop further immunoglobulin gene rearrangements are dependent on Syk and also on Lyn. That the Syk^oLyn^o double knockout mice have higher percentages of B cells expressing

the endogenous chains, would suggest that Lyn can act partly independently of Syk in this process. Bcl-2 transgene is needed to detect IgM^b and Igλ expression probably to allow these cells to survive long enough to complete the rearrangement and/or transcription/translation process.

4.2.2.3 Negative selection

I also wanted to analyse the role of Syk and Lyn in B cell development in the presence of the cognate ligand for the Ig transgene. The class I-reactive 3-83 transgene has high affinity for H2-K^k, lower for H2-K^b and virtually none for H2-K^d. To investigate potentially small, or stepwise changes brought about by changing levels of signalling molecules, H2^{bd} background was used for which the transgene has rather low reactivity.

Analysis of IgM^a (transgenic heavy chain) expression is shown on Figure 16.

As expected, B220^{hi}IgMa^{hi} and B220^{hi}IgMa^{lo}IgD⁺ (not shown) cells are missing in the wild type and Lyn^o chimaeras, due to receptor downmodulation. In all four genotypes we find similar levels of IgMa (IgMa^{lo}) with intermediate expression of B220 in Wt and Lyn^o and low levels of B220 in Syk^o and Syk^oLyn^o mice. These results suggest, that for deletion Syk and Lyn are dispensable.

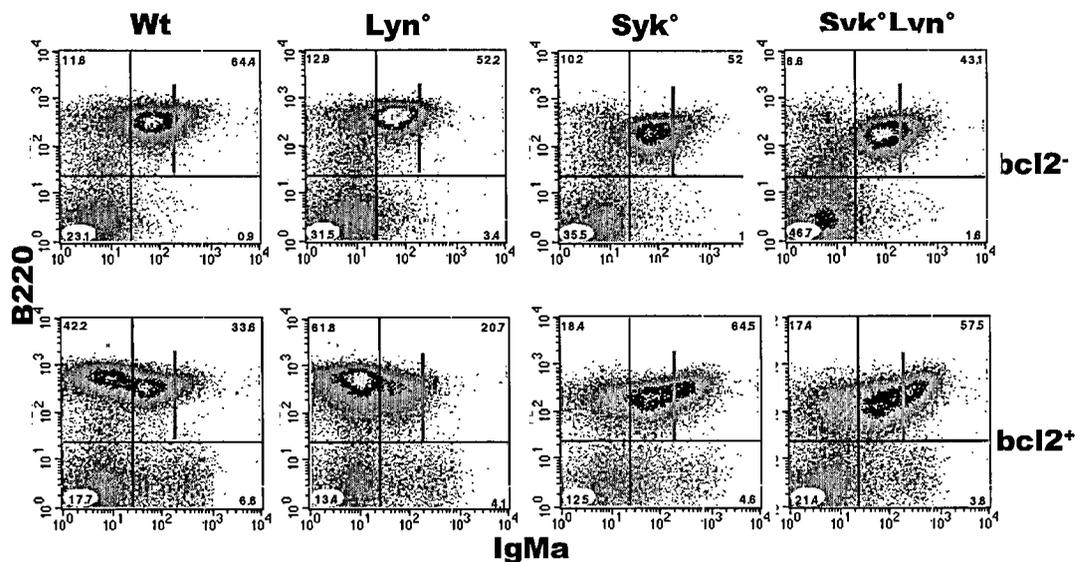


FIGURE 16. DEVELOPMENT OF 3-83 IG TRANSGENIC B CELLS IN THE PRESENCE OF COGNATE LIGAND.

Irradiated (C57BL/10 x B10.D2)F1 [H2^{b/d}, Ly-9.2/9.2] recipients were reconstituted with 3-83⁺ H-2^{d/d} Ly-9.1/9.1 fetal liver cells of Syk/Lyn genotypes shown. 8 weeks later bone marrow cells were analysed. Cells were stained with anti-Ly-9.1-FITC, anti-IgM^a-PE and B220-APC. Plots shown are gated on Ly-9.1⁺ donor-derived cells. Vertical lines separate IgMa^{hi} and IgMa^{lo} cells. See also Figure 13.

As mentioned before, in the anti-HEL/sHEL system Syk^o B cells appeared to be hypersensitive to the cognate ligand, suggesting that Syk can also act as negative regulator [161]. Increased responsiveness in the absence of Syk would have resulted in lower levels of IgM^a in the Syk^oLyn^o double knockout bone marrow than in the others. On the contrary, if there is any difference in IgM^a levels, than it is Lyn^o<Wt = Syk^o < Syk^oLyn^o. This appears to be consistent with Lyn being a negative and Syk a positive regulator of BCR signalling in immature B cells (Figure 17).

(The IgMa^{hi} cells detected in syk^o and syk^olyn^o chimaeras carrying the Bcl-2 transgene are also expressing Igλ, that renders them unresponsive to H2^b.)

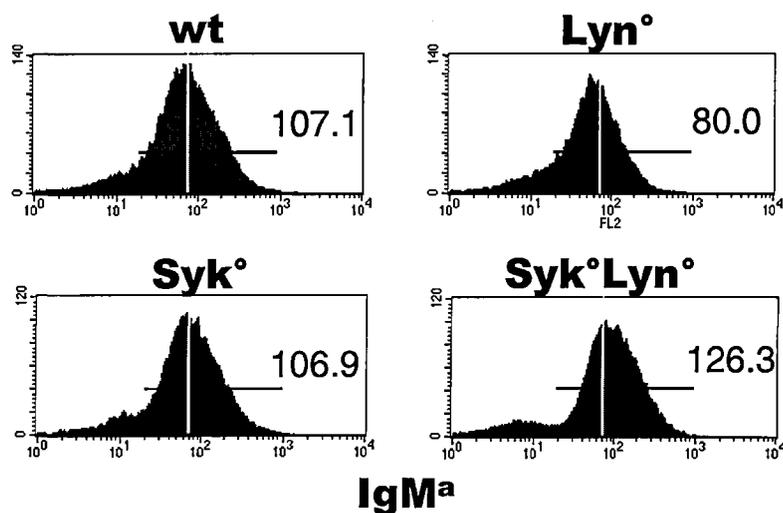


FIGURE 17. IgM^A LEVELS ON $B220^+$ BONE MARROW CELLS.

Irradiated (C57BL/10 x B10.D2)F1 ($H2^{b/d}$, Ly-9.2/9.2) recipients were reconstituted with $3-83^+$ $H-2^{d/d}$ Ly-9.1/9.1 fetal liver cells of Syk/Lyn genotypes shown. 8 weeks later bone marrow cells were analysed. Cells were stained with anti-Ly-9.1-FITC, anti-IgM^a-PE and B220-APC. Plots shown are gated on (Ly-9.1⁺ B220⁺) cells. Mean fluorescence intensity of positive cells (black line) is shown. White bars added for easier comparison.

Interestingly, if we compare Figure 13 with Figure 16, we see significant expansion of $IgMa^{lo}$ cells in all four *syk/lyn* genotypes examined in $H2^{b/d}$ chimaeras. In addition, the presence of Bcl-2 transgene further expands the $IgMa^+$ cells in Syk° and in Syk°Lyn° chimaeras, mostly due to the expansion of $Ig\lambda$ expressing cells. In the wild type (Syk^{+/+}Lyn^{+/+}) and Lyn° chimaeras, however, forced Bcl-2 expression leads to increased numbers of $IgMa^-$ (also $IgMb^-$, not shown) cells at the expense of the $IgMa$ expressing compartment.

One could speculate that the receptor levels on these $IgMa^{lo}$ cells are too low to signal differentiation/survival/expansion in $H2^{d/d}$ mice, therefore they die of neglect.

Increasing signal strength by providing a somewhat higher affinity ligand ($H2-K^b$) leads to their proliferation, but this signal is not appropriate (qualitatively or

quantitatively) to induce differentiation. Cells with higher IgM^a levels in the same environment would receive a strong enough signal for differentiation, resulting in upregulation of BCR on their surface, that strengthens the signal enough to cause negative selection.

Since the expansion of IgM^{lo} cells is seen in all four genotypes examined, this proliferative signal presumably does not require Lyn and Syk.

Preliminary experiments have shown that providing H2-K^k ligand for 3-83 transgenic cells (using H2^{kd} hosts in fetal liver radiation chimaera experiments) results in complete deletion of transgene bearing cells regardless whether Syk and/or Lyn is present (data not shown). This suggests that strong signals can induce negative selection mediated by Syk and/or Lyn independent pathways.

4.3 *Syk and ZAP-70 in B cell development*

Gene targeted mutant mice have frequently revealed redundancies among signal transduction molecules known to perform similar functions [163]. Syk protein tyrosine kinase also has a 'sibling', ZAP-70, and these two family members have been observed to compensate for each other's absence [164-166]. ZAP-70, however, has only been shown to be expressed in T cells and NK cells, and has never been detected in B lineage cells. In light of the phenotype of Syk° animals - B cell development is severely, but incompletely blocked at the pro-B \rightarrow pre-B checkpoint (Figure 9 in *Introduction* and [127, 128]) - I intended to revisit this issue. In order to conduct the genetic experiment Syk° and $ZAP-70^{\circ}$ [167] gene targeted mutant mice were inter-crossed, to generate 'double knockout' (DKO) animals lacking both members of the Syk/ZAP-70 family.

As expected - and similarly to the single mutant animals only missing Syk -, the double mutation also leads to perinatal lethality. While T cell development in DKO mice can be and has been studied in newborn mice [165], for the analysis of B cell development, fetal liver radiation chimaeras were needed.

Day 16.5 fetal liver cells from wild type ($Syk^{+}ZAP-70^{+/+}$), Syk° ($Syk^{-/-} ZAP-70^{+/+}$), $ZAP-70^{\circ}$ ($Syk^{+} ZAP-70^{-/-}$) and DKO ($Syk^{-/-} ZAP-70^{-/-}$) embryos were injected into lethally irradiated recipients. The resulting chimaeric mice were analysed 8-12 weeks after transfer. To also make use of the 3-83 transgene (see below) and to differentiate between donor- and host-derived B lineage cells, all the donor mice were on $H2^{d/d}$,

Ly-9.2/9.2, IgH^{b/b} background (backcrossed 6 generations unto B10.D2), whereas the recipients were H2^{d/d} or H2^{b/d}, Ly-9.1/9.2, IgH^{a/b} ((BALB/c x B10.D2)F1 or (BALB/c x C57BL/10)F1) mice.

These chimaeras confirmed the T-cell development phenotype seen in intact newborn animals [165] : in the absence of Syk and ZAP-70 thymocyte development is arrested at the pre-TCR checkpoint, resulting in a block at the CD4⁺CD8⁻, DN3 stage (Figure 18), similarly to RAG-1^o mice. This phenotype demonstrates that Syk and ZAP-70 perform essential but overlapping functions at the pre-TCR checkpoint.

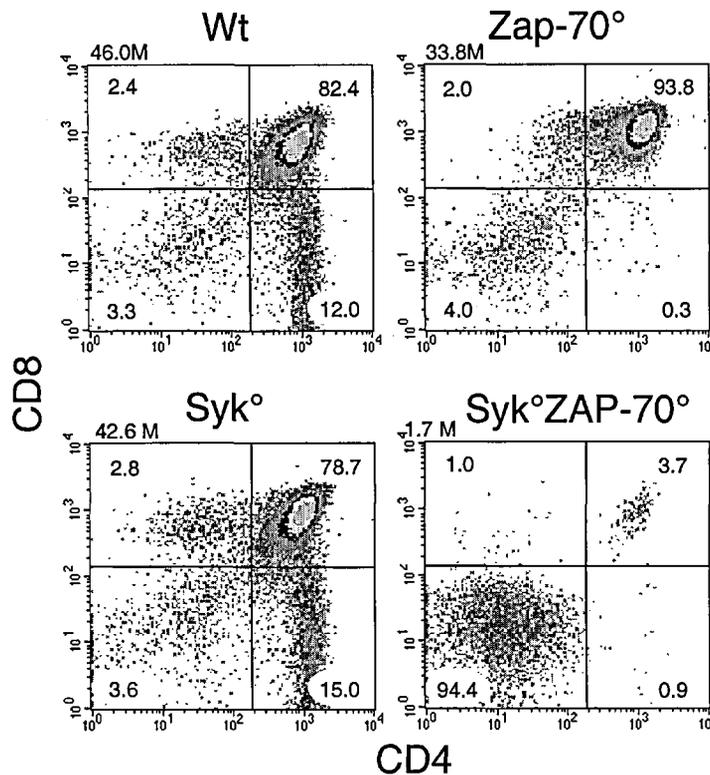


FIGURE 18. T CELL DEVELOPMENT IN THE ABSENCE OF SYK AND ZAP-70 IS BLOCKED AT CD4⁺CD8⁻ STAGE

Thymi from chimeric mice generated from fetal livers of the shown genotypes are compared. Plots show Ly-9.1⁺ thymocytes labelled with anti-CD8 and anti-CD4 antibodies. Numbers within plots refer to percentage of Ly-9.1⁺ thymocytes falling into each quadrant. Numbers above plots show total thymocyte numbers in millions of cells.

4.3.1 B cell development in the absence of Syk and ZAP-70

As reported before [159] and also shown in Figure 9, in the absence of Syk kinase B cell maturation appears to be blocked at two developmental checkpoints: (1) incompletely at the pre-BCR checkpoint, resulting in generation of lower numbers of pre-B cells, and (2) completely at the immature \rightarrow mature transition, resulting in the absence of mature B lymphocytes in these animals.

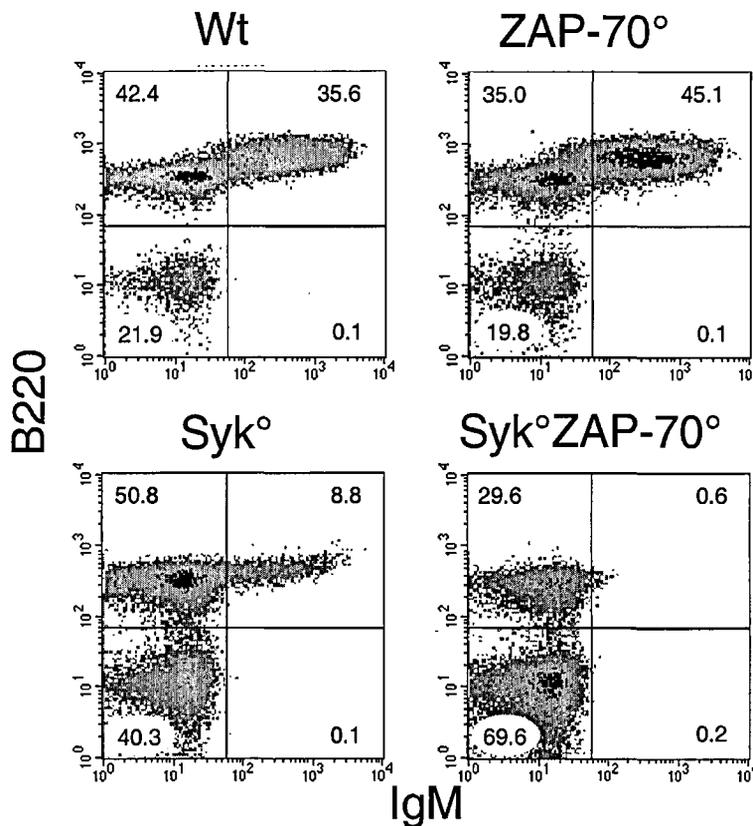


FIGURE 19. NO IGM⁺ B CELLS DEVELOP IN THE ABSENCE OF SYK AND ZAP-70

Fetal liver radiation chimaeras were generated using day 16.5 fetal livers of the four shown genotypes. 8-12 weeks after cell transplantation bone marrow cells were harvested. Ly-9.1⁺ donor-derived cells are shown that were stained with anti-B220 and anti-IgM. Numbers refer to percentage of Ly-9.1⁺ bone marrow cells falling into each quadrant.

Similar phenotypic analyses were performed on the DKO (Syk^oZAP-70^o) chimaeric mice to determine whether removal of the other Syk/ZAP-70 family member changes the developmental pattern of B lymphocytes.

Figure 19 shows that while ZAP-70^o chimaeras show normal B cell development, removal of ZAP-70 in addition to Syk has a surprisingly strong effect on B cell development: in the DKO chimaeric animals no IgM expressing donor-derived bone marrow cells develop, resulting in a complete lack of immature and mature B cells.

Further analysis of surface marker expression shows that in DKO mice B cell maturation is completely blocked before the pre-B cell stage. Figure 20 shows expression of CD2 on B lineage donor-derived cells, using CD19 as a marker this time to limit our analysis to B lineage cells, especially since CD2 is not restricted to B lymphocytes. CD2 is not detectable on pro-B cells, but is expressed on pre-B, immature and mature B cells, and as such is a valuable marker of cells that successfully passed the pre-BCR checkpoint. The lack of expression of CD2 on DKO CD19⁺ bone marrow cells suggest that without Syk and ZAP-70 B cells cannot transmit signals from the pre-BCR for developmental progression.

Further phenotypic analysis in Figure 21 shows that DKO B lineage bone marrow cells are arrested at the CD19⁺CD43⁺ large pro-B cell stage. In comparison, a quarter of single knockout Syk^o B cells are CD43⁻ and smaller in size, confirming again that ZAP-70 is partially compensating for the lost Syk function at the pro-B → pre-B transition.

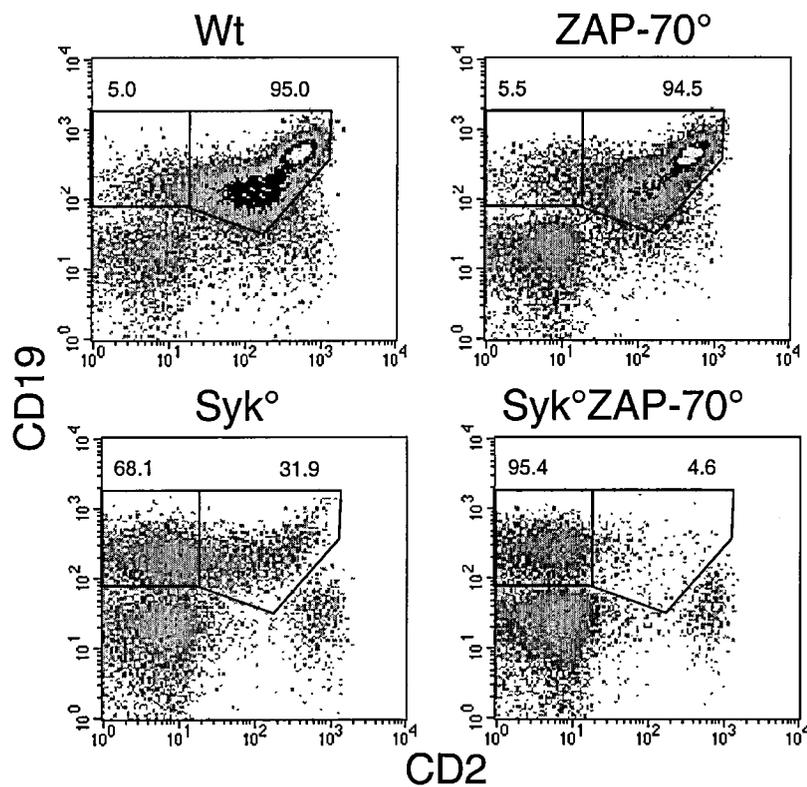


FIGURE 20. B CELL DEVELOPMENT IS BLOCKED BEFORE THE PRE-B CELL STAGE IN SYK° ZAP-70° MICE

As in Figure 19, plots show donor-derived Ly-9.1⁻ bone marrow cells. Numbers refer to percentage of donor-derived CD19⁺ bone marrow cells that do or do not express CD2, respectively.

