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Analysis of B Lymphocyte Activation and Differentiation by Expression Profiling

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Abbreviations

Ab	antibody
APC	allophycocyanin
APS	ammonium persulfate
BASH	B cell adaptor containing SH2 domain
BCR	B cell antigen receptor
BLNK	B cell linker of activated B cell
bp	base pair
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
CalA	calgranulin A
CCR2	chemokine C-C motif receptor 2
cDNA	copy DNA
C-terminus	carboxy-terminus
Da	Dalton
DEPC	diethylpyrocyrbonate
DMSO	dimethylsulfoxid
dNTP	deoxynucleotide triphosphate
DTT	dithiotreitol
ECL	enhanced chemoluminescence
EDTA	ethylenediamine-tetraacetic acid
EGR1	early growth response 1
EtOH	ethanol
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein-isothiocyanat
HRPO	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
IL	interleukine
IRF	interferon regulatory factor
ITAM	immuno-tyrosine-based activation motif
kb	kilobase pair
kDa	kilodalton
LAT	linker for activated T cells
LPA	linear polyacrylamid
LPS	lipopolysaccharide
Ly-6	lymphocyte 6 antigen
Lyn	v-yes-1Yamaguchi sarcoma viral related oncogene homologue
M	molar
MAPK	mitogen-activated protein kinase
MAPKK	MAPK-kinase
MHC	major histocompatibility complex
mIg	membrane bound Ig
Na	sodium
NF-κB	nuclear factor of kappa light chain gene enhancer in B cells
N-terminus	amino-terminus
Oct1	octamer-binding transcription factor 1

ON	over night
PAGE	polyacrylamide-gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PKC	protein kinase C
PLCγ	phospholipase Cγ
PMSF	phenylmethylsulfonylfluoride
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PVDF	polyvinylidene fluoride
rpm	revolutions per min
RT	reverse transcription
SA	streptavidin
SDS	sodium dodecyl sulfate
SH	Src-homology
SLFN	schlafen
SLP-65	SH2 domain-containing leukocyte protain of 65 kDa
SLP-76	SH2 domain-containing leukocyte protain of 76 kDa
Src	sarcoma
Syk	spleen tyrosine kinase
TAE	Tris-acetate - EDTA
TAF10	TATA-binding box protein-associated factor, 30 kDa
TBE	Tris-borate - EDTA
TE	Tris-EDTA
TEMED	N,N,N',N' - tetramethylethylendiamine
Tris	Tris (hydroxymethyl) aminomethan
tRNA	transfer RNA
V_H	variable domain of the immunoglobulin heavy chain
V_L	variable domain of the immunoglobulin light chain

1 Introduction

In the mouse, B cells are generated throughout life by differentiation from hematopoietic stem cells (HSCs), in the liver before birth and in the bone marrow of adult animals. Differentiation process of HSCs can be viewed as a progression from very primitive progenitors with multiple lineage potentials (for example, B-cell, T-cell, NK-cell, myeloid and erythroid cells) through more restricted progenitors, such as the common lymphoid progenitor (CLP) (Kondo et al. 1997), finally to a B lineage restricted stage. Thereafter, B lineage cells execute a programmed development, rearranging immunoglobulin heavy chains (IgH) at the pro-B stage, then undergoing multiple rounds of clonal expansion at the pre-B stage and finally rearranging the light chain to yield newly formed surface Ig^+ B cells.