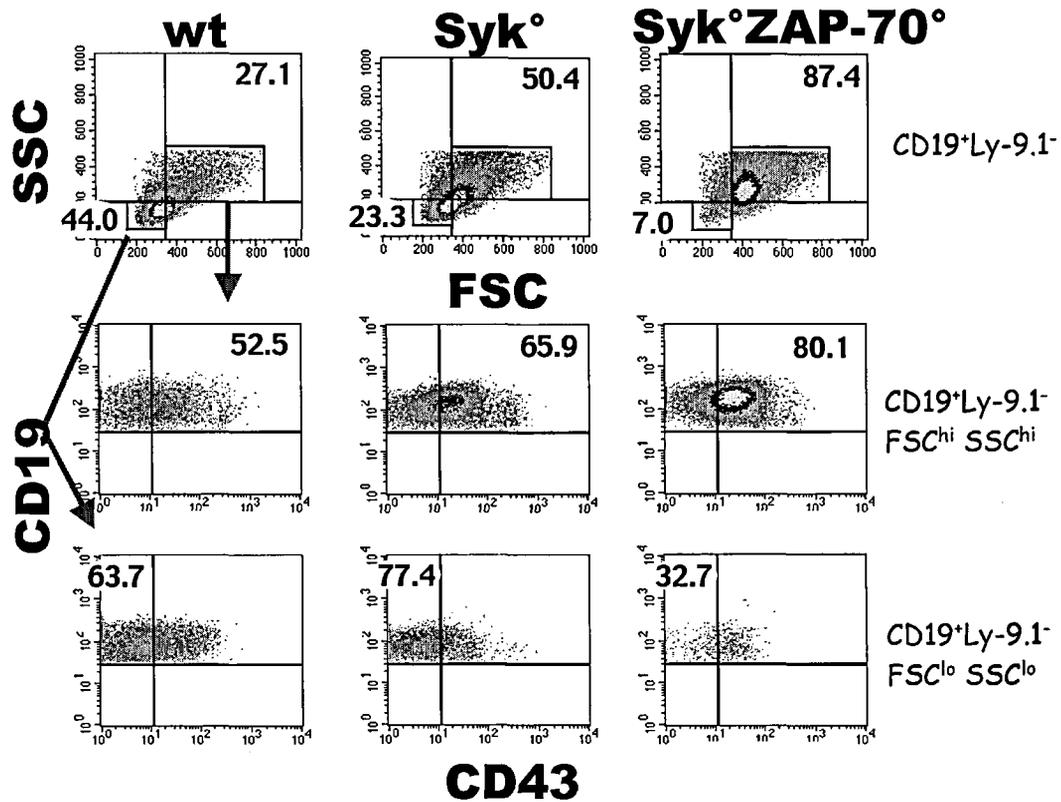


FIGURE 21. B CELLS DO NOT DEVELOP INTO SMALL PRE-B CELLS IN THE ABSENCE OF SYK AND ZAP-70

(top panel) FSC/SSC analysis of all donor-derived B lineage bone marrow cells. Numbers shown refer to percent of cells displaying FSC^{high}SSC^{high} and FSC^{low}SSC^{low} phenotypes.

(middle panel) FSC^{high}SSC^{high} cells gated as shown on the top panel, then CD19/CD43 expression displayed. Numbers shown refer to percentage of cells in the CD19⁺CD43^{+/int} quadrant;

(bottom panel) FSC^{low}SSC^{low} cells gated as shown on the top panel, then CD19/CD43 expression displayed. Numbers shown refer to percentage of cells in the CD19⁺CD43^{-/low} quadrant. No difference has been seen between wild type and ZAP-70[°] animals.

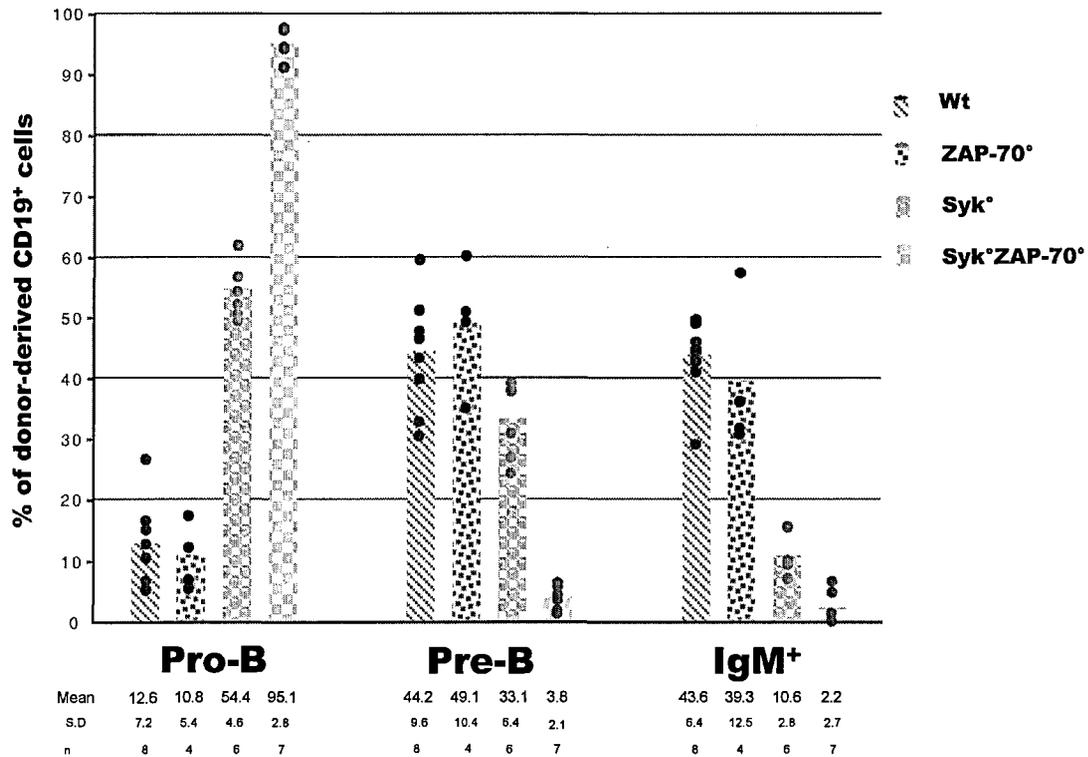


FIGURE 22. SUMMARY OF B CELL DEVELOPMENT IN THE PRESENCE AND ABSENCE OF SYK AND/OR ZAP-70

Donor-derived (Ly-9.1⁺) CD19⁺ B cells are subdivided into pro-B cells (CD2⁺IgM⁻), pre-B cells (CD2⁺ IgM⁻) and IgM⁺ cells. Values refer to percentage of CD19⁺ donor-derived cells falling into each subpopulation. Filled circles show individual chimeric mice, while columns show average values for each group. Below each column mean percentages are shown, with standard deviations and the number of mice analysed.

The above results suggest that in the absence of Syk and ZAP-70 B lymphocytes cannot progress beyond the CD19⁺CD43⁺CD2⁺IgM⁻ pro-B cell stage. The question arises whether these cells are capable of producing a pre-BCR, but incapable of transducing the signal emanating from this receptor, or whether these cells are impaired at an earlier stage and cannot even produce a pre-BCR. To distinguish between these two possibilities intracellular heavy chain expression was analysed.

As shown in Figure 23, pro-B cells in the absence of Syk and ZAP-70 are capable of expressing heavy chain in their cytoplasm. The proportion of DKO cells with μ chain in their cytoplasm, however, stays well below that of Syk^o single mutant mice. A likely explanation for this difference is that, as seen above, a portion of Syk^o B lymphocytes do reach the pre-B/immature stage, providing these mice with an opportunity to expand their pre-B cells with a productive/functional heavy chain. In contrast, if the DKO mice are incapable of transmitting a pre-BCR signal, as the phenotypic analysis leads us to think, there would be no selective proliferation of cells with productive heavy chain rearrangement.

Detection of μ heavy chain protein in the cytoplasm of DKO pro-B cells also suggests that heavy chain rearrangement, including locus accessibility and rearrangement initiation is unimpaired in the absence of Syk and ZAP-70 (see analysis of rearrangement later in this chapter). This phenotype is different from the one seen in Ig β ^o mice, where DJ_H, but not VDJ_H rearrangement is seen [10]. This difference would imply that while we propose that Syk/ZAP-70 is crucial in transducing signals from the pre-BCR that supposedly would also involve Ig β , earlier in development Ig β may function without Syk or ZAP-70.

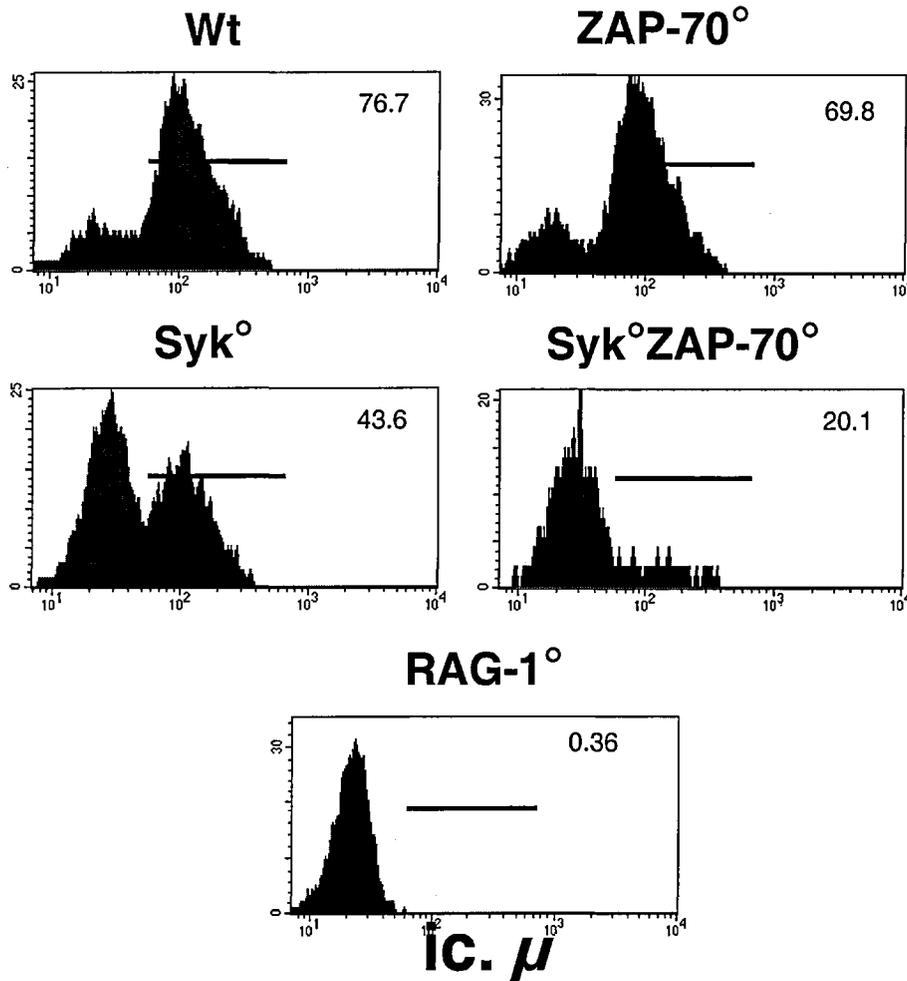


FIGURE 23. SYK^oZAP-70^o PRO-B CELLS CAN EXPRESS CYTOPLASMIC μ HEAVY CHAIN

Plots show Ly-9.1⁺ B220^{low} IgM⁻ donor-derived pro- and pre-B cells from chimaeric mice. Numbers refer to percentage of cells showing intracellular heavy chain expression. As a negative control, RAG-1^o pro-B cells are shown.

In addition to the μ heavy chain, $\lambda 5$ and Vpre-B must also be present for successful pre-BCR assembly. Figure 24 shows that both of the surrogate light chain components are detected on the surface of B lineage cells in the absence of Syk

and/or ZAP-70. In fact, most of the CD19⁺ cells in Syk^o and Syk^oZAP-70^o mice express λ 5 and Vpre-B after 1 hour incubation at 37°C, further emphasizing the lack of progression beyond the pre-BCR stage.

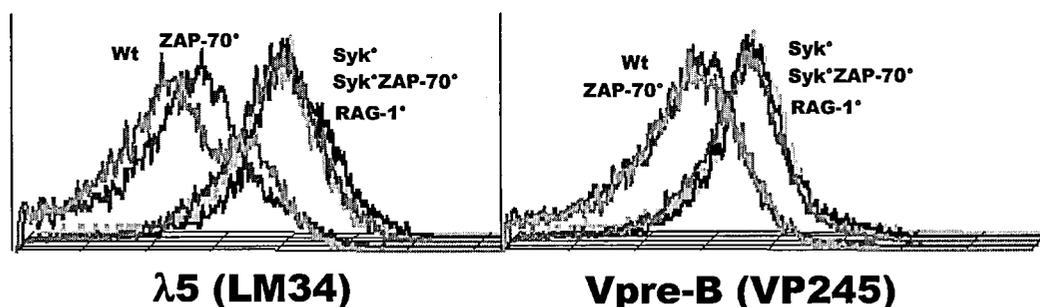


FIGURE 24. SURROGATE LIGHT CHAIN EXPRESSION IN THE ABSENCE OF SYK AND ZAP-70

Bone marrow cells from chimeric mice generated with Wt, ZAP-70^o, Syk^o and Syk^oZAP-70^o fetal livers were incubated at 37°C for 1 hour before staining. Donor-derived CD19⁺ cells are shown. As control, CD19⁺ bone marrow cells from intact RAG-1^o animals are also shown.

4.3.2 Cell-autonomous effect of Syk and ZAP-70 deficiency

Results presented above show that ZAP-70 and Syk are required within haemopoietic cells for the pro-B → pre-B transitions. One possible explanation could be that ZAP-70 can affect B cell development in trans, i.e. ZAP-70 is not required in B lineage cells themselves, but rather in another cell type that can influence the maturation of B cells, for example by secreting a cytokine, or expressing a surface protein that can

interact with the B cell precursors. To confirm or exclude this possibility, mixed radiation chimaeras were generated.

4.3.2.1 Generation of mixed radiation chimaeras

As Figure 25 schematically depicts, by mixing Syk° and $\text{Syk}^\circ\text{ZAP-70}^\circ$ donor fetal liver cells, development of B cells from Syk° precursor with or without ZAP-70, respectively, can be studied in the same animals. If ZAP-70 has a cell-autonomous role, i.e. is needed within the developing B lineage cells themselves, the observed differences in B cell maturation between Syk° and DKO animals should also be seen in the mixed chimaeras. If, however, ZAP-70 acts in trans, all B cells should develop as they do in Syk° mice, since there is ZAP-70 expression in other (non-B) haemopoietic cells contributed by the Syk° fetal liver.

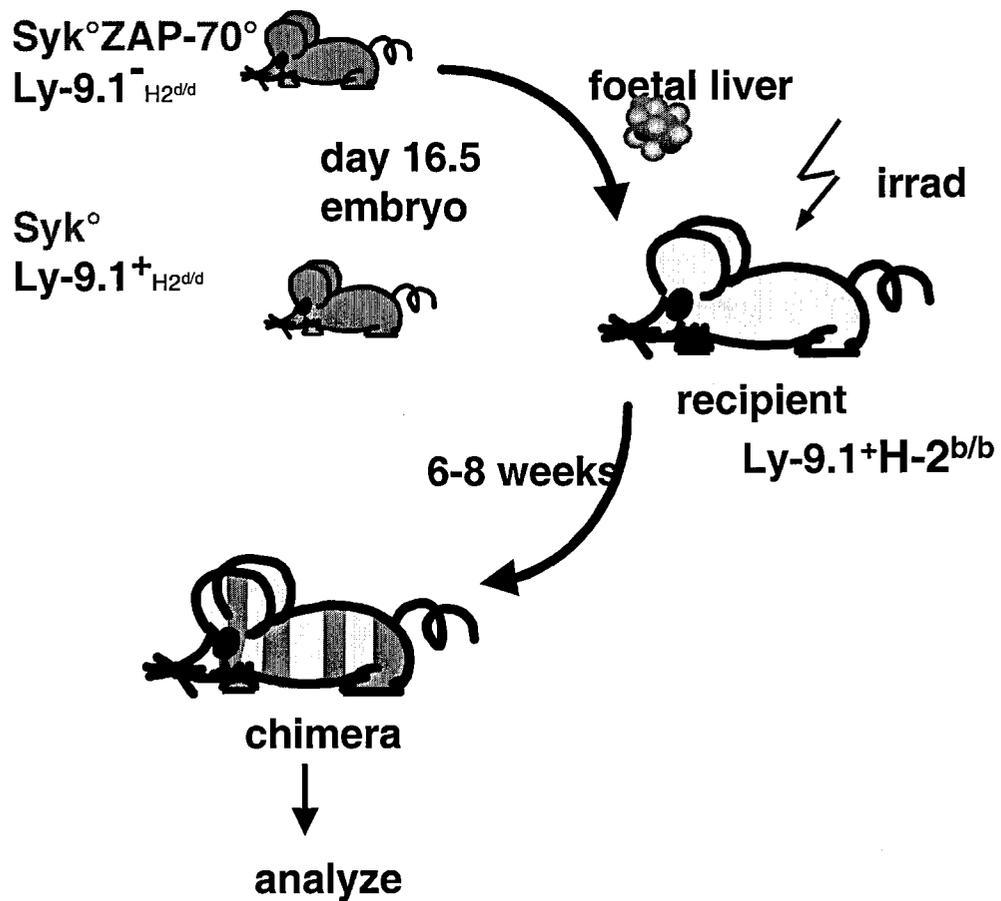


FIGURE 25. GENERATION OF MIXED FETAL LIVER CHIMAERAS

Fetal livers were harvested as before. The two different donors (Syk[°] and Syk[°]ZAP-70[°]) and the host can be distinguished by a combination of Ly-9.1 and H2 expression. In the host only a few mature B lineage cells survive long-term following the irradiation. These express high levels of H2^b and high levels of Ly-9.1, enabling us to exclude them from the analysis of B lineage cells of donor origin(s).

4.3.2.2 Results from mixed radiation chimaeras

As seen in Figure 19 and Figure 20 the incomplete vs. complete block in B cell maturation in Syk° mice in the presence or absence of ZAP-70, respectively, can be reliably characterised by IgM and CD2 expression on B lineage bone marrow cells: both IgM and CD2 can be found on a portion of Syk° B cells, whereas neither of these two markers are expressed on DKO cells.

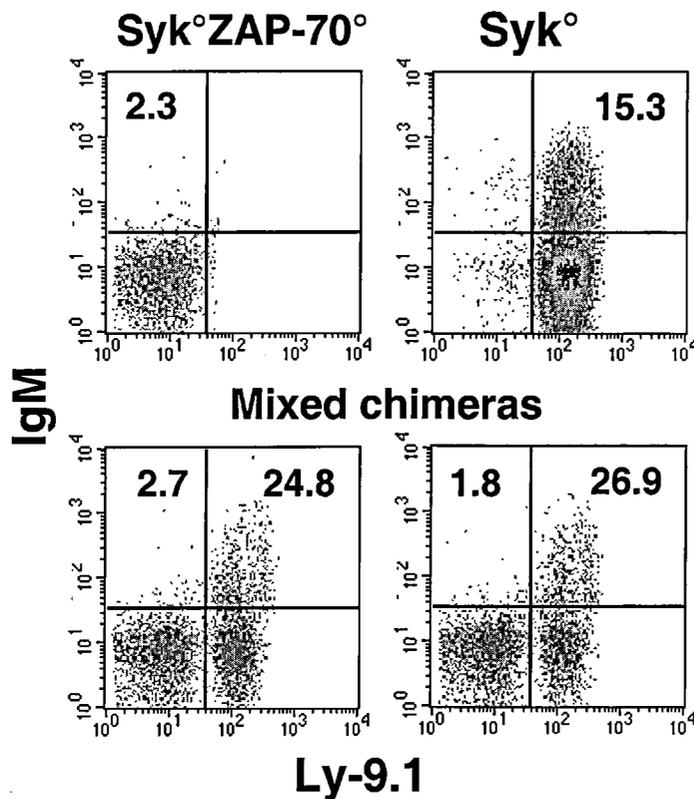


FIGURE 26. THE ABSENCE OF ZAP-70 IN SYK° MICE AFFECTS B CELL DEVELOPMENT CELL-AUTONOMOUSLY – IGM EXPRESSION

(top) chimaeras generated with unmixed fetal liver cells as donors

(bottom) two examples of mixed chimaeric mice

Plots show donor-derived CD19⁺ cells - host cells (Kb⁺, Ly-9.1^{high}) are excluded from the analysis.