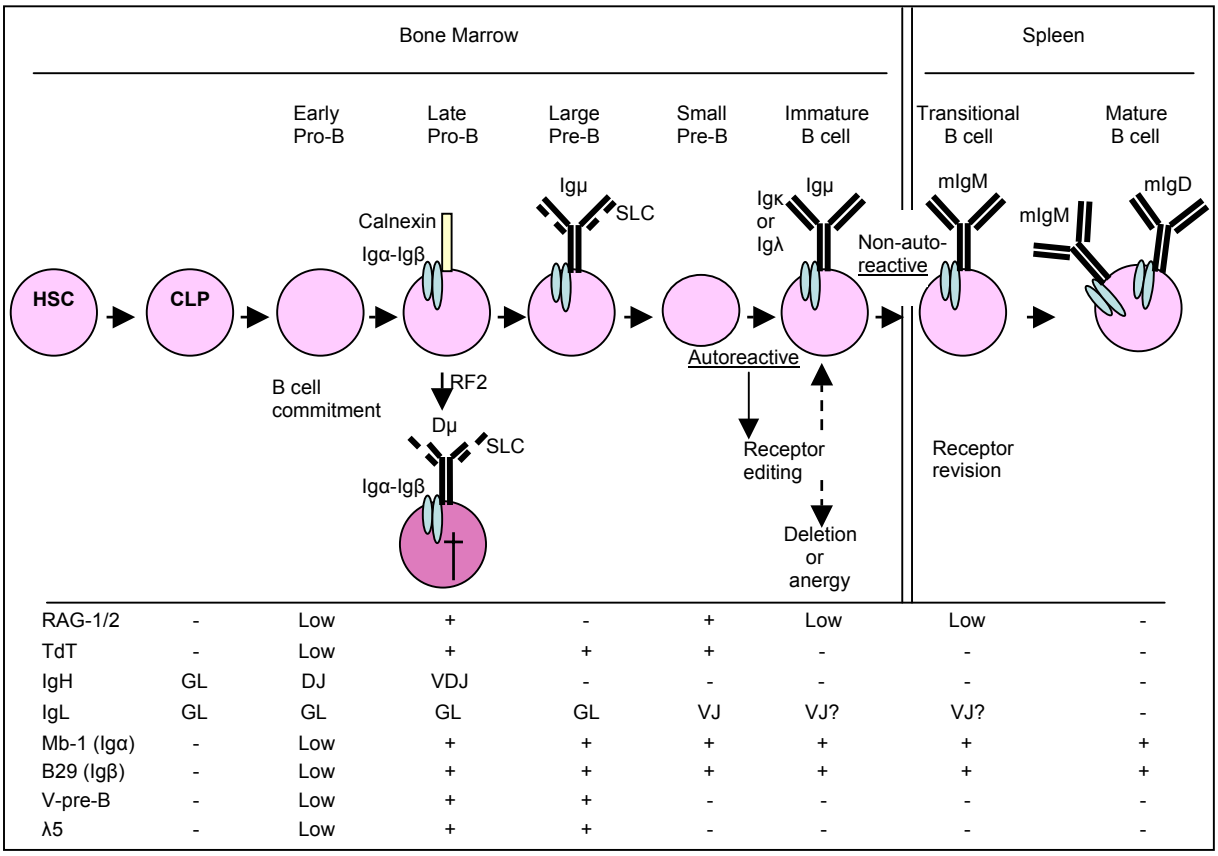
1.1 B cell development

1.1.1 Pro-B cells and early immunoglobulin gene rearrangements

The earliest B-lineage cells are known as pro-B cells, as they are progenitor cells. They are identified by the appearance of characteristic cell surface markers, such as B220, CD43 and c-kit (Osmond et al. 1998; Carsetti 2000). They express recombination activating genes (RAG-1 and RAG-2) (Schatz et al. 1989; Oettinger et al. 1990) and rearrangement of the immunoglobulin (Ig) heavy-chain (H-chain) locus takes place in pro-B cells. IgH-chain gene rearrangement proceeds in a precise order, recombination of diversity (D_H) gene segment to the joining (J_H) segment occurs at the early pro-B cell stage (Ehlich et al. 1994; ten Boekel et al. 1995) (Fig. 1). After DJ_H rearrangement, in late pro-B cells variable (V) gene segments of H-chain locus become accessible to the V(D)J recombinases, RAG-1 and RAG-2, and complete heavy chain transcription units are assembled (Tonegawa 1983; Alt et al. 1984). Pro-B cells express a precursor form of the BCR composed of Ig alpha (Igα), Igβ and calnexin (pro-BCR) (Hermanson et al. 1988; Hombach et al. 1988; Nagata et al. 1997) (Fig. 1).