Numbers shown represent percentage of CD19⁺ cells of (each) donor origin expressing IgM.

Figure 26 and Figure 27 show analyses of IgM and CD2 expression on the B lineage cells in the bone marrow of mixed radiation fetal liver chimaeras, respectively. The chimaeras shown were generated by mixing equal numbers of fetal liver cells from the two donor genotypes. Similar results were seen using 90%-10%, 75%-25%, 25%-75% and 10%-90% mixtures, as well.

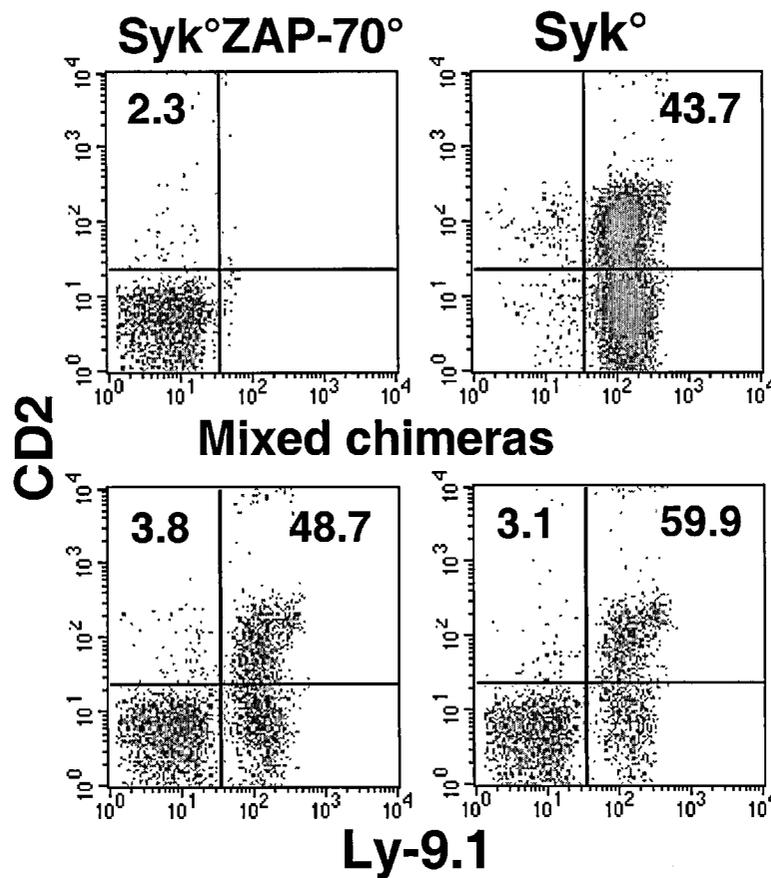


FIGURE 27. THE ABSENCE OF ZAP-70 IN SYK⁰ MICE AFFECTS B CELL DEVELOPMENT CELL-AUTONOMOUSLY – CD2 EXPRESSION

See Figure 26.

Numbers shown represent percentage of CD19⁺ cells of (each) donor origin expressing CD2.

Both Figure 26 and Figure 27 reveal the same outcome for the experiment: the differences in IgM and CD2 expression seen in the non-mixed chimaeras (top panels) can still be observed after mixing the two donor fetal liver population (bottom panels). These results confirm that ZAP-70 must be expressed within the B lineage cell themselves in order to allow progression beyond the pre-BCR checkpoint in the absence of Syk, since ZAP-70 expressing non-B lineage haemopoietic cells provided by the Syk^o (ZAP-70^{+/+}) fetal liver cannot rescue the complete developmental block in DKO B cells.

Mixed chimaeras were also generated using wild-type/Syk^o and wild-type/Syk^oZAP-70^o fetal liver cell mixtures. Not surprisingly, however, B lineage cells developing from the wild type donor outcompeted the mutant precursors resulting in no detectable B lineage cells of mutant donor origin. I was, therefore, unable to analyse the developmental progression of the mutant cells in these chimaeras. Whether this is due to defective expansion of the mutant B cells after the pre-BCR checkpoint would need further investigation.

4.3.3 ZAP-70 protein expression in B lineage cells

Results from the mixed fetal liver radiation chimaera experiments (Figure 26 and Figure 27) strongly suggest that ZAP-70 must be expressed by the B lineage cells themselves. Formally the mixed chimaera experiment shows that ZAP-70 is required cell autonomously within the lineage leading to pro-B cells, but it might be required

not in the pro-B cell itself, but at an earlier stage, e.g. in the hemopoietic stem cell.

To differentiate between these possibilities, ZAP-70 protein expression was analysed by Western blotting experiments.

I wanted to examine protein expression in B lineage cells of different maturational stages. Special emphasis was put on pro-B cells for the following reasons:

(1) Due to the complete block at the pro-B→pre-B transitional step, analyses of DKO chimaeric mice (above) have only addressed the overlapping function of Syk and ZAP-70 in pro-B cells.

(2) Syk expression has been reported to be downregulated upon pre-TCR signalling in developing thymocytes [168]. If a reciprocal situation were to be true in B lineage cells, one might expect downregulation of ZAP-70 in pre-B cells relative to pro-B cells.

(3) Syk^o mutant mice show complete block at the immature→mature B cell transition [127, 128], suggesting that ZAP-70, even if expressed in immature B cells, cannot efficiently substitute for the missing activity of Syk.

(4) No impairment of mature B cell function has been described in ZAP-70^o animals. Based on this, if ZAP-70 is expressed in B lineage cells, it might be most abundant in pro-B cells.

4.3.3.1 Sorted population of B lineage cells for analysis

The most informative protein expression analysis would have been done using flow cytometry and detecting intracellular ZAP-70 expression at a single cell level. My

attempts using four different anti-ZAP-70 antibodies, and different intracellular staining methods, however, failed to detect any ZAP-70 expression even in thymocytes or mature T cells. I resorted, therefore, to Western blot analysis of protein expression.

Since Western blotting detects protein expression in bulk cell populations without the possibility of extrapolating expression levels in single cells, cell samples used had to be homogenous, and most importantly, devoid of non-B lineage cells. FACS sorting was therefore performed to obtain pure pro-B, pre-B and peripheral B cells.

As a source of pro-B cells RAG-1^o bone marrow cells were used, where B cell differentiation is blocked at the pre-BCR step owing to the absence of Ig heavy (and light) chain rearrangement [7]. Since CD2 expression starts only in pre-B cells, cells were sorted using anti-CD19 and anti-CD2 antibodies. Pro-B cells collected were 96% CD19⁺CD2⁻.

Pre-B cells were sorted from the bone marrow of RAG-1^o mice carrying the HC186, heavy chain only transgene that drives differentiation of B lineage cells through the pre-BCR signalling stage, and allows the accumulation of pre-B cells. These cells cannot, however progress any further in the absence of light chain rearrangement [169]. Pre-BCR can be expressed in these mice, but the levels are too low to be detected by our anti- μ antibody. Cells were sorted using anti-CD19 and anti-CD2 antibodies. Pre-B cells collected were 98% CD19⁺CD2⁺.

Peripheral B cells were sorted from spleens of wild type mice using only CD19 as a marker of B cells, therefore these samples contain all subpopulations of B lymphocytes found in murine spleen, including immature, transitional, recirculating mature follicular and marginal zone B cells. Cells collected were 99% CD19⁺.

T lineage cells are known to express ZAP-70, therefore total thymocytes from wild type and ZAP-70^o mice served as a positive and negative control, respectively.

4.3.3.2 Western blot analysis

As predicted by the mixed chimaera experiments and shown in Figure 28, ZAP-70 expression can indeed be detected in B lineage cells. Analysing graded numbers of cells or graded amounts of protein suggests that the difference between the two lineages is less than expected. Pro-B cells express 3-5-fold less ZAP-70 than total thymocytes, whereas expression in pre-B cells is a further 3-fold lower. In contrast, peripheral B cells show comparable expression to pro-B cells.

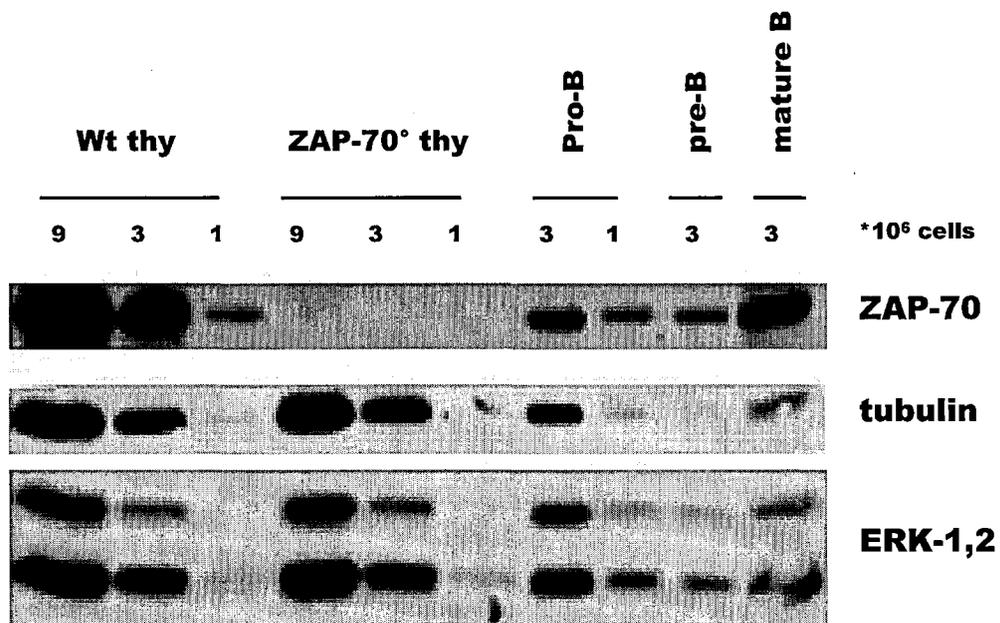


FIGURE 28. ZAP-70 EXPRESSION IN B-LINEAGE CELLS OF DIFFERENT MATURATIONAL STAGES

Cytoplasmic lysates were prepared from sorted B cell populations and unsorted total thymocytes. The numbers above each lane refer to the number of cells analysed.

4.3.4 Syk/ZAP-70 function in pro-BCR/pre-BCR signalling *in vivo*

The above mixed chimaera and western blot experiments demonstrate that ZAP-70 is expressed in the B lineage of mice. These results raise the question whether ZAP-70 is functional in B lymphocytes.

In the absence of ZAP-70, Syk appears to be sufficient for unimpaired B lymphocyte development and function, serving as a warning that in the presence of Syk, ZAP-70 activity might be difficult to assess. A more suitable system for this purpose, therefore, would be provided by the Syk^o mice, with or without ZAP-70 expression, respectively.

As shown before (Figure 20), in the absence of Syk and ZAP-70, B lymphocyte development is arrested at the pro-B cell stage. Hence the only B lineage cells that we can identify and analyse for ZAP-70 function in these mice are pro-B cells.

As Figure 23 displays, these pro-B cells are capable of synthesizing μ heavy chain, so we can assume that at least a fraction of them can also express pre-BCR (μ heavy chain in complex with the surrogate light chain) on their surface.

In addition, a fraction of these pro-B cells would also express the 'pro-BCR', Ig α/β in complex with calnexin [11, 122].

In order to study signal transduction through these receptors, one can take advantage of a recently described system [122] [133], that uses anti-Ig β antibody to crosslink the Ig β component of these receptors and induce signalling.

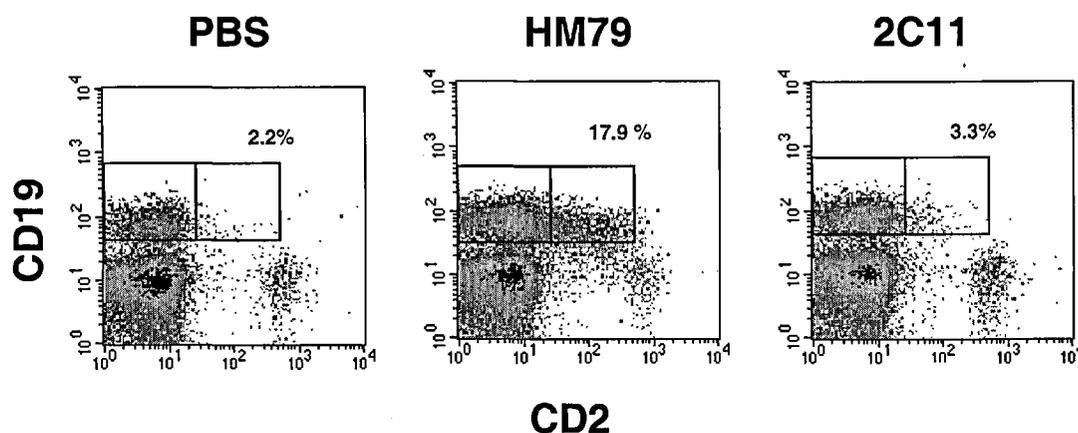


FIGURE 29. HM79 (ANTI-Ig β) TREATMENT INDUCES DIFFERENTIATION OF RAG-1[°] PRO-B CELLS

RAG-1[°] mice were injected intraperitoneally with 1 mg purified HM79 (anti-Ig β) antibody, with 0.5 mg 2C11 (anti-CD3) antibody (both in 400 μ l volume), or with 400 μ l PBS. 9 days later bone marrow from these mice were analysed. Numbers show percentage CD2⁺ cells among CD19⁺ bone marrow cells.

As reported, and also shown on Figure 29, intraperitoneal injection of 1 mg purified HM79 (anti-Ig β) antibody induced differentiation of RAG-1[°] pro-B cells characterised by induction of e.g. CD2, CD25 and increased BP-1 expression. In contrast, i.p. injection of anti-CD3 ϵ (2C11) had no effect on B cell development in RAG-1[°] mice.

2C11 (anti-CD3 ϵ) was chosen as negative control not only because it is a hamster-anti-mouse antibody as is HM79, but also because it has been shown to induce differentiation of RAG[°] thymocytes [123], thereby performing a potentially

analogous function in the T-lineage to that of HM79 in the B-lineage. This well-characterised effect of 2C11 on RAG^o thymocytes serves as a useful control: it confirms the effectiveness of the injected 2C11 and shows the specificity of both antibodies (Figure 30).

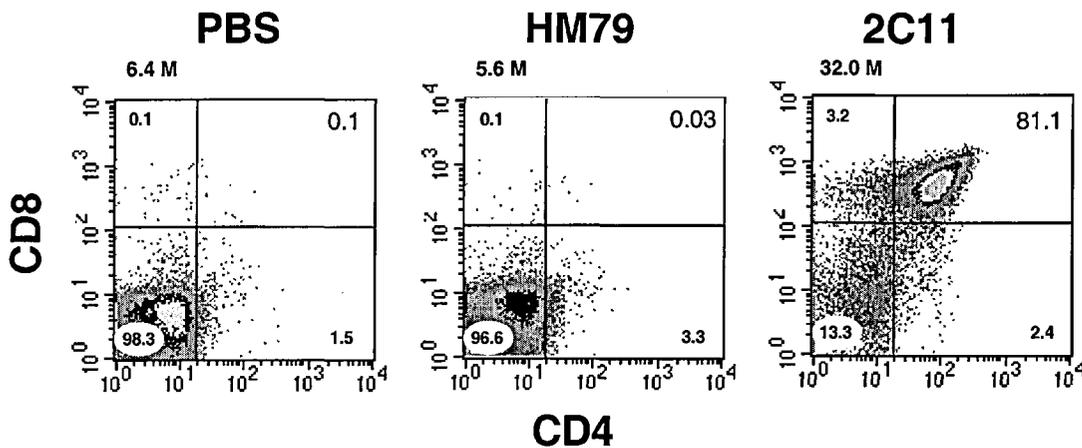


FIGURE 30. 2C11 (ANTI-CD3), BUT NOT HM79 (ANTI-Ig β) TREATMENT INDUCES DIFFERENTIATION OF RAG-1^o THYMOCYTES

See legend to Figure 29. 9 days after i.p. injections thymi were analysed. Numbers within each plot represent percentage of thymocytes falling into each quadrant. Numbers above plots show total thymocyte cell numbers in millions.

Figure 29 and Figure 30 confirm reported findings: HM79 (anti-Ig β) can induce differentiation of RAG-1^o pro-B cells, presumably by crosslinking the Ig β component of the pro-BCR, whereas this antibody has no discernible effect on thymocyte differentiation in RAG-1^o mice. 2C11 (anti-CD3) on the other hand induces developmental progression of RAG^o pro-T cells and an increase in thymocyte cell number presumably by stimulating the clonotype-independent CD3 complex (CIC). However, it does not affect B cell development in RAG-1^o mice.

In order to assess the functionality of ZAP-70 in the B lineage, the effect of HM79 injection on Syk^oZAP-70^o pro-B cells was determined. Radiation fetal liver chimaeras were generated as before, using Syk^o or Syk^oZAP-70^o fetal livers as donors, injected with 1 mg purified HM79 intraperitoneally and compared with similarly treated intact RAG-1^o animals.

Figure 31, Figure 32 and Figure 33 show analyses performed 13 days after antibody injections. Similar data were obtained 7 and 9 days after injections (not shown).

As seen before, RAG-1^o pro-B cells can be induced to differentiate into CD2 and CD25 expressing 'pre-B-like' cells. BP-1 expression is also detectable on a higher percentage of cells. HM79 injections do not induce differentiation of all RAG-1^o pro-B cells, probably because not all cells express the pro-BCR at the same time and at the same level and also because new pro-B cells are continuously generated.

In contrast to RAG-1^o animals, HM79 injections do not have any effect on CD2, CD25 and BP-1 expression on Syk^oZAP-70^o pro-B cells. As mentioned above, these pro-B cells can potentially express pro-BCR and pre-BCR, as well – though not necessarily at the same time. This result shows that without Syk and ZAP-70 these receptors cannot transmit a differentiation signal, suggesting that ZAP-70 can transduce signals from either the pro-BCR or pre-BCR in B lineage cells.

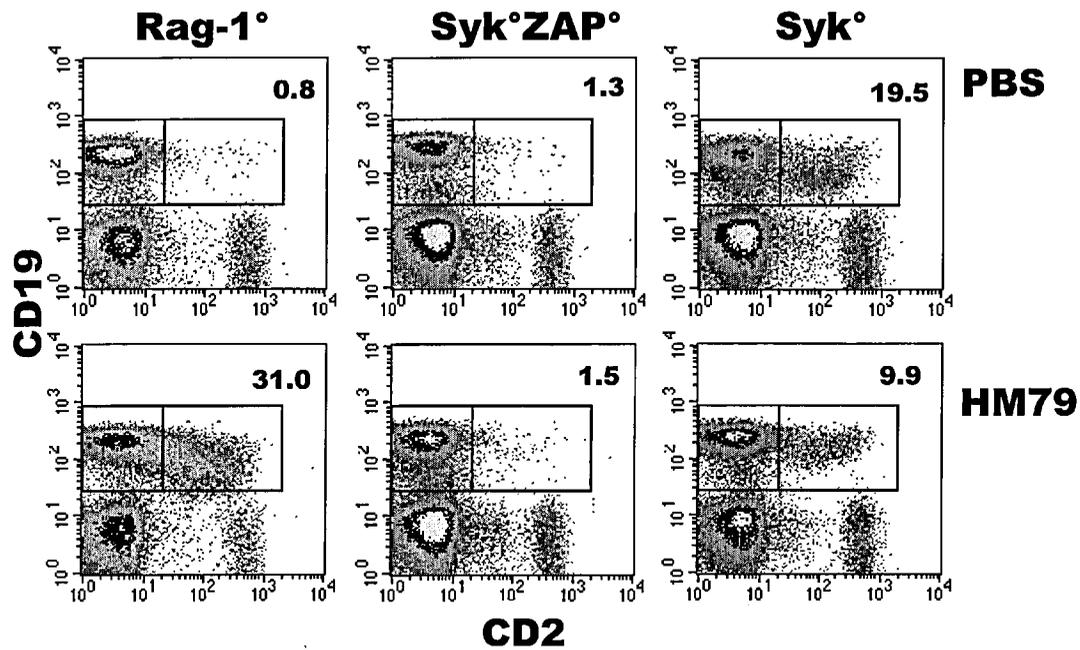


FIGURE 31. HM79 INDUCES DIFFERENTIATION OF RAG-1^o BUT NOT SYK^oZAP-70^o PRO-B CELLS (I)

Radiation fetal liver chimaeras were generated as before, using Syk^o or Syk^oZAP-70^o fetal livers as donors (all Ly-9.2/9.2, H2^{bb}), and 129/Sv (Ly-9.1/9.1, H2^{bb}) mice as lethally irradiated recipients. 8 weeks after cell transplantation chimaeras and intact RAG-1^o (Ly-9.2/9.2, H2^{dd}) mice were injected intraperitoneally with either 400 μ l PBS or 1 mg purified HM79 in 400 μ l volume. 13 days later mice were sacrificed and bone marrow cells analysed. Plots show Ly-9.1⁻ (donor-derived) bone marrow cells. Numbers refer to percentages of Ly-9.1⁻ CD19⁺ cells expressing CD2.

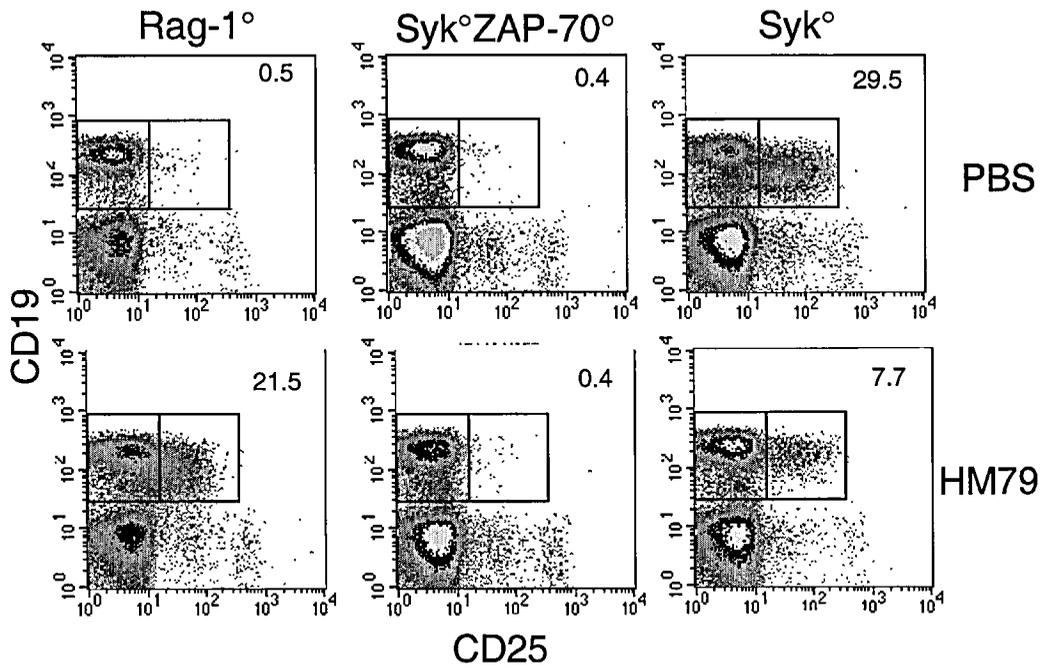


FIGURE 32 HM79 INDUCES DIFFERENTIATION OF RAG-1[°] BUT NOT SYK[°]ZAP-70[°] PRO-B CELLS (II) See legend to Figure 31.

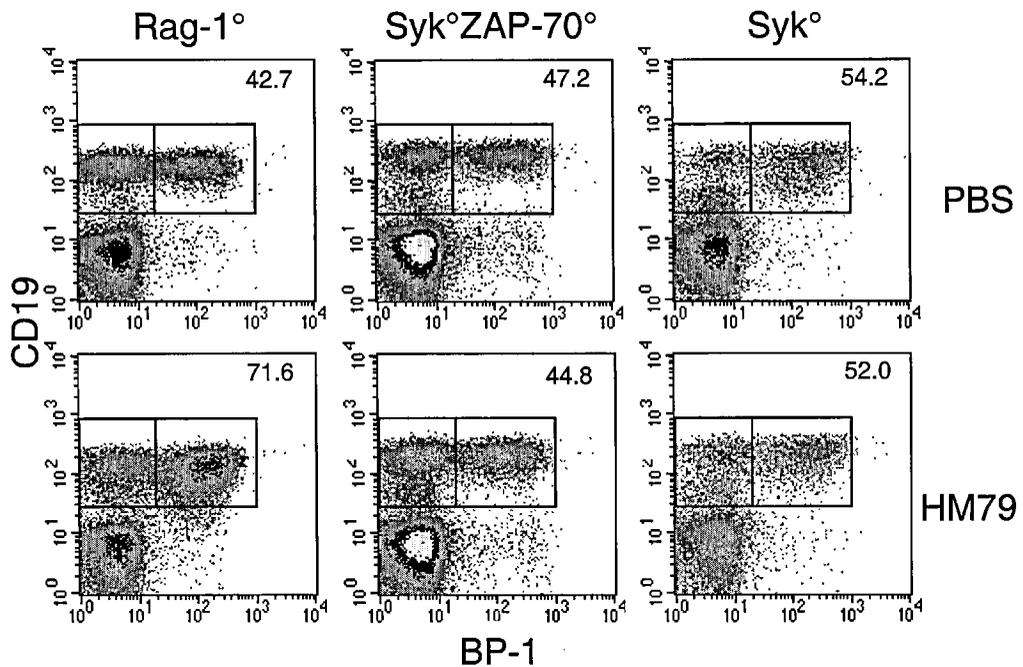


FIGURE 33. HM79 INDUCES DIFFERENTIATION OF RAG-1[°] BUT NOT SYK[°]ZAP-70[°] PRO-B CELLS (III) See legend to FIGURE 31.

Syk^o chimaeric mice were also injected with HM79 and analysed. These mice have a more complicated receptor makeup. B lineage cells in Syk^o bone marrow can potentially express pro-BCR, pre-BCR, and some of them also express a 'real' BCR. The predicted effects of Ig β crosslinking, therefore could be induced differentiation (through pro-BCR and pre-BCR), proliferation (via pre-BCR) and negative selection/deletion (via BCR). As Figure 34 shows there is a trend for loss of CD2 expressing cells in HM79-injected Syk^o chimaeras, suggesting that deletion dominates. There is, however, large variability among the mice analysed.

An even more controlled experiment is currently in progress to assess whether ZAP-70 can play a role in (early) B cell signalling: three types of chimaeric mice are being generated, RAG-1^o, Syk^o RAG-1^o and Syk^oZAP-70^oRAG-1^o. B cell development is expected to be blocked at the pro-BCR stage in all three types of mice. They are all expected to express only the pro-BCR, so Ig β injections would reveal the importance of Syk/ZAP-70 in pro-BCR signalling.

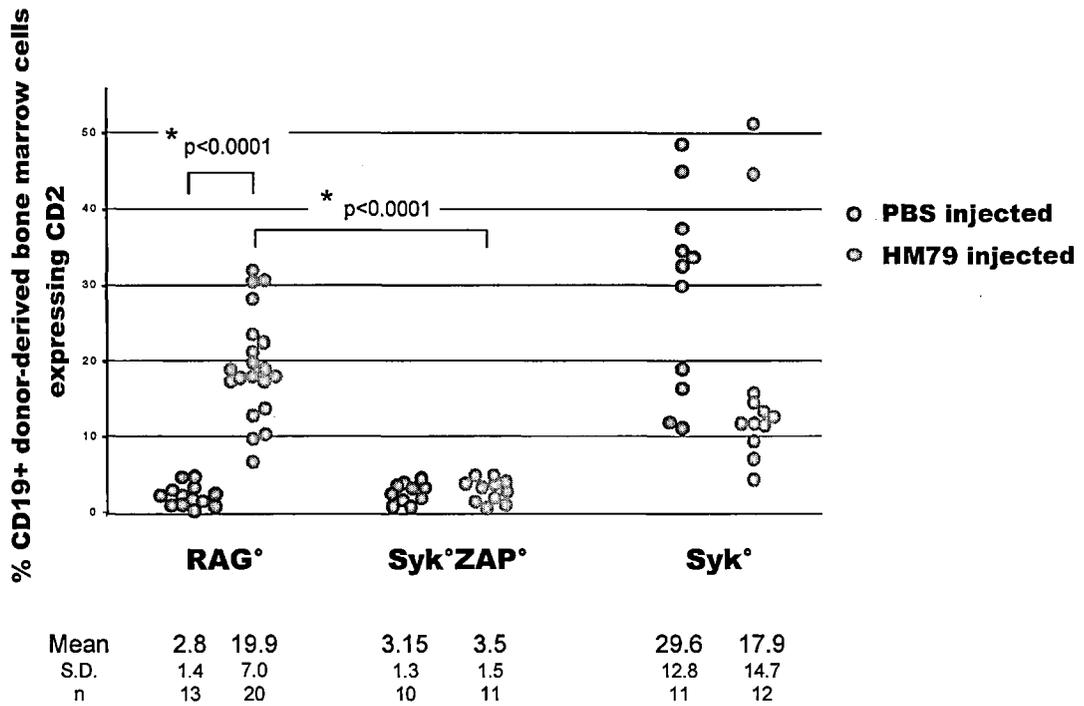


FIGURE 34 HM79 INDUCES DIFFERENTIATION OF RAG-1° BUT NOT SYK°ZAP-70° PRO-B CELLS (IV)

See legend to Figure 31. Each symbol represents an individual mouse. Data from analyses 7, 9 and 13 days after antibody injection are combined. Below each group of mice mean values, standard deviation and number of mice analysed are shown. Values for RAG° and Syk°ZAP-70° mice were also analysed by the Mann-Whitney test. Significant differences are shown by the asterisk.

Figure 35 shows another control for the above experiments. Similarly to the results seen in Figure 30, injection of HM79 does not affect T cell differentiation in Syk° or Syk°ZAP-70° mice.

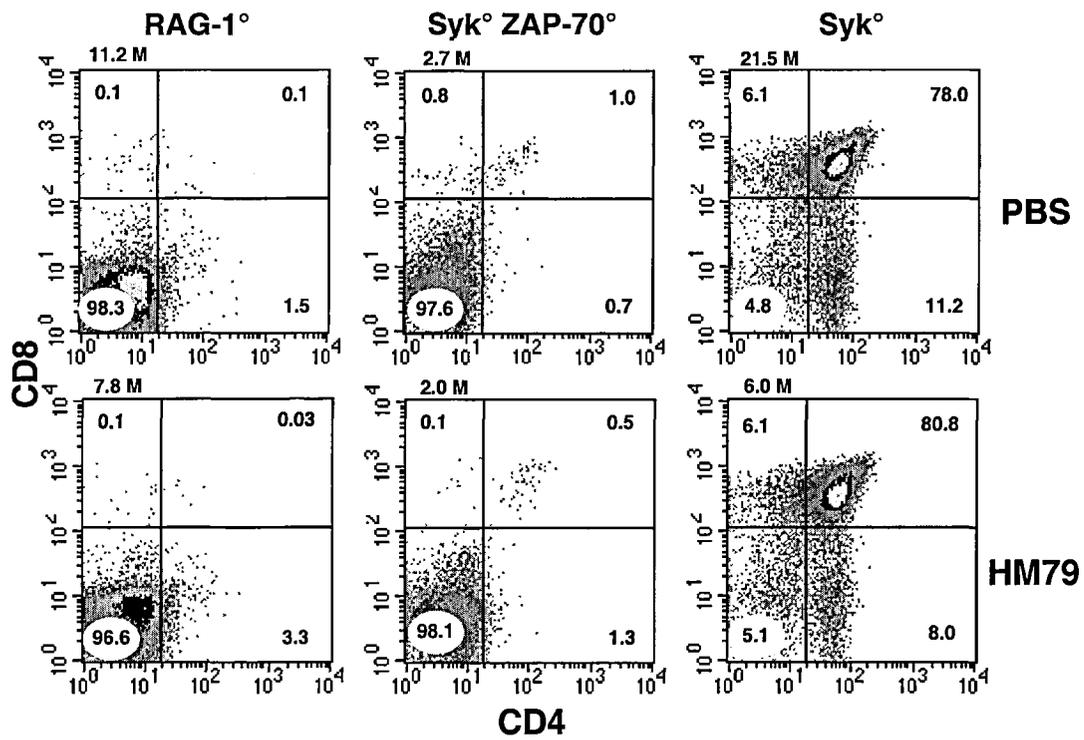


FIGURE 35. HM79 TREATMENT DOES NOT AFFECT THYMOCYTE DIFFERENTIATION

See legend to Figure 31. Ly-9.1⁻ donor-derived thymocytes are shown. Numbers within each plot represent percentage of thymocytes falling into each quadrant. Numbers above plots show Ly-9.1⁻ thymocyte cell numbers in millions.

4.3.5 Introduction of the 3-83 BCR transgene

Intracellular heavy chain expression in Syk^oZAP-70^o pro-B cells (Figure 23) suggests that immunoglobulin heavy chain rearrangement can proceed in the absence of Syk/ZAP family protein kinases at least to some extent. Introduction of a pre-rearranged BCR transgene that bypasses the need for endogenous recombination is a useful tool to probe for any rearrangement deficiencies. The 3-83 BCR transgene [59] was chosen (see also: Syk/Lyn chimaeras), since it allowed us to analyse not only B cell development across the pre-BCR checkpoint and associated allelic exclusion, but also negative selection, since the cognate ligand (MHC class I, H-2K^{k,b}) is easily introduced into our chimaeric system.

4.3.5.1 Effect of a rearranged transgene on B cell development in the absence of Syk and ZAP-70

As seen in Figure 19, no IgM-expressing cells develop in the bone marrow of DKO (Syk^o ZAP-70^o) mice. In contrast, Figure 36 shows that a pre-rearranged BCR that is introduced as a transgene can be easily detected on the surface, suggesting that the lack of IgM expression in non-transgenic mice is not due to any problems related to problems with carrying the protein on the surface (e.g. lack of stabilizing components [Ig α / β], enhanced internalization etc.).

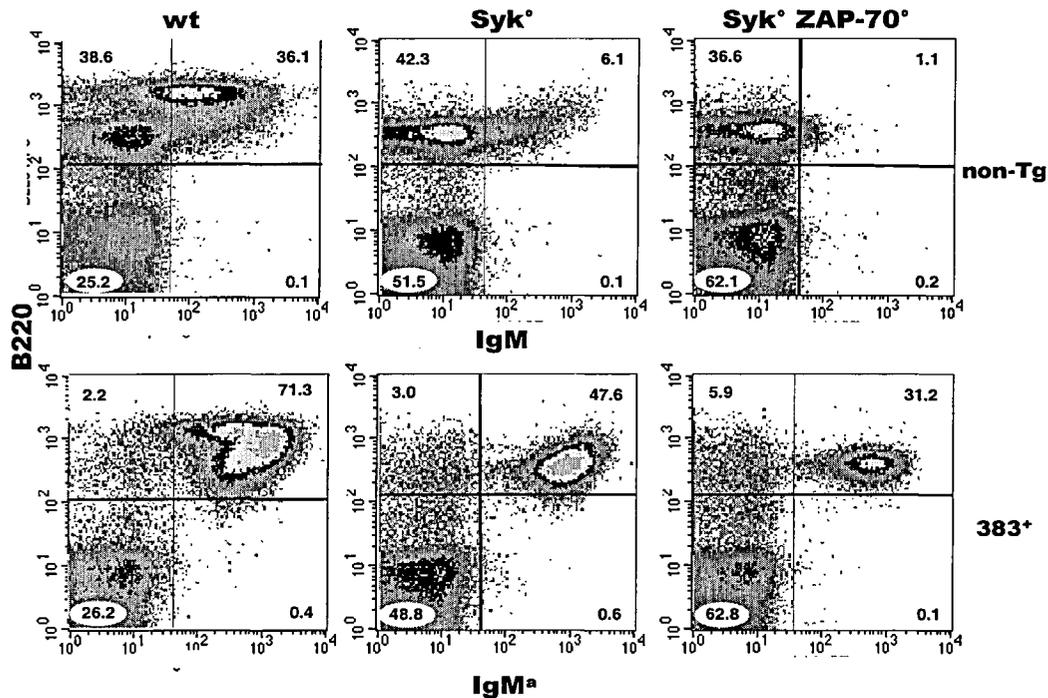


FIGURE 36. TRANSGENIC (3-83) BCR IS EXPRESSED ON THE SURFACE OF SYK^{-/-}ZAP-70^{-/-} BONE MARROW CELLS

Plots show Ly-9.1⁻ (donor) gated bone marrow cells. Top panels: Non-transgenic chimaeric mice. Anti-IgM antibody used (b7.6) recognises all IgM allotypes. Bottom panels: Chimaeric mice generated using 3-83 BCR transgene-carrying fetal liver cells. Anti-IgM^a binds to the transgene-encoded BCR. All donors are Ly-9.2/9.2, H2^{d/d}, IgH^{b/b}, the transgene is IgH^a, the recipients are Ly-9.1/9.2, H2^{d/d}, IgH^{a/b}. Numbers refer to percentage of Ly-9.1⁻ bone marrow cells falling into each quadrant.

Levels of BCR expression on DKO B lineage cells are lower than on wild type cells.

Also, B220^{high} cells can only be detected wild type, but not Syk^{-/-} or Syk^{-/-}ZAP-70^{-/-} mice. These findings raise the question about the maturational stage of cells expressing the transgenic BCR in the single and double knockout animals.

Given that no B220^{high} cells were found in the knockout animals, suggesting that Syk^o and Syk^o ZAP-70^o B lineage cells do not reach the recirculating mature stage, it is not surprising that no IgD expression can be detected on the transgenic BCR-carrying (IgM^{a+}) cells (Figure 37).