Because of the random nucleotide loss and addition, D_H segments can be joined to J_H in any one of three reading frames, but there are a few mature B cells in the mouse that have D_H segments in reading frame 2 (RF2), a phenomenon referred to as RF2 counterselection (Meek 1990). DJ_H joins in RF2 encode a truncated form of membrane bound Igμ (mIgμ) – (Dμ) that associates with Igα-Igβ and surrogate light chains (SLCs, consisting of V-pre-B and λ5 proteins) (Melchers 1999) to produce a defective pre-BCR that inhibits V_H to DJ_H recombination and is unable to support further B cell differentiation (Reth and Alt 1984; Gu et al. 1991). B cells that express Dμ are therefore arrested at the pro-B cell stage where they are

either deleted or their truncated receptors are replaced by continuing recombination (Reth et al. 1986). Dμ signaling through Igα-Igβ is required for RF2 counterselection because in the absence of the transmembrane domain of mIgμ or Igβ there is no counterselection (Gu et al. 1991; Gong et al. 1996) (Fig. 1).



RAG-1/2	-	Low	+	-	+	Low	Low	-
TdT	-	Low	+	+	+	-	-	-
IgH	GL	DJ	VDJ	-	-	-	-	-
IgL	GL	GL	GL	GL	VJ	VJ?	VJ?	-
Mb-1 (Igα)	-	Low	+	+	+	+	+	+
B29 (Igβ)	-	Low	+	+	+	+	+	+
V-pre-B	-	Low	+	+	-	-	-	-
λ5	-	Low	+	+	-	-	-	-

Figure 1. B cell differentiation and maturation scheme. Developmental stages of B lymphopoiesis. RAG, TdT expression and rearrangements on both immunoglobulin heavy (IgH) and light (IgL) chain loci as well as expression of B cell specific proteins are shown. GL, locus is in germline configuration. Cell surface expression of pro-B (calnexin and Igα-Igβ), pre-B (Igμ, SLC (V-pre-B + λ5) and Igα-Igβ) or B (Igμ, Igκ or Igλ and Igα-Igβ) cell receptors is shown. (Figure adapted from Meffre et al., 2000/ Nature Immunology).

Expression of mIgμ results in the assembly of the pre-BCR and marks the transition to the pre-B cell stage. The pre-BCR is composed of mIgμ, SLC, Igα and Igβ (Pillai and Baltimore 1987; Tsubata and Reth 1990) and is a key checkpoint regulator in B cell development (Fig. 1). Its primary functions are to trigger B cell differentiation, clonal expansion and heavy chain allelic exclusion.

1.1.2 Pre-B cells and IgH chain allelic exclusion and selection

Burnet proposed in his clonal selection theory that lymphocytes carry unique cell surface receptors for antigen (Burnet 1959). This one-cell-one-antibody hypothesis was confirmed and phenomenon is referred to as allelic exclusion (Nossal and Lederberg 1958; Pernis et al. 1965). Two models were proposed to explain allelic exclusion: the "stochastic" model suggested that random joining of Ig genes would rarely produce two in-frame IgH-chains (Coleclough et al. 1981), whereas the "regulated" model proposed feedback control of recombination by a productively rearranged antibody (Alt et al. 1984). Early transgenic experiments with intact pre-recombined antibody genes were controversial in that exclusion was always incomplete and varied between different transgenic mouse strains (Rusconi and Kohler 1985; Weaver et al. 1985). Variations in these early results may have been due to the nature of the transgene or their site of integration. The regulated model was finally confirmed by experiments with a transgene expressing only the membrane bound form of Igμ, which showed that expression of mIgμ in pre-B cells inhibits further $V(D)J_H$ recombination (Nussenzweig et al. 1987; Kitamura et al. 1991).

In addition to allelic exclusion, expression of mIgμ activates a developmental program that includes clonal expansion and pre-B cell differentiation. Gene targeting experiments in mice and naturally occurring mutations in humans showed that deletion of components of the pre-BCR, including the transmembrane domains of Igμ or Igα-Igβ, blocks B cell development at the pro-B cell stage (Kitamura et al. 1991; Gong and Nussenzweig 1996; Yel et al. 1996; Minegishi et al. 1999; Schiff et al. 2000). Thus, mIgμ and Igα-Igβ are essential for pre-B cell development *in vivo*. In contrast, mutation of λ5, one of the components of SLC in pre-BCR, produces a leaky phenotype. $\lambda5^{-/-}$ mice show only a partial block in B cell differentiation and allelic exclusion (Kitamura et al. 1992; Rolink et al. 1993; ten Boekel et al. 1998). This leaky phenotype is probably explained by substitution of the SLCs in the pre-BCR by early rearrangements of conventional light chains (Ehlich et al. 1993; Ghia et al. 1996; Novobrantseva et al. 1999). These early rearranging light chains can combine with newly assembled mIgμ to induce allelic exclusion and pre-B cell development. In the absence of both λ5 and conventional light chains mIgμ fails to induce pre-B cell development (Papavasiliou et al. 1996; Pelanda et al. 1996). Whether pre-BCR cross-linking by an unknown ligand or simple surface expression of pre-BCR is sufficient to trigger allelic exclusion and pre-B cell development is still debated. However, it is likely that some form of receptor aggregation is required for signaling. Since, deletion of Syk, SLP65 (also called BLNK or BASH), or PI3K, which are nonredundant components of the BCR signaling

pathway impairs pro-B cell development and RF2 counterselection (Turner et al. 1995; Fruman et al. 1999; Jumaa et al. 1999; Minegishi et al. 1999). In pre-B cells, mIgμ signaling down-regulates the expression of components of the V(D)J recombinases RAG-1 and RAG2, and expression of mIgμ appears to make V_H genes less accessible to recombinases (Stanhope-Baker et al. 1996). Thus expression of mIgμ and the pre-BCR produces signals that regulate heavy chain allelic exclusion and induce pre-B cell development. Lymphocytes that fail to assemble a pre-BCR fail to progress in development and are deleted.

It has been proposed that the mechanism that regulates heavy chain selection in pre-B cells involves mIgμ pairing with SLCs. In this model, mIgμs that combine well with SLCs would be positively selected by inducing pre-B cell development and a burst proliferation in adult bone marrow, whereas there would be selection against mIgμs that pair poorly with SLCs (ten Boekel et al. 1998; Wasserman et al. 1998).

1.1.3 Light chain gene recombination and allelic exclusion

After clonal expansion of mIgμ producers, pre-B cells arrest in G_1 phase of cell cycle. RAG and Igκ germline transcripts are expressed and pre-B cells undergo light chain gene recombination (Reth et al. 1987; Schlissel and Baltimore 1989). Successful light chain (L-chain) gene rearrangement leads to BCR assembly and replacement of the SLCs in the pre-BCR by Igκ or Igλ. To maintain the one-cell-one-antibody rule, allelic exclusion would have to be imposed on L-chains as well as H-chains. However, L-chain allelic exclusion has never been measured precisely and in contrast to the H-chain, many of the experiments with transgenic mice showed only partial L-chain allelic exclusion (Ritchie et al. 1984; Rusconi and Kohler 1985). In addition, normal human B cells and myelomas that synthesize two L-chains have been described (Kwan et al. 1981; Giachino et al. 1995) and single cell sequencing experiments showed two or more in-frame L-chain genes in approximately 10% of all mature B cells (Yamagami et al. 1999). Nevertheless, some form of regulation is likely because the great majority of mouse and human B cell lines produce only a single antibody even when they can synthesize two L-chains (Kwan et al. 1981; Berinstein et al. 1989). One of the mechanisms likely to contribute to L-chain allelic exclusion is asymmetric demethylation of the Igκ alleles. Asymmetric demethylation is thought to render one of the two Igκ alleles preferentially accessible for VJ_K rearrangement (Mostoslavsky et al. 1998). The idea that undermethylation is required for recombination is further supported by experiments with V(D)J recombination substrates in cell lines (Hsieh and Lieber 1992; Cherry and Baltimore 1999). Later, it was proposed that an additional mechanism, which regulates