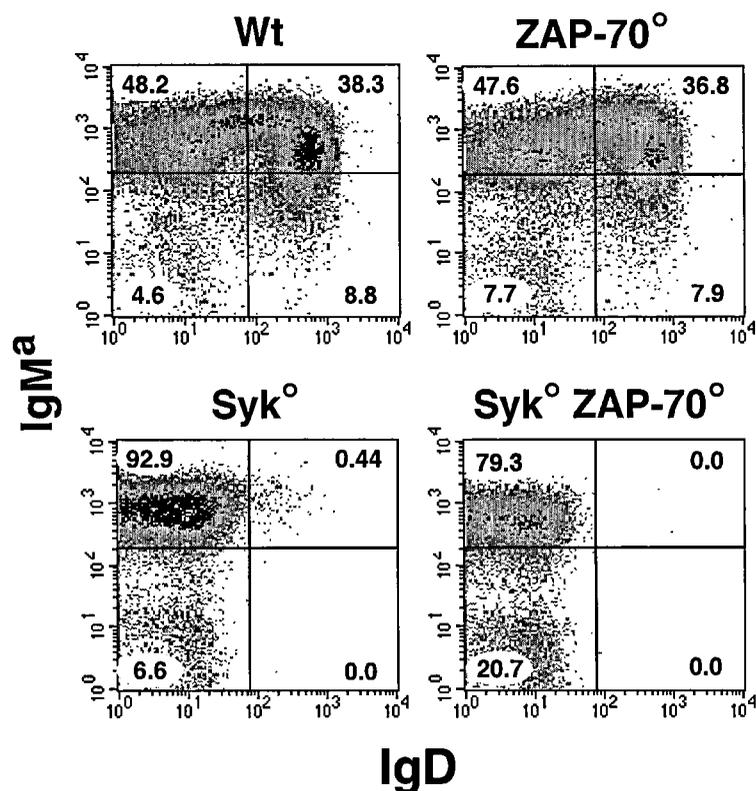


FIGURE 37. NO IGD EXPRESSION IN THE ABSENCE OF SYK AND ZAP-70 IN BCR TRANSGENIC CHIMAERIC MICE

Plots show Ly-9.1⁻ B220⁺ donor-derived B lymphocytes from bone marrow of chimaeric mice. IgM^a identifies B cells expressing transgene-encoded BCR on the surface. Numbers refer to percentage of Ly-9.1⁻ B220⁺ cells falling into each quadrant.

To further assess whether introduction of a BCR transgene lead to *any* developmental progression in Syk^oZAP-70^o pro-B cells, CD2, CD25 and CD43 expression was analysed in these mice (Figure 38).

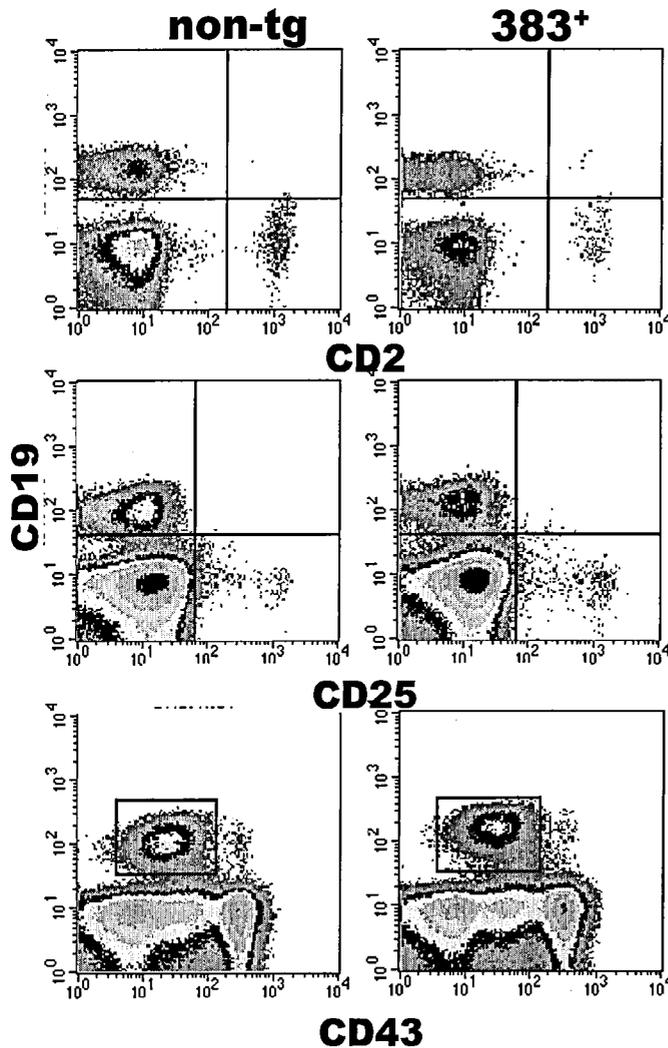


FIGURE 38. INTRODUCTION OF BCR TRANSGENE DOES NOT ALLOW FOR DEVELOPMENTAL PROGRESSION IN SYK^oZAP-70^o PRO-B CELLS

Bone marrow cells from non-transgenic (left column) and 3-83 BCR transgenic (right column) Syk^oZAP-70^o chimaeric mice are compared. Plots show Ly-9.1- (donor-derived) bone marrow cells.

CD2 and CD25 expression starts in pre-B cells. The absence of both of these markers from both non-transgenic and 3-83 transgenic Syk^oZAP-70^o cells suggests that B cell development has not progressed beyond the pro-BCR stage. This is confirmed by the

expression levels of CD43: all CD19⁺ (B-lineage) bone marrow cells express CD43, a marker of pro-B cells, which is turned off as cells mature into pre-B cells.

The fact that introduction of a pre-rearranged BCR transgene does not influence the developmental block seen in the absence of both Syk and ZAP-70, and that (as seen in Figure 23) Syk^oZAP-70^o pro-B cells can express intracellular heavy chain suggest that the developmental arrest is not due to a defect in immunoglobulin heavy chain rearrangement, but rather to a signalling defect from the pre-BCR. This also suggests, that if there is any signal needed to initiate rearrangement (e.g. from Ig β , as suggested by the Ig β -deficient mice[10]), that signal is independent of Syk/ZAP-family of protein tyrosine kinases.

4.3.5.2 Allelic exclusion

One of the important tasks a pre-BCR has to accomplish is to establish allelic exclusion at the heavy chain locus, which ensures that a developing B cell only expresses one heavy chain protein on the surface. Introduction of a BCR transgene allows for an easy assay of this process: since this BCR comes on early in development, it stops all endogenous heavy (and light) chain rearrangement as detected by surface expression or by DNA recombination.

4.3.5.2.1 Cell surface receptor expression

4.3.5.2.1.1 *Heavy chain expression*

The heavy chain construct used for the generation of the 3-83 transgene is derived from BALB/c (IgH^a) mice, therefore it can be distinguished from the B10.D2 (IgH^b) derived endogenous heavy chain locus by allotype-specific antibodies. As previously described [53] and seen in Figure 39, wild type mice carrying the 3-83 transgene do not express μ heavy chain encoded by the endogenous IgH^b locus, showing intact allelic exclusion. In contrast, in the absence of Syk and ZAP-70, a sizeable portion of IgM^a expressing cells also expresses IgM^b μ chain, demonstrating a failure of allelic exclusion.

This experiment suggests that without Syk and ZAP-70, the allelic exclusion process is incomplete, showing that at least this aspect of pre-BCR signalling is dependent of Syk and/or ZAP-70.

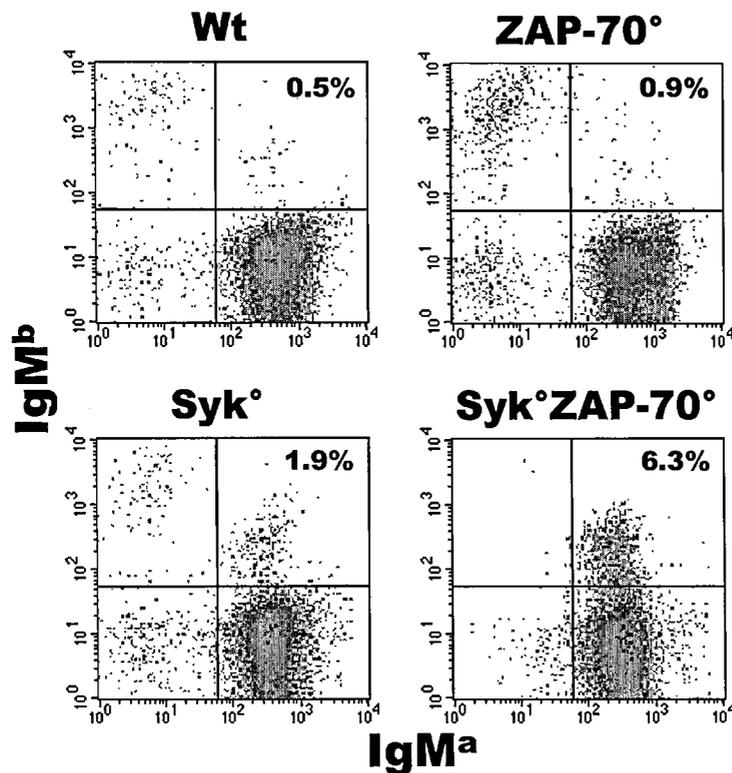


FIGURE 39. LACK OF ALLELIC EXCLUSION IN THE ABSENCE OF SYK AND ZAP-70

Plots show Ly-9.1⁻ CD19⁺ donor-derived B lineage cells from bone marrow samples of chimaeric mice reconstituted with fetal liver cells from Wt, Zap-70^o, Syk^o and Syk^oZAP-70^o embryos, respectively, all carrying the 3-83 BCR transgene. IgM^a is the heavy chain allotype of the transgene, whereas IgM^b is the endogenous heavy chain allotype. Numbers represent percentage of double-expressors among IgM^a⁺ cells.

4.3.5.2.1.2 Light Chain expression

The 3-83 transgene encodes not only a μ/δ heavy chain, but also a κ light chain.

While the pre-BCR (heavy chain plus surrogate light chain) ensures allelic exclusion at the heavy chain locus, the BCR (heavy chain plus conventional light chain) stops endogenous light chain rearrangement. κ exclusion is difficult to assess by FACS

analysis, since anti- κ antibodies cannot differentiate between the transgenic and endogenous κ chains. A good marker for light chain allelic exclusion in this case is expression of λ light chain, since that must be encoded by the endogenous locus.

Figure 40 shows endogenous λ expression in transgenic mice. Less than 1% of the IgM^a-expressing cells show λ expression confirming the existence of allelic exclusion in these transgenic mice. In contrast to the heavy chain results (Figure 39) there is less, rather than more λ expression in the absence of Syk and ZAP-70. This result could suggest perfect light chain allelic exclusion without Syk and ZAP-70, however, a more likely explanation is that Syk^oZAP-70^o3-83⁺ cells are still pro-B cells (as seen in Figure 38), and have not received any signals initiating light chain rearrangement/expression. DNA analysis (see below) could confirm this possibility.

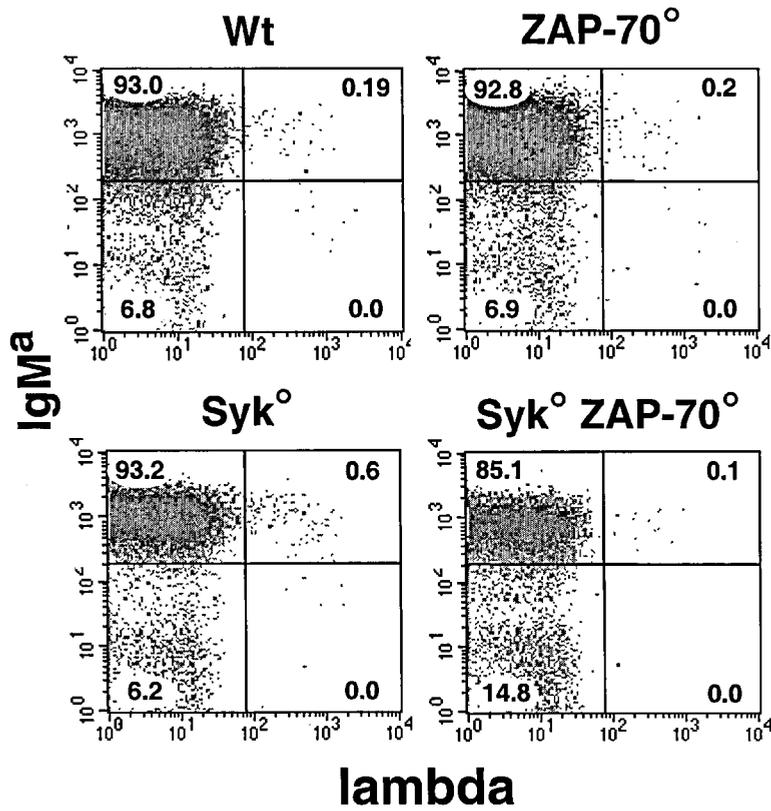


FIGURE 40. EXPRESSION OF ENDOGENOUS LIGHT CHAIN IN BCR TRANSGENIC MICE

Plots show Ly-9.1- B220+ donor-derived B lineage cells from bone marrow samples of chimaeric mice. IgMa is the heavy chain allotype of the transgene, l denotes endogenous light chain expression. Numbers represent percentage of B220+ Ly-9.1- cells falling into each quadrant.

4.3.5.2.2 Rearrangement of immunoglobulin heavy chain genes

DNA rearrangement analyses in $\text{Syk}^{\circ}\text{ZAP-70}^{\circ}$ mice could answer the following questions:

- do VDJ_{H} rearrangements of the heavy chain locus use 3' as well as 5' V_{H} families, suggesting that the initiation of rearrangement/locus accessibility is intact in these mice?
- is VDJ_{H} rearrangement detectable in $3\text{-}83^{+}\text{Syk}^{\circ}\text{ZAP-70}^{\circ}$ mice confirming the lack of allelic exclusion at the heavy chain locus?

4.3.5.2.2.1 Bulk fetal liver PCR

Bulk fetal liver DNA was analysed for heavy chain rearrangement. To assess usage of 5' V_{H} -families, which is impaired in $\text{IL-7R}\alpha^{\circ}$ mice [12], VDJ rearrangement using V-segments from the $\text{V}_{\text{H}}\text{J558}$ family was analysed. As shown in Figure 42, all non-transgenic samples show rearrangements, suggesting that no signal through Syk and/or ZAP-70 is needed for the initiation and completion of heavy chain rearrangements.

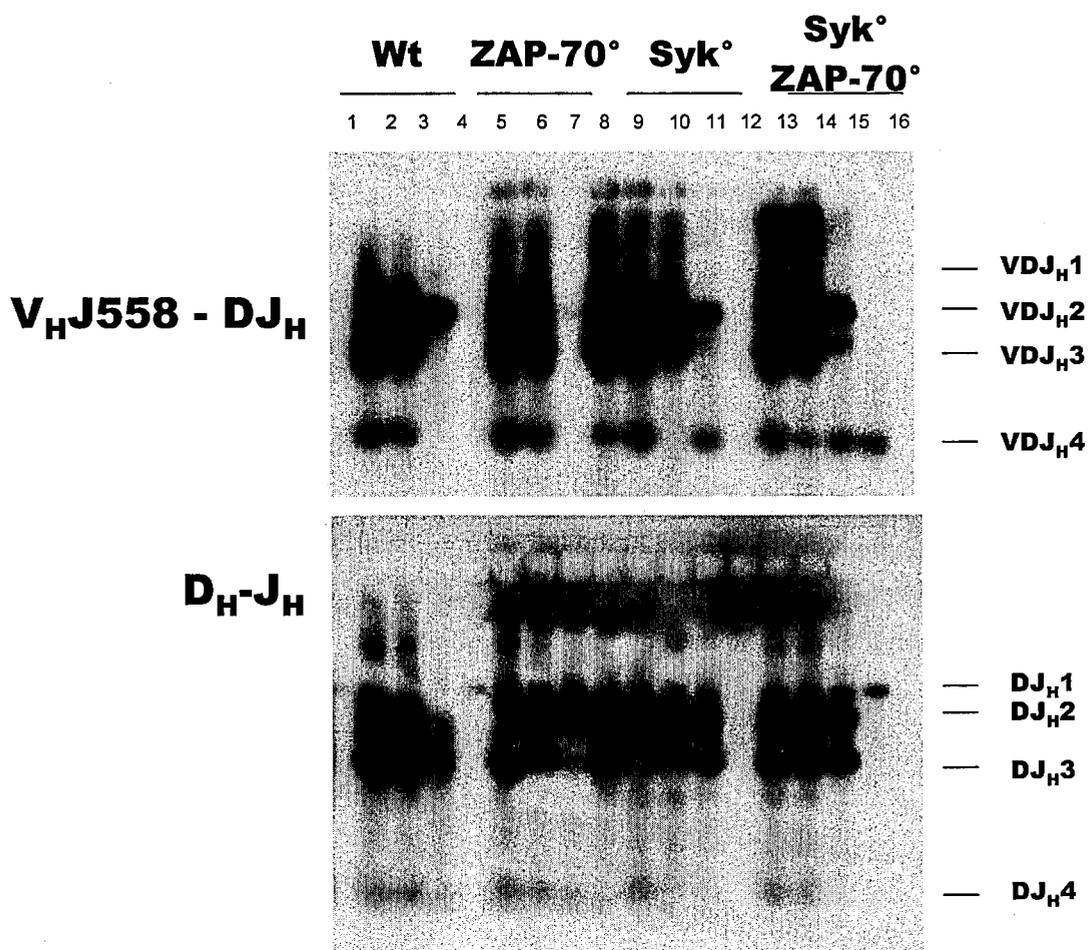


FIGURE 41. UNIMPAIRED HEAVY CHAIN REARRANGEMENTS IN THE ABSENCE OF SYK AND ZAP-70

DNA from day 16.5 fetal liver cells was prepared and analysed by PCR using upstream primers for (top panel) V_HJ558 and (bottom panel) D_{FL/SP2} families in combination with downstream primers hybridizing downstream of JH₄. DNA from 20,000 (lane 1,5,9,13), 4,000 (lanes 2,6,10,14), 800 (lanes 3,7,11,15) and 160 (lanes 4,8,12,16) cell-equivalents was used. PCR products were analysed on agarose gel, transferred onto nylon membrane and hybridized with a probe encompassing JH_{3,4}. RAG-1° fetal liver DNA was used as negative control.

Transgenic samples allow the evaluation of allelic exclusion in the presence and absence of Syk and/or ZAP-70. Figure 42 shows VDJ rearrangement in Syk° and Syk°ZAP-70°, but not in wild type or ZAP-70° transgenic samples, showing impaired

allelic exclusion in the absence of Syk and/or Zap-70. DJ_H rearrangements serve as control showing that there is DNA in the samples analysed and that allelic exclusion stops V-DJ_H but not D-J_H recombinations. These results are consistent with the FACS-analysis shown before (Figure 39): Syk and/or ZAP-70 is needed for complete allelic exclusion at the heavy chain locus.

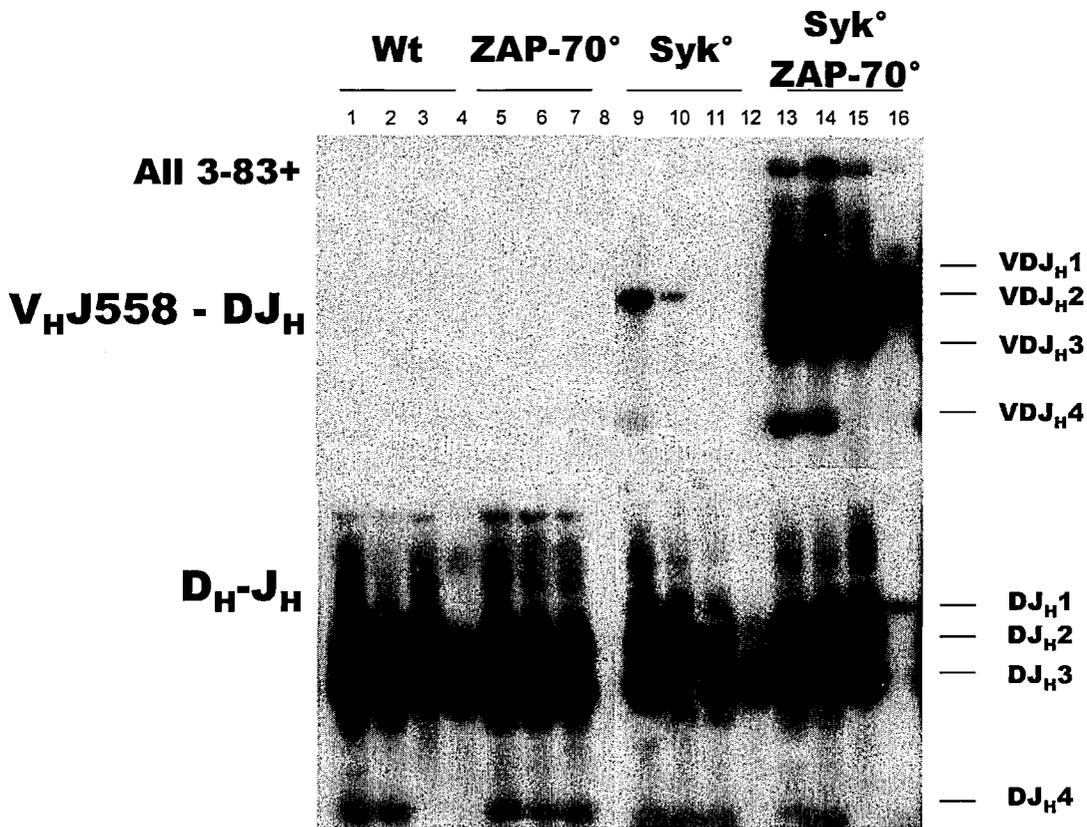


FIGURE 42. LACK OF IMMUNOGLOBULIN HEAVY CHAIN ALLELIC EXCLUSION IN THE ABSENCE OF SYK AND ZAP-70

See legend to Figure 41. All samples from embryos carrying the 3-83 BCR transgene.

4.3.5.3 *Negative selection*

Previous analyses of negative selection in Syk° 3-83⁺ H2^{kd} mice suggested that clonal deletion proceeds unimpaired in the absence of Syk (M. Turner and V. Tybulewicz, unpublished). Since *in vivo* Syk is indispensable for full B cell maturation, finding unimpaired deletion in Syk° mice was rather unexpected.

Introduction of the 3-83 BCR transgene allows for studies of negative selection, since the cognate ligand, MHC class I^{k,b} is easily introduced into our chimaeric system. Since the 3-83 BCR has higher affinity for K^k than for K^b, and it does not appear to react to K^d, in order to make the assay more sensitive, H2^{b/d} mice were used as recipients since these are expected to show much less negative selection than H2^{k/k}, or H2^{b/b} mice. In addition, the cognate ligand was only expressed on the radioresistant elements of the irradiated host animals, thereby decreasing the amount of antigen in the system, and also potentially restricting ligand expression to cells that are less efficient in causing negative selection. This strategy might allow detection of subtle differences in the knockout animals, that would go unnoticed if the deletion was rampant.

4.3.5.3.1 BCR expression

Negative selection can take multiple shapes: deletion of autoreactive cells, receptor downmodulation [66], receptor editing or anergy. In the 3-83 system clonal deletion, receptor downmodulation and receptor editing are induced by the presence of the negatively selecting ligand [53, 60, 170]. Figure 43 shows that wild type chimaeras generated using irradiated H2^{bd} recipients show lower IgM^a expression, where IgM^a is the IgH allotype of the transgenic BCR. Whether this phenomenon is truly receptor downmodulation or/and deletion of high transgene expressors with concomitant expansion of low expressors, cannot be distinguished from these data. Importantly, a similar level of receptor downmodulation can be seen both in Syk^o and Syk^oZAP-70^o chimaeras in the presence of H2^b on the irradiated host animals. One possible explanation for these results is that negative selection uses different signalling pathways than positive selection (that is blocked in Syk^o mice), and it is also different from the 'wiring' through the pre-BCR.

Also, as shown in Figure 38, DKO cells remain at the pro-B cell stage even in the presence of the BCR transgene. Pro-B cells do not normally express the complete BCR, and therefore would not be expected to be physiological targets for negative selection. These results are informative, however, since they show that pro-B cells in the absence of Syk and ZAP-70 are not inert, some signals can be transmitted from the receptors even without these two kinases.

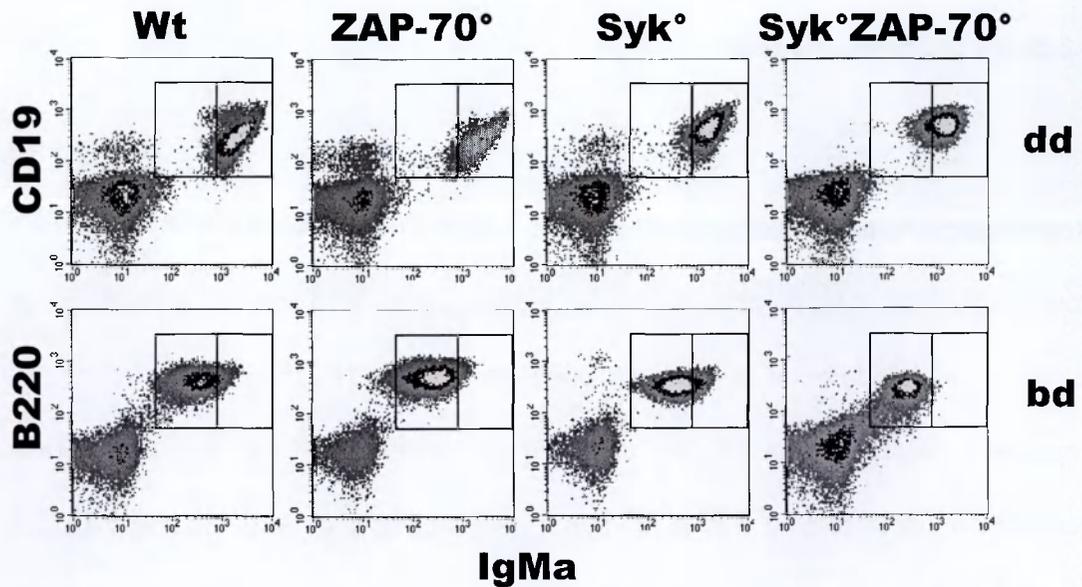


FIGURE 43. $H\text{-}2^b$ (3-83 LIGAND) CAUSES NEGATIVE SELECTION EVEN IN THE ABSENCE OF SYK AND ZAP-70

Plots show Ly-9.1⁺ donor-derived bone marrow cells from chimaeras generated from 3-83⁺ fetal livers. Donors are H2^{d/d}, recipients are either H2^{d/d} or H2^{b/d} as labelled. IgM^a identifies cells carrying the BCR transgene.

4.3.5.3.2 CD19 downmodulation

As seen on Figure 43, BCR/IgM^a levels are downmodulated upon cognate ligand recognition whether or not Syk and/or ZAP-70 is present in the cells. One difference, however, became apparent during analyses of negative selection in these chimaeric mice: CD19 is downmodulated in wild type H2^{b/d} chimaeras, but less so in Syk^o and Syk^oZAP-70^o mice (Figure 44).

These results show a link between BCR crosslinking and CD19 downregulation, that is not unconceivable, given CD19's role as a positive regulator of BCR signalling

[135]. This phenomenon could be specific for immature cells, where negative selection would be expected to take place.

Moreover, the defective CD19 down-modulation in H2^{bd} Syk^o or Syk^oZAP-70^o mice suggests that different signals are needed for BCR and CD19 down-modulation, respectively. Syk/ZAP-70 appears to be needed for the latter, but not the former process. Again, the question of maturational stage (immature B cells in wt mice, vs. pro-B cells in Syk^oZAP-70^o animals) means that we cannot tell if the failure to down-modulate CD19 in Syk^oZAP-70^o chimeras is due to defective BCR signalling or to an intrinsic inability of pro-B cells to downregulate CD19 following BCR engagement.

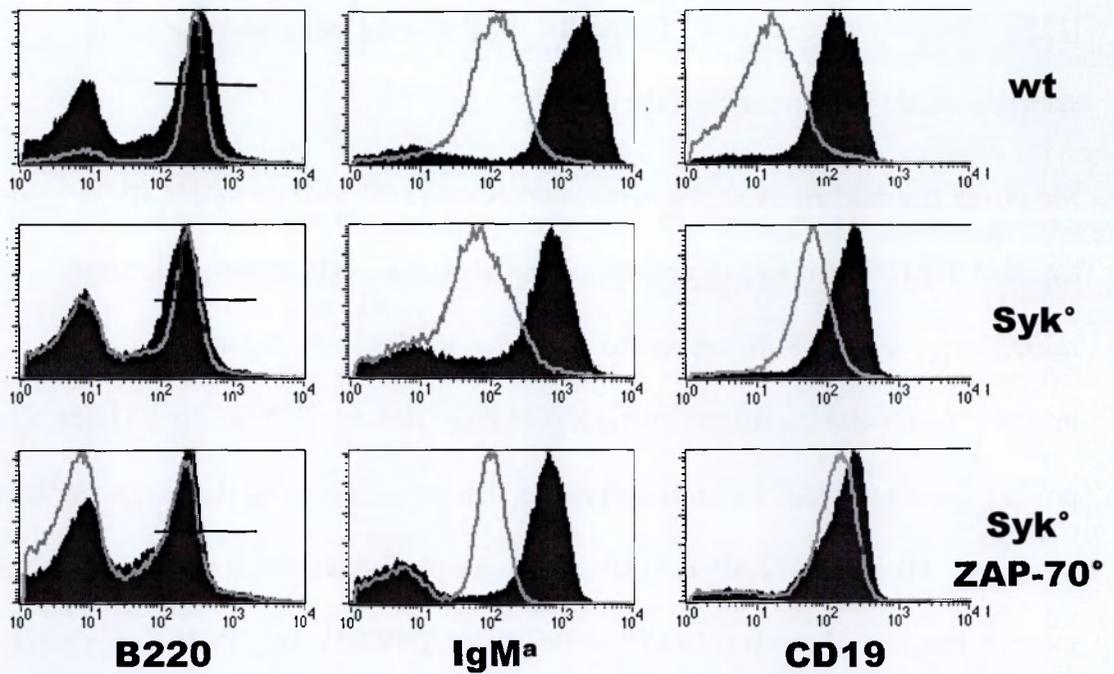


FIGURE 44. CD19 DOWNMODULATION UPON NEGATIVE SELECTION IS PARTLY DEPENDENT ON SYK AND ZAP-70

3-83 transgenic wild type, Syk[°] and Syk[°]ZAP-70[°] chimaeras were generated. Recipients were either H2^{d/d} (no negative selection, solid histograms) or H2^{b/d} (cognate ligand present, leading to negative selection, open histograms) mice. (left column) Plots show B220 expression on Ly-9.1⁻ donor-derived bone marrow cells.

(middle and right column) Plots show Ly-9.1⁻ B220⁺ (as gated on left panel, horizontal line) donor-derived B lineage cells.

5 Discussion

One of the major checkpoints in B cell development is the transition from pro-B cells to pre-B cells, mediated – at least partly – by the pre-BCR. Extensive studies have revealed that this signalling step results in differentiation and proliferation, but the exact mechanism and signal transduction machinery of this process has not been fully explored yet.

Based on experiments conducted using mature B cells and B cell lines, tyrosine kinases are prime ‘suspects’ in the early steps of signalling from the pre-BCR. The aim of this work has been to further elucidate the role of Syk/ZAP family of tyrosine kinases in early B cell development.

Gene targeted Syk mutant animals had been generated [128, 159], and analysis of these mice showed that in the absence of Syk B cell development is blocked incompletely at the pre-BCR step, and completely at the transition from immature to mature B cells. The partial block at the pre-BCR suggests that either there are different parallel pathways leading from the pre-BCR to the nucleus bringing about differentiation, or that other molecule(s) can perform the same functions as Syk, but maybe less efficiently. In either case, the complete block at the immature→mature transition shows that Syk is non-replaceable at that step.

In order to understand the relationship of Syk to other signalling molecules expected to play a role in early B cell development, I took a genetic approach and generated different combination mutant mice, missing either Lyn or ZAP-70 tyrosine kinases in addition to Syk.

5.1 The role of Lyn tyrosine kinase in early B cell development

Lyn was chosen as the most abundant representative of Src-family tyrosine kinases (SFK), shown to be associated with the resting BCR and activated with fast kinetics after receptor crosslinking [94]. SFKs are thought to contribute to the tyrosine phosphorylation of the Ig α / β subunits of the B cell receptor, thereby creating binding sites for the tandem SH2 domains of Syk. As a next step Syk becomes tyrosine phosphorylated either by autophosphorylation or by transphosphorylation, potentially by Lyn. This is, however, not the only role of Lyn in BCR signalling. As other positive effects, Lyn can contribute to Btk activation, and thereby influencing Ca⁺⁺-flux, phosphorylate (and be further activated by) CD19, thus contributing to PI3K and Vav activation. In addition, Lyn can also curtail BCR signalling by activating negative regulatory molecules, e.g. CD22-SHP-1 and FC γ RIIB-SHIP.

Based on these findings in mature B cells, the combination mutants missing both Syk and Lyn could:

- show the same phenotype as the Syk^o mice, suggesting, that at the pre-BCR step, the only downstream effector of Lyn is Syk; alternatively, that other SFKs can replace Lyn in every aspect;
- complete block at the pre-BCR step in the double mutant animals would suggest that other pathways bypassing the need for Syk are entirely dependent on Lyn, or that the other molecule(s) compensating for the absence of Syk cannot be called into action without Lyn;

- if removing Lyn was to restore differentiation in Syk^o animals, one would have to postulate a mechanism where the role of Syk is to counteract a pathway from Lyn that blocks differentiation, i.e. in the absence of Syk, the negative effect of Lyn dominates, that has to be removed to restore maturation;

Introduction of a pre-rearranged BCR transgene (3-83) with known specificity (H-2^{k,b}) allowed testing of negative selection in the absence of Syk [Turner, M. and Tybulewicz, V.L.J., unpublished]: deletion of autoreactive B cells proceeds even without Syk. In addition, in the HEL-transgenic system [161] in the absence of Syk the antigen-binding capacity of B lineage cells in the presence of cognate ligand (HEL) is more diminished than in control animals. This result is reminiscent of the hyper-responsiveness seen e.g. in the absence of the negative regulatory phosphatase SHP-1 [171], suggesting, that Syk may potentially play a negative role in the signalling from the BCR.

While Lyn was shown to exert positive as well as negative effect on BCR signalling, its negative influence predominates in B cell tolerance: removal of Lyn makes developing B cells more sensitive to negative selection [120]. Using the 3-83 system in combination with Syk and/or Lyn mutation(s) allows us to assess the relative contribution of the two kinases to negative selection.

Homozygous Syk mutant animals die around birth, so in order to assess B cell development in the absence of Syk, radiation chimaeras had to be produced from fetal livers harvested from wild type embryos or embryos deficient in Syk, Lyn or both. Chimeric Lyn^o mice showed similar phenotype to the published one [137]: in Lyn^o

mice the proportion of B220^{high} mature cells in the bone marrow is dramatically reduced, since Lyn appears to be required for successful B cell maturation and/or survival in the periphery[172]. In contrast, the generation of immature B cells in the bone marrow appears unaffected in the absence of Lyn.

Syk^o mice, as demonstrated before, show impaired development into immature B cells, and a complete block at the immature → mature B cell transition [127, 128].

Double ‘knockout’ chimaeric mice show further impairment in B cell development: the proportion of bone marrow cells expressing IgM is even lower than in the single mutants. This suggests that Lyn, in addition to contributing to Syk activation either directly or by phosphorylating Igα/β, thereby enabling Syk binding and activation, also has a positive role in early B cell development that is independent of Syk.

Introduction of an anti-apoptotic transgene (e.g. bcl-2) may help to reveal whether promoting survival is the only important role Lyn plays at these early stages.

Further investigation is currently underway to determine the relative contribution of Syk and Lyn to the pre-BCR signalling and their role at even earlier steps in B cell maturation.

Introduction of the 3-83 pre-rearranged transgene into Syk^o animals allows the expression of – transgene-derived – IgM^a on the surface of B lineage cells, proving that there is no inherent problem of expressing BCR on the surface. These IgM⁺ cells, however, are still pro-B cells that do not express CD2 (data not shown). This result confirms, that Syk^o B cell maturation is not blocked because of lack of rearrangement, which was expected, since there are some IgM expressing cells in Syk^o mice even in the absence of the BCR transgene.

Interestingly, in the bone marrow of Syk^o Lyn^o mice there are very few IgM^a expressing cells. Addition of a Bcl-2 transgene, however, appears to correct this defect (Figure 13), suggesting that in this BCR transgenic case either Syk or Lyn must be present for a survival signal.

Similar analyses in Syk^oZAP-70^o mice show only mildly decreased percentages of IgM^a expressing bone marrow cells in the double knockouts relative to Syk^o (Figure 36). This result taken together with the Syk^oLyn^o phenotype suggests that Lyn and Syk can transmit signals for survival independently of each other, but at least one of these pathways has to be active.

Cell survival defect is only one component in the B cell developmental block seen in Syk^o and Syk^oLyn^o mice, however, since addition of the Bcl-2 transgene – while increases cell numbers – does not lead to developmental progression, as assessed by lack of IgD expression.

Using a BCR transgene of a different allotype (IgM^a) from that of the endogenous heavy chain (IgM^b) is a convenient tool to assay allelic exclusion at the heavy chain locus, and similarly, analysis of endogenous λ light chain expression allows the evaluation of light chain allelic exclusion in the presence of a κ transgene.

3-83⁺Syk^o mice show minimal endogenous HC/LC recombination; however, expression of IgM^b and λ from the endogenous loci is significantly increased in the presence of the Bcl-2 transgene (Figure 14, Figure 15). Since Bcl-2 transgene has been shown to extend the half-life of IgM⁺ B cells in this system [159], one explanation is that in the absence of Syk allelic exclusion is inefficiently maintained, but increased lifespan of cells is needed to accumulate easily detectable numbers of cells with two receptors. The fact, that in the absence of both Syk and ZAP-70 heavy

chain 'inclusion' is easily detectable (without a Bcl-2 transgene) (Figure 42) says that ZAP-70 can sustain allelic exclusion in the absence of Syk, albeit less efficiently. Since the removal of Lyn has not changed endogenous heavy and light chain expression in the presence or absence of Syk, Lyn does not appear to be needed for allelic exclusion. I have not addressed whether other SFKs can replace Lyn in this process.

The 3-83 BCR confers MHC Class I (H-2^{k,b}) reactivity to transgenic B cells, that allows for testing of negative selection by using different MHC backgrounds. The 3-83 BCR has low affinity for H-2^{b/d}, especially if it is expressed only on the irradiated host cells in fetal liver radiation chimaeras. In this system the presence of the cognate ligand results in the disappearance of B220^{hi} cells from the bone marrow, and in the enlarged B220^{low} compartment IgM^a expression is about 10-fold lower than in H2^{dd} mice. This phenotype can be brought about by either deletion of all cells expressing high levels of IgM^a, or by IgM^a receptor downmodulation that does not allow for any differentiation into a B220^{hi} compartment, or by the combination of these two mechanisms. In addition, the increased proportion of B220⁺ IgM^{a+} cells in the bone marrow of H2^{bd} chimaeras may be explained by suggesting that the ligand-receptor interaction that is not strong enough to cause deletion of IgM^a^{low} cells, induces their proliferation instead.

Removal of Lyn leads to somewhat stronger receptor down-modulation (Figure 17), which is not unexpected, since Lyn^o B cells have been shown to be hypersensitive to tolerance induction previously [120].

Syk^o mice, however, appear to show unaffected negative selection as assayed by IgM^a levels in bone marrow cells of H2^{bd} chimaeric mice. This finding is somewhat surprising, given the requirement for Syk at the pre-BCR step and at the immature →

mature transition. We do have to keep in mind, however, that although these cells express IgM, and therefore look like immature B cells, they may have different characteristics from conventional immature B cells due to the impaired development in the absence of Syk.

Syk^oLyn^o mice show somewhat higher IgM^a levels, than Wt, or single mutant animals (Figure 17). This could suggest that a small positive contribution of Syk to negative selection can be revealed only in the absence of Lyn, because without Lyn, ZAP-70 may not be competent to replace Syk in this process. However, these effects are only marginal; moreover, Syk^oZAP-70^o mice can also downregulate IgM^a efficiently (Figure 43).

In summary, removal of Lyn in addition to Syk resulted in

- development of immature B cells (in contrast to Syk^oZAP-70^o mice);
- B lineage cells comprising much smaller fraction of the bone marrow both in non-transgenic and BCR transgenic animals
- Bcl-2 transgene correcting the deficit of B lineage cells in the presence of the BCR transgene
- Mildly enhanced effect on allelic exclusion (relative to removal of Syk alone)
- Similar or mildly smaller receptor downmodulation in the presence of a BCR transgene and its cognate ligand.

The conclusion that is emerging from these data – although further experiments are no doubt necessary – is that the most important role of Lyn in early B cell development is in supporting cell survival acting together with and/or in parallel to the Syk/ZAP family of tyrosine kinases. In this task Lyn cannot be efficiently

substituted for by other Src family members. If Src family kinases are important in promoting differentiation, Lyn can be substituted for by other family members.

5.2 The role of ZAP-70 tyrosine kinase in B cell development

As discussed in the previous chapter, the incomplete block in B cell development at the pre-BCR step seen in Syk^o mice became more severe by additional removal of Lyn SFK. B cell maturation in these double knockout Syk^o Lyn^o animals, however, is not completely arrested at the pro-B → pre-B transition, suggesting that additional molecule(s) can replace the function of Syk at this step, albeit inefficiently.

Redundancy among tyrosine kinase family members has been repeatedly seen in different aspects of lymphocyte development and function [163]. Since Syk is a member of the Syk/ZAP-70 protein tyrosine kinase family that has only two known members, Syk and ZAP-70, this prompted an investigation of the role of ZAP-70 in B cell development, especially in the absence of Syk. Whereas Syk is known to be widely expressed throughout the haemopoietic system (and possibly beyond), ZAP-70 has only been detected in T and NK lineage cells and cell lines.

To test whether ZAP-70 contributes in any way to early B cell maturation, mice carrying mutant Syk and ZAP-70 alleles, respectively, were intercrossed to generate double mutant animals. As in the case of the Syk^o single mutants, homozygous double knockout (DKO) Syk^oZAP-70^o mice also die perinatally, necessitating the

generation of radiation chimaeras from wild type or different mutant fetal livers as donors.

Syk^oZAP-70^o DKO mice have been generated before, and newborn animals were analysed for thymic T cell development [165]. These studies demonstrated that T cell development beyond the DN (CD4⁺8⁻) stage requires at least one of these kinases.

In chimaeric mice I found similar thymic phenotype (Figure 18) confirming that radiation chimaeras are useful models for studying lymphocyte development.

5.2.1.1.1 Complete block at the pre-BCR step in the absence of Syk and ZAP-70

Importantly, analyses of early B cell development in the bone marrow revealed a complete absence of IgM⁺ immature B cells in the DKO animals, in contrast to ZAP-70^o single mutants, that are indistinguishable from wild type mice, or to the Syk^o mice, which do contain immature B cells, though in severely reduced numbers (Figure 19, Figure 22).

Further dissection of B cell maturational steps in DKO animals using cell surface antigen-expression-based staging revealed that in the absence of both of these kinases B lineage cells cannot express CD2 (Figure 20), and are thus arrested at the IgM⁻ CD2⁻ CD43⁺ pro-B cell stage (Figure 21). In addition, cell size analyses show that all these cells remain large (Figure 21), consistent with their pro-B cell stage .

This phenotype suggests that in the absence of Syk and ZAP-70 pro-B → pre-B differentiation cannot be induced, which is one of the major tasks for the pre-BCR signalling pathway.

The reason for this missing signal could be either

- that ZAP-70 is not required cell-autonomously within the B cell lineage, but is must be present in other haemopoietic cell(s);
- lack of pre-BCR assembly/expression, or
- failure to transmit a signal from a properly expressed pre-BCR

5.2.1.1.2 The requirement for ZAP-70 (in the absence of Syk) is B cell-autonomous

Given that pre-BCR expression does not appear to be impaired in the Syk^oZAP-70^o DKO mice, lack of differentiation into the pre-B cell compartment is probably due to lack of proper signal transduction from this receptor. Alternatively, since these analyses were done on radiation chimaeras, and ZAP-70 was absent in all donor-derived fetal liver cells, it is possible, that ZAP-70 is not needed within the B lineage cells themselves, but rather in other haemopoietic cells that influence B cell development in *trans*, by secreting soluble factors or by expressing surface antigens needed for proper interactions with B cell progenitors.

To distinguish between these two possibilities mixed fetal liver radiation chimaeras were generated (Figure 25). The aim of these experiments was to allow development of Syk^oZAP-70^o B lineage progenitors surrounded by other, non-B lineage fetal liver-

derived cells that are ZAP-70⁺. As an internal control, the B lineage cells from the ZAP-70⁺ fetal liver can also be analysed. The maturation of these ZAP-70⁺ B cells should not be influenced by the presence of Syk^oZAP-70^o haemopoietic cells.

At first, wild type and DKO fetal liver cells were mixed in different ratios and injected into irradiated recipients, but the resulting chimaeric mice contained almost undetectable numbers of DKO-derived B lineage cells. This is probably caused by the lack of proliferative capacity of DKO pro-B cells.

In order to try to match competitiveness between the two donor fetal livers, DKO cells were mixed with Syk^o(ZAP-70^{+/+}) cells in different ratios. No significant competitive difference was seen using these two donor populations. Analysis of bone marrow cells from the resulting chimaeras revealed that mixing fetal livers did not change the phenotype of the developing B cells, i.e. B cells derived from DKO donors in the mixed chimaeras looked identical to those in unmixed chimaeras generated from DKO livers (Figure 26, Figure 27) This result demonstrates that the effect of ZAP-70 removal is cell-autonomous, i.e. ZAP-70 must be present within the B lineage cells themselves to allow some developmental progression in the absence of Syk across the pre-BCR checkpoint. Whether ZAP-70 exerts this function at the pre-BCR step, or it is needed at earlier developmental stages cannot be concluded from these experiments.

Syk and ZAP-70, though homologous in domain structure and primary sequence, have distinct expression pattern in the haemopoietic system and beyond. While Syk is found in T, B, NK cells, macrophages, dendritic cells, platelets, eosinophils, vascular endothelium, mammary gland tissue, etc., ZAP-70 protein has only been detected in T and NK lineage cells. Results discussed above from mixed chimaera

experiments suggest that ZAP-70 must be expressed within the B lineage cells themselves.

To address this issue at single cell level, I attempted to analyse ZAP-70 protein expression by intracellular flow cytometry. I used different monoclonal and polyclonal antibodies with different permeabilization and fixing protocols but could not detect any reproducible difference between wild type and ZAP-70^o thymocytes.

Short of intracellular staining I resorted to Western blot analysis on populations of cells. Since, ZAP-70 is expressed in T and NK lineage cells, it was necessary to use sorted cells of well-defined cell surface phenotype. If ZAP-70 is expressed in B cells, it may not be present, or present at the same levels throughout maturation. The developmental studies of the DKO animals demonstrated that ZAP-70 plays a role at the pro-B→pre-B transition, therefore pro-B cells and pre-B cells appeared obvious candidates for B lineage cells expressing ZAP-70. Also, in the T lineage, Syk expression is downregulated upon pre-TCR signalling [168], therefore if ZAP-70 were to play somewhat analogous role in B cells to that of Syk in T cells, ZAP-70 expression may be higher at earlier stages. Pure, uncontaminated pro-B cell population can be obtained from RAG-1^o bone marrow, since in the absence of recombination, these cells cannot progress into the pre-B cell compartment.

Progression to the pre-B stage can be achieved, however, by the introduction of a heavy chain only transgene into RAG^o mice, which allows pre-BCR signalling, but in the absence of any endogenous light chain rearrangement no further differentiation is possible. For comparison splenic CD19⁺ B cells were also included. This latter population is heterogenous, containing a mixture of transitional, mature and marginal zone B cells.

Western blot analysis of different sorted B cell populations demonstrated that ZAP-70 is indeed expressed in the B lineage. Comparison with expression levels found in total thymocytes revealed that while pro-B and pre-B cells have somewhat less ZAP-70 expression than thymocytes, splenic B cells express comparable amounts. This was surprising, since previously ZAP-70 was not detected in peripheral B cells [156].

5.2.1.1.3 Pre-BCR can be assembled and expressed in DKO B cells

The pre-BCR is composed of a μ heavy chain (HC) complexed with the so-called surrogate light chain (SLC), which in turn contains two proteins, Vpre-B and $\lambda 5$. Only a very small portion of all produced μ heavy chain actually gets to the surface as pre-BCR, most of the μ protein stays in the cytoplasm, where it is easily detectable by intracellular flow cytometry. Analysis of intracellular heavy chain expression proves that μ heavy chain can be produced in the absence of Syk and ZAP-70, that is detectable in chimaeric bone marrow pro-B cells as well as in fetal liver B lineage cells (Figure 23 and data not shown). Importantly, the portion of cells expressing heavy chain in the cytoplasm is much lower in the DKO mice, than in Wt, ZAP-70^o or even Syk^o animals, consistent with the hypothesis that the expansion of pre-B cells following pre-BCR signalling, which is yet another role for this receptor, is blocked. Detection of heavy chain protein in the cytoplasm proves that there is at least some level of heavy chain rearrangement 'allowed' in the absence of Syk and ZAP-70. This in turn suggests, that if there is a pro-BCR signal needed to allow/initiate HC

recombination, as implied by the phenotype of Igb° mice [10] (see Introduction), this does not require either of the Syk-family tyrosine kinases.

Rearrangement at the HC locus was also assessed using a PCR assay. Rearranged alleles were detected in fetal liver DNA samples from all four genotypes (Wt, ZAP-70 $^{\circ}$, Syk $^{\circ}$ and Syk $^{\circ}$ ZAP-70 $^{\circ}$) using upstream primers specific for a very frequently used 5' VH family, VHJ558 and downstream primers binding to sequences 3' of JH4 (Figure 41). These data confirm that heavy chain rearrangement can be initiated and completed in the absence of any Syk/ZAP family protein kinases. The detection of recombinations using the VHJ558 family of V-region gene segments was important, since a signal appears to be required for progression from 3' VH segments to more D-distal 5' VH families as shown by the phenotype of the IL-7Ra $^{\circ}$ animals. These mice can rearrange D-proximal, but not D-distal VH genes (such as VHJ558) [12]. The Syk $^{\circ}$ ZAP-70 $^{\circ}$ recombination results therefore show that Syk/ZAP-70 are not required for the pathway that leads from IL-7Ra and influences locus accessibility and/or the recombination machinery.

In addition to heavy chain rearrangement and cytoplasmic expression, surrogate light chain expression on the surface of pro-B cells was also detectable without Syk and/or ZAP-70 (Figure 24), suggesting that a pre-BCR can indeed be formed on Syk $^{\circ}$ ZAP-70 $^{\circ}$ B lineage cells, and it is the function of this receptor that is impaired without these two kinases.

5.2.1.1.4 ZAP-70 can function in B lineage cells

Results discussed in the previous chapter provide evidence that ZAP-70 is present in B lineage cells, but can it also function in these cells? To answer this question, I made use of a recently described system, showing that RAG^o pro-B cells can be induced to differentiate by administering anti-Ig β antibody intraperitoneally to crosslink Ig β in vivo [122]. If both Syk and ZAP-70 function downstream of Ig β , as was shown for Syk in mature B cells, then Syk^o ZAP-70^o DKO pro-B cells should not be able to mature further upon anti-Ig β injection. This is indeed the result we obtained: RAG^o pro-B cells show surface phenotype changes consistent with differentiation into pre-B cells, whereas DKO pro-B cells are not influenced by the injections. RAG^o pro-B cells cannot express any pre-BCR on their surface, since they cannot rearrange the heavy chain, therefore the effect of Ig β crosslinking is understood to be due to the stimulation of the so-called pro-BCR, composed of – among others – Ig α / β and calnexin. This signalling is assumed to model a pre-BCR signal, since the phenotypic changes are similar to those found at the pro-B→pre-B transition. Whether a ‘real’ pre-BCR reacts similarly to Ig β crosslinking has not been possible to test yet, since as soon as a pre-BCR assembles on the surface it appears to induce differentiation of pro-B cells into pre-B cells. Anti-Ig β injection into the Syk^oZAP-70^o chimeras is likely to have cross-linked both a pro-BCR and pre-BCR on the surface of the Syk^oZAP-70^o pro-B cells.

Instead of comparing pro-BCR signalling on RAG^o B cells with pro-BCR+pre-BCR signalling on Syk^oZAP-70^o DKO cells a more appropriate experiment would be a three-way comparison between RAG^o, SYK^oRAG^o and Syk^oZAP-70^oRAG^o mice, testing the pro-BCR in all three cases and directly assessing the relative contributions

to signalling by Syk and/or ZAP-70. The mice needed to perform this experiment are currently being generated.

A possible test for pre-BCR (instead of pro-BCR) signalling using the Ig β crosslinking could be to introduce an inducible Syk transgene into Syk $^{\circ}$ ZAP-70 $^{\circ}$ pro-B cells and induce Syk expression in vitro before anti-Ig β treatment, then assay biochemical events.

5.2.1.1.5 The 3-83 BCR transgene

Use of a BCR transgene makes the analysis of a cohort of developing B cells with the same specificity possible, and in the case of the 3-83 transgene that was used during these studies the cognate ligand is also known (MHC Class I, H2k,b), which can easily be introduced into the system.

The questions I wanted to answer using this transgene were:

- does a pre-rearranged BCR transgene relieve the developmental block seen in Syk $^{\circ}$ and Syk $^{\circ}$ ZAP-70 $^{\circ}$ DKO mice? If it does, then either the rearrangement process is impaired or – since this HC+LC transgene comes onto the surface earlier than a normal BCR – a BCR can use different pathways for inducing differentiation than a pre-BCR;
- is Syk and/or ZAP-70 involved in allelic exclusion at the heavy and/or light chain locus? The process of allelic exclusion can be conveniently analysed in the presence of a transgenic BCR, since transgenic and endogenous heavy chains can be distinguished by choosing different allotypes for the two, and the transgene

codes for κ light chain, therefore any λ light chain has to be coded for by endogenous genes.

- is Syk and/or ZAP-70 needed for negative selection of autoreactive B cells?

The presence of the cognate ligand converts B cells expressing the transgene-encoded BCR into autoreactive cells, the fate of which can be studied in the presence or absence of Syk/ZAP-70.

5.2.1.1.6 Transgenic BCR cannot relieve the maturational block in DKO B cells

As discussed above, Syk^oZAP-70^o DKO B cell development is arrested at the pro-B cell stage. Introduction of the 3-83 BCR transgene resulted in surface expression of transgene-encoded IgMa in the bone marrow of DKO cells, giving these cells the appearance of immature B cells. Further phenotypic analysis, however, established, that – despite BCR expression – these 3-83⁺ Syk^oZAP-70^o DKO B cells are still pro-B cells.

Rearrangement analysis of DKO B cells – in the absence of the transgene – appeared comparable to wild type cells (see above), which already suggested that recombination defect is probably not the cause of the developmental block. The lack of maturational progress upon transgenic BCR expression confirms this, and in addition suggests that even if a HC + conventional LC is used instead of a pre-BCR, which is known to allow progression from pro-B cells to pre-B cells in e.g. RAG^o

mice, signalling from this receptor still needs Syk and/or ZAP-70 to induce differentiation.

Moreover, detection of the transgenic BCR on the surface also demonstrates that there is no inherent problem in DKO B cells with synthesis of immunoglobulin and its transport to the cell surface.

5.2.1.1.7 Impaired allelic exclusion In DKO B cells

Allelic exclusion at the heavy chain locus is understood to be one of the consequences of pre-BCR signalling. Since a functional pre-BCR requires μ HC protein synthesis and complex formation with the surrogate light chain, a signal through this receptor means that the heavy chain has passed 'quality control'. A logical step, therefore is to stop any further rearrangement at the heavy chain locus of the other allele, known as allelic exclusion.

One way of testing this process is to use mice heterozygous at the heavy chain locus, carrying two different allotypes at the two different alleles, then assess the fraction of cells expressing μ protein from both alleles. These mice are currently being generated.

Alternatively, introduction of a pre-rearranged transgene has been shown to induce allelic exclusion, resulting in no endogenous VH-DJH rearrangement, and as a consequence no endogenous μ HC allotype expression. Introduction of the 3-83 transgene allowed this type of analysis. As seen in the phenotypic studies, this

transgene can be expressed already in pro-B cells, it is expressed probably as early in ontogeny as the pre-BCR would be.

Allelic exclusion at rearrangement level was assessed similarly to the assay used for nontransgenic samples. PCR analysis of VH-DJH recombinations using VHJ558 and 3' of JH4 primers revealed that the 3-83 transgene is indeed very effective in preventing any endogenous rearrangement in wild type and ZAP-70^o mice. In contrast, the Syk mutation already impairs allelic exclusion and the presence of endogenous rearrangement is even more marked in the DKO fetal liver cells. These results show that a signal from the pre-BCR (which in this case is replaced by a BCR expressed on pro-B cells) that shuts down endogenous rearrangement requires Syk or ZAP-70, and that ZAP-70 is less effective in mediating this effect than Syk.

Analyses at the protein level showed that a significant fraction of DKO B cells can express endogenous heavy chain on the surface in addition to the transgenic BCR. This result further confirms that there is endogenous rearrangement in these mice carrying the transgene. Since we cannot detect μ expression on DKO pro-B cells in the absence of the transgene (as a pre-BCR), this result also suggest that endogenous μ heavy chain probably pairs with transgenic light chains, that allows high levels of surface expression.

Light chain allelic exclusion is rather difficult to assay in these mice. The transgene encodes a κ light chain, and in the absence of allotype-specific antibodies, surface expression of endogenous κ chains cannot be determined. λ light chain expression was not detected even in the absence of the transgenic BCR on DKO B cells. The reason for this could be that DKO pro-B cells cannot reach the stage (pre-B cells) when λ rearrangement would be initiated. In contrast, by PCR, κ rearrangement can

be detected in the absence of the transgene, but κ is known to start recombination before λ , and a small fraction of κ rearrangement can even start before any HC recombination is complete. Alternatively, Syk and or ZAP-70 is directly involved in initiating/allowing λ light chain rearrangement, as it was also suggested for Btk [132].

5.2.1.1.8 Negative selection can proceed without Syk and ZAP-70

Introduction of the cognate ligand (H-2K^b) for the 3-83 transgene allows the analysis of negative selection with or without Syk and ZAP-70. Restricting this low affinity ligand to the radio-resistant elements of the host in fetal liver radiation chimaeras induces negative selection as detected by a lack of idiotype⁺ cells in the periphery and IgM^a downmodulation in the bone marrow.

In the absence of Syk and both Syk and ZAP-70 the same effect of the ligand can be seen as in wild type mice: only IgM^{low} bone marrow B cells can be detected. Thus IgM^{high} B cells are being eliminated even in the absence of Syk and ZAP-70.

However, CD19 down-modulation in response to H-2K^b seen in Wt mice was absent in Syk^oZAP-70^o animals. These results suggest that at least some signal transduction pathways can proceed in the absence of both Syk and ZAP-70. This is in contrast to the complete block seen in pre-BCR signalling in Syk^oZAP-70^o pro-B cells as read out by failure of differentiation to the pre-B cell stage, proliferative expansion and allelic exclusion.

In the case of negative selection an external ligand is implicated, whereas an external ligand may not be necessary for pre-BCR signalling. This ligand difference may translate into completely different signal propagation machinery.

Alternatively, if receptor downmodulation is due to internalisation, this process may be independent of Syk and ZAP-70.

The disadvantage of using the 3-83 model to study negative selection in DKO mice is that B cell development in these mice is blocked at the pro-B cell stage, a compartment in which negative selection does not usually occur. One approach to overcome this would be to generate a mouse strain with a conditional Syk gene, allowing Syk to be turned off in immature B cells, the normal target of negative selection

6 Conclusion

The work presented here tried to explore the relative role of Syk/Lyn and Syk/ZAP-70 kinases, respectively in early B cell development.

From the results discussed the following conclusions can be drawn:

- as published before, in the absence of Syk there is an incomplete block at the pre-BCR step, and a complete block at the immature → mature transition stage of B cell maturation
- the incomplete block at the pro-B → pre-B cell transition becomes complete by the additional removal of ZAP-70
- ZAP-70 is present within B lineage cells and is required for B cell development in the absence of Syk
- ZAP-70 can function in B lineage cells, and can – albeit inefficiently – replace Syk
- Signalling through the pre-BCR absolutely requires Syk or ZAP-70 for (at least) changes in surface protein expression, expansion and allelic exclusion
- Even though the immature → mature B cell transition absolutely requires Syk, and at this stage Syk cannot be replaced by ZAP-70, not all aspects of the BCR signalling are impaired in Syk⁰ZAP-70⁰ mice
- Removal of Lyn – in addition to Syk – revealed that in early B cell development Lyn plays an important role in supporting cell survival, whereas

other Src family member kinases can possibly substitute for Lyn in differentiation.

Future work will address the role of Syk and Zap-70 at later stages of development, by generating inducible/conditional Syk mutant or transgenic models.

7 Materials and Methods

7.1 Solutions and Media

TE	10 mM Tris.Cl, pH 7.4, 1 mM EDTA, pH 8.0
ACK lysis buffer	0.15 M NH ₄ Cl + 1.0 mM KHCO ₃ + 0.1 mM Na ₂ EDTA pH 7.2-7.4
Tail lysis buffer	0.1 M Tris.Cl pH 8.5 + 5 mM EDTA + 0.2% SDS + 0.2 M NaCl
FACS buffer	PBS + 0.1% Na-azide + 0.5% BSA
for Southern blotting:	
50 x TAE	40 mM Tris-acetate + 2 mM Na ₂ EDTA.2H ₂ O
20 x SSC	3 M NaCl + 0.3 M Na ₃ citrate.2H ₂ O (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
for Western blotting:	
4 x Lower Tris Buffer	1.5 M Tris-Cl + 0.4% SDS pH 8.8
4 x Upper Tris Buffer	0.5 M Tris-Cl + 0.4% SDS pH 6.8
10 x Running Buffer	3.03% Tris-Cl + 1 % SDS + 14.42% Glycine pH 8.3
2xRSB	20% glycerol + 6% SDS + 1x upper Tris buffer + 0.005% bromophenol blue
10% polyacrylamide gel	10% Acrylamid/Bis-Acrylamide + 1x Lower Tris Buffer+0.033 % ammonium-persulfate + 0.066% TEMED
Stacking gel	3.75% Acrylamide/Bis-acrylamide + 1x Upper Tris Buffer + 0.05% ammonium-persulfate + 0.1% TEMED
CAPS-buffer	2.21% CAPS in H ₂ O, pH 11.0
Stripping buffer	62.5 mM Tris.Cl pH 6.7 + 100 mM 2-mercaptoethanol + 2% SDS
Blot-wash	0.05% Tween-20 in PBS

7.2 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 [127], 3-83 transgenic mice were provided by David Nemazee [59], *Bcl-2* transgenic mice by Andreas Strasser [162], *Lyn*-mutant mice by Ashley Dunn [137], *Rag-1* knockout mice by D. Baltimore [7], *Zap-70*-mutant mice by Art Weiss [167], *HC-186* and *E μ l* transgenic mice by Fred Alt [169]. 129/Sv, B10.D2/o, BALB/c, C57BL/10, (BALB/c x B10.D2)F1 and (BALB/c x C57BL/10)F1 mice were provided by the SPF (specific pathogen free) facility of the Institute.

7.3 Antibodies and Cell Lines:

The following antibodies were purchased for use for flow cytometry or Western blotting:

COMPANY	ANTIBODY	CLONE/CAT.NO.
Becton-Dickinson	B220-CyChr	RA3.6B2
Becton-Dickinson	BP-1-PE	6C3
Becton-Dickinson	CD19-biotin	1D3
Becton-Dickinson	CD2 (LFA-2)-PE	RM2-5
Becton-Dickinson	CD24 (HSA)-PE	M1/69

Becton-Dickinson	CD25-PE	PC61
Becton-Dickinson	CD43(leukosialin)-biotin	S7
Becton-Dickinson	CD4-PE	H129.19
Becton-Dickinson	Ig β -purified	HM79b
Becton-Dickinson	IgD-FITC	11-26c.2a
Becton-Dickinson	IgMa-FITC	IgH6 ^a
Becton-Dickinson	IgM-FITC	R6-60.2
Becton-Dickinson	IL-7R α -PE	SB/14
Becton-Dickinson	kappa-PE	02155A
Becton-Dickinson	lambda _{1,2} -biotin	R26-46
Becton-Dickinson	Ly-9.1-biotin	30C7
Becton-Dickinson	Ly-9.1-FITC	30C7
Becton-Dickinson	Streptavidin-PE	
Becton-Dickinson	ZAP-70	Z24820
Caltag	B220-APC	RA3-6B2
Caltag	CD19-Tricolor	RM7706
Caltag	CD8 α -APC	RM2205
Caltag	Streptavidin-PECy7	SA1012
Santa Cruz	ERK-1	K23
Santa Cruz	ZAP-70	LR
Santa Cruz	phospho-ERK	E4
DAKO	rabbit-anti-mouse-Ig-HRP	PO260
Cell Signalling Techn.	ZAP-70	rabbit polyclonal Ig
Life Technologies	Streptavidin-Red613	

7.3.2 Labelling of antibodies

7.3.2.1 FITC (*fluorescein-5' isothiocyanate*)

Protein G-purified 54.1 mAb at 1 mg/ml concentration was dialysed against 0.25 M carbonate buffer (pH 9.3) for at least 1-2 hours. FITC (Molecular Probes, Oregon) was dissolved in DMSO to yield 10 mg/ml stock concentration, then 100 μ g FITC was added to each mg protein, and rotated overnight at 4°C. Conjugated antibody was dialysed against PBS, then stored at -20°C or at 4°C in 0.1% Sodium azide.

7.3.2.2 Biotin

Protein G (Hi-Trap, Pharmacia) purified HM79b mAb was conjugated to biotin using EZ-Link Sulfo-NHS-LC-biotinylation kit (Pierce, Rockford, IL). 0.2 mg sulfo-NHS-LC-biotin was added to 2 mg protein in 1 ml PBS, and incubated for 2 hours on ice. Using the column provided by the kit, conjugated protein was desalted then concentration determined by spectrophotometer. Conjugated antibody is stored at 4°C in 0.1% sodium azide.

7.3.2.3 APC

APC-conjugation of anti-mouse CD19 (1D3) was performed using the Phycolink APC labelling kit (ProZyme) according to manufacturer's protocol. APC-conjugated antibody was then stored at 4°C in 0.1% sodium azide protected from light.

Serotec	Goat-anti-mouse-IgM-FITC	STAR86F
Amersham-Pharmacia	Protein-A-HRP	NA 9120

24G2 (Fc-block) hybridoma was a gift from B. Stockinger, 54.1 (anti-clonotype for 3-83 transgenic BCR) from D. Nemazee, 1.19 (anti-IgD) and B76 (anti-mouse μ) from G. Klaus, HM-79b (anti-Ig β) from T. Nakamura [11], biotinylated LM-34 (anti- λ 5) and VP-245 (anti-VpreB) from L. Mårtensson (with permission from H. Karasuyama), TAT-1 (anti-tubulin) from S. Ley, K^b-biotin (MV3) from R. Zamoyska.

7.3.1 Purification of antibodies

Hybridoma supernatants were cleared of debris by centrifugation at 10,000 rpm in Beckman JA-25 centrifuge for 10 min, and the pellet discarded. Protein-G column (connected to Pharmacia LKB fraction collector) was pre-washed with PBS-Azide, then supernatant added. Unbound material washed off with excess amount of PBS, then specifically bound protein eluted with 0.2M acetic acid. Eluted fractions were neutralized with 1M Tris.HCl (pH 11), protein concentration determined by spectrophotometer. Peak fractions were pooled, then dialysed overnight against PBS. If needed, Centricon10 (Amicon) concentrators were used to increase protein concentration.

7.4 FACS analysis and FACSorting

Bone marrow cells were obtained by flushing out cells from the femur and tibia of both hind legs. Spleens were pressed through a 75 μm nylon mesh (John Stanier) to obtain a cell suspension. Red blood cells and dead cells were removed by spinning the cell suspension through an equal volume of Lympholyte (Cedarlane laboratories), then collecting live lymphocytes from the interface. Live cells were counted in trypan blue solution. 10^6 cells were stained in 50 μl FACS-buffer, containing the appropriate, pre-titered antibodies. To avoid non-specific binding of antibodies through Fc receptors, cells were pretreated with 24G2 (anti-FcR) antibody, before adding specific antibodies. Three-colour analysis was done on FacScan, four colour analysis on FacVantage and FacsCalibur, sorting on FacStar Plus and MoFlo(Cytomation) using Cellquest software (all Becton Dickinson, Mountain View, CA). Plots shown represent at least three independent experiments.

7.4.1 Intracellular 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 1 hour to overnight. Samples were washed twice in PBS, then autofluorescence from paraformaldehyde was quenched by incubation in 50 mM NH_4Cl . After another wash in PBS, cells were permeabilised using 1% NP-40 (in PBS) for 3 min, followed by a PBS wash, staining with the intracellular antibody and a final PBS wash.

7.5 Genotyping of Mice

7.5.1 FACS analysis

FACS analysis for genotyping purposes was done using peripheral blood collected with heparinized capillaries. 35 μ l FACS-buffer containing the appropriate antibodies was added to 15 μ l blood, incubated for 20 minutes on ice, then 1 ml ACK lysis buffer was added to lyse the red blood cells. After 2 minutes (hemolysis visible) cells were washed with FACS buffer before analysis on FACScan.

7.5.2 Southern Blot

2-5 mm tail-snips were taken from mice between 16-21 days of age using local anaesthetics. Tails were incubated overnight in 500 μ l tail-lysis buffer containing 0.1 mg/ml Proteinase K (Boehringer Mannheim) in a shaking waterbath at 55°C. Next day tubes were spun down for 20 min at 13,000 rpm and supernatant transferred to fresh tubes. 350 μ l isopropanol was added and mixed well. DNA becomes visible as a white precipitate. Tubes were spun down for 15 min at 13,000 rpm, supernatant removed, pellet washed once with 70% ethanol. Supernatant was removed as completely, as possible, then pellet was allowed to air-dry. 100 μ l TE was added to the DNA and placed in a 37°C incubator for a few hours to facilitate resuspension. 15 μ l of this DNA solution was cut with the appropriate restriction enzymes in a 40 μ l reaction, and incubated at 37°C overnight. Digests were separated on a 0.8% agarose

gel at low speed overnight. Gels were then denatured in Denaturing solution, and DNA transferred onto Hybond N⁺ nylon membrane (Amersham) via capillary transfer in 20xSSC. DNA was fixed on the membrane with 0.4M NaOH. Probes were labelled with ³²P-dCTP using Random Primed Oligonucleotide Labelling Kit (Boehringer Mannheim), then purified on NICK columns (Pharmacia). Hybridization was carried out at 65°C for 16 hours. Blots were washed in 2xSSC/0.5%SDS for 2x20 min, then in 0.1xSSC/0.5%SDS for 2x20 min. Blots were then placed into autoradiograph cassettes with Kodak Xomat AR films. Films were developed 2-7 days later. Alternatively, blots were placed into phosphoimager cassettes and scanned 1-3 days later on Storm 860 phosphoimager (Dynatech).

7.5.3 Earpunch-PCR

DNA was prepared from earpunches by adding 58.5 μ l dH₂O, 10 μ l 10x PCR buffer II (without MgCl₂), 1 μ l 10 mg/ml Proteinase K, 0.5 μ l Tween-20 and 20 μ l Chelex (Bio-Rad Instagene DNA Matrix) to each sample. After 30 min incubation at 55°C, 10 μ l 25mM MgCl₂ is added to each sample, then incubated for a further 2 hours at 55°C. In order to inactivate Proteinase K, samples are incubated for 15 min at 95°C, then spun down at 13,000 rpm for 10 sec. 10 μ l of the supernatant is used for 50 μ l PCR reactions. The 40 μ l PCR reaction mix contains 4 μ l 10xPCR buffer II (without MgCl₂), titrated amounts of 25mM MgCl₂, 0.8 μ l 25mM dNTP mix, 1 μ l (12.5 pmol) of each primer, 0.2 μ l Taq polymerase (Perkin-Ellmer) and water. PCR reactions are run on PE-6700 PCR machine with annealing temperatures and cycle numbers optimised for each primer set.

7.5.4 Primer sets used for genotyping by PCR

Syk	A	AGAGAAGCCCTGCCCATGGAC
	B	GTCCAGGTAGACCTCTTTGGGC
	C	GAGACTAGTGAGACGTGCT
Lyn	A	TGCTACTTCCATTTGTCACGTCC
	B	CAGGTGGAGCATACCTGGCTGTTT
	C	ACAGAGCTAGACCGTTCTTTCCCTC
RAG-1	A	GACCAGCTGCAAGCATCTATTCTG
	B	TTCCAGACTCACTTCCTCATTGCA
	C	ACCCGTGATATTGCTGAAGAGCTT
3-83	A	CAGCTTCCTGCTAATCAGTGCC
	B	TGGTCCCCCCTCCGAACGTG
Bcl-2	A	TTCCACAAGGCCATCCCAGCCT
	B	ACTTCGCCGAGATGTCCAGG
ZAP-70	A	GCA CAT ATG CAC TGT CCC TGG TCT A
	B	TGG CTA CCC GTG ATA TTG CTG AAG A
	C	GGG TCG CTG TAG GGA CTC TCG TAC A
HC-186	A	CCGGAATTCTCCTGCAAGGCTTCTGGC
	B	CCGGTACCGAGACTGTGAGAGTGGTGC

7.6 Generation of Radiation Chimaeras

Recipient mice are treated with 0.1 M HCl in their drinking water for 1 week prior to irradiation. On the day of the transfer, recipient mice receive 2x5 Gy total body irradiation, administered 3 hours apart to minimise GI tract damage. Donor fetal livers are washed in serum-free media three-times, and filtered through a 75 μ m nylon mesh before injecting on average 5 million cells (as determined by Casyton Cell Counter, Schärfe Systems, Reutlingen) in 0.2 ml volume iv. (tail-vein) into the irradiated recipients at any time up to 24 hours following the second irradiation. Mice

are treated with 0.16% Neomycin-sulfate (Sigma) in their drinking water for 4 weeks post-transfer. Mice are analyzed between 8-14 weeks after transfer.

7.7 Freezing Fetal Livers

Timed matings are set up, and vaginal plugs checked every morning. The day the plug is found is considered day 0.5. Embryos are sacrificed at day 16.5 of gestation, fetal liver removed, made into cell suspension in RPMI media, using 75 μ m nylon mesh or 70 μ m cell strainers (Falcon), spun down for 5 min at 1,400 rpm. Supernatant is removed, cell pellet is then resuspended in 900 μ l FCS and transferred into freezing vials. 100 μ l DMSO is added, mixed, then frozen in -70°C freezer.

For genotyping purposes, part of the embryonic brain is removed and DNA prepared.

7.8 Western Blotting

Cytoplasmic lysates are prepared by lysing cells in 1xCell lysis buffer (NEB) containing small peptide inhibitors (Sigma) and 1 mM Na₃VO₃. Lysis is allowed to proceed for at least 30 min on ice, which is followed by centrifugation for 30 min. at 13,000 rpm at 4°C to remove nuclei and debris. Supernatant is transferred into fresh tubes, and equal amount of 2xRSB (reducing sample buffer) is added, mixed well, then denatured for 5 min at 100°C.

Samples are loaded onto a 10% polyacrylamide gel, then transferred onto PVDF membrane by electroblotting in CAPS solution. Blots are blocked in 15% dry milk in PBS/Tween for at least four hours, then washed, incubated with the primary antibody (in PBS/Az/milk) for 1 hour, washed extensively, incubated with the secondary antibody for 1 hour and washed again. Signal is revealed by ECL (Amersham) using Kodak XB200 or X-OMAT UV film. Before reprobing, blots are stripped for 25 min at 50°C, washed extensively and blocked again.

7.9 Rearrangement analysis – Bulk fetal liver PCR

Day 16.5 fetal livers were harvested, red blood cells removed by differential lysis using ACK lysis buffer. Cell concentration was determined using Casyton cell counter. 10 million fetal liver cells were pelleted, then resuspended in 500 μ l tail lysis buffer containing 200 μ g/ml Proteinase K (Boehringer Mannheim). Digestion was carried out for at least 2 hours but not more than 24 hours at 55°C. 350 μ l isopropanol was added to precipitate the DNA. DNA was collected as a pellet after centrifugation at 13,000 rpm for 20 min. Pellet was washed once with 70% ethanol, then air-dried, resuspended in 500 μ l TE and left overnight at 37°C to allow for complete resuspension. DNA content was determined by spectrophotometer (OD_{260}) and sample volumes were adjusted to yield equal DNA concentration for each sample.

First round PCR reactions were set up using 1 μ l of 1:1, 1:5, 1:25 and 1:125 dilutions of the stock DNA samples, that corresponds to 20,000 - 4,000 – 800 – 160 cells. For the second round reaction 1 μ l of the first round PCR product was used.

The 50 μ l reaction mix for both rounds contained 1xPCR buffer, 3 mM MgCl₂, 0.4 mM of each of the four dNTPs, 12.5 pmol primers and 1U Taq polymerase (AB Systems).

Primers used [12, 48]:

VHJ558-1	ACCATGGGATGGAGCTGKATCWTBC
VHJ558-2	GTGARGCCTGGGRCTTCAGTGAAG
DHFLSP-1	ACAAGCTTCAAAGCACAATGCCTGGCT
DHFLSP-2	ACGTCGACTTTTGTSAAGGGATCTACTACTGT
JH4-1	AGGCTCTGAGATCCCTAGACAG
JH4-2	GGGTCTAGACTCTCAGCCGGCTCCCTCAGGG

PCR reactions:

First round:

Second round:

94°C 3'	94°C 3'
94°C 45" }	94°C 40" }
65°C 45" } 2x	67°C 45" } 3x
72°C 1'45" }	72°C 1'45" }
94°C 40" }	94°C 40" }
63°C 45" } 3x	64°C 45" } 27x
72°C 1'45" }	72°C 1'45" }
94°C 40" }	72°C 7'
60°C 45" } 20x	24°C ∞
72°C 1'45" }	
72°C 7'	
24°C ∞	

30 μ l of PCR products were separated on 2% agarose gels in 1x TAE, then transferred onto Hybond N+ (Amersham-Pharmacia) membranes as for Southern blots (see above). The probe for hybridization contains a JH3-JH4 fragment (a kind gift from K. Rajewsky).

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