L-chain allelic exclusion, involves feedback control of B cell development by the BCR (Meffre et al. 2000). In this model a nonself-reactive BCR would produce a signal that induces transition from the pre-B cell stage to the immature B cell stage where expression of the RAG genes and V(D)J recombination are down-regulated (Monroe et al. 1999; Yu et al. 1999; Meffre et al. 2000). Cross-linking self-reactive BCRs, or lack of signaling due to failure to assemble a BCR, would be expected to trap developing B cells in a compartment where they would continue to undergo secondary recombination (Yamagami et al. 1999). This BCR regulated cellular model for L-chain allelic exclusion could explain the observation that some B cells make two L-chains only one of which pairs with the H-chain as well as phenomenon of receptor editing. The idea that BCR signaling regulates the checkpoint between pre-B cell and immature B cell stage is supported by gene targeting experiments where it has been shown that in the absence of cytoplasmic domain of Igα immature B cell development is inefficient (Torres et al. 1996).

1.1.4 Receptor editing

Early studies with B cell lines showed that Ig gene rearrangement could continue even after assembly of functional BCRs (Feddersen and Van Ness 1985; Kleinfield et al. 1986; Levy et al. 1989). In addition, DNA episomes excised from the genome by V(D)J recombination showed occasional in-frame VJ_κ genes that had been displaced by nested recombination between upstream V_κs and downstream J_κs (Yamagami et al. 1999). These secondary rearrangements can occur at both heavy and light chain genes, but the mechanism of replacement is different at the two antibody loci. Cryptic recombination signal sequences (RSSs) embedded in many heavy chain genes allow upstream V_H genes to recombine with the pre-existing V(D)Js to produce hybrid heavy V_H genes (Kleinfield et al. 1986; Reth et al. 1986). In contrast, secondary V_κ rearrangement entirely replaces pre-existing VJ_κ genes by recombination with downstream J_κs (Harada and Yamagishi 1991). The potential physiological significance of secondary recombination was first appreciated from studies on transgenic mice that carried antibodies to double-stranded DNA or MHC. In these mice self-reactive antibodies on B cells were replaced by secondary L-chain gene recombinations (Gay et al. 1993; Tiegs et al. 1993). In contrast to anergy or deletion, this new mechanism of tolerance, termed “receptor editing”, spares autoreactive B lymphocytes by replacing their receptors and is therefore an example of molecular selection.

Whether receptor editing is due to specific RAG reinduction or is simply the result of random recombination and selection is yet to be determined. Support for specific RAG reinduction

comes from experiments with bone marrow-derived immature transgenic B cells, where *in vitro* BCR cross-linking results in increased RAG expression and secondary L-chain rearrangements (Tiegs et al. 1993; Hertz and Nemazee 1997; Melamed and Nemazee 1997). However, antigens that mediate clonal deletion of transgenic receptors *in vivo* appear to arrest B cell development in a recombination competent B cell stage (Nemazee and Burki 1989; Hartley et al. 1993; Tiegs et al. 1993; Chen et al. 1995). Studies with gene-targeted mice identified the pre-B cell compartment as the site for gene replacement suggesting that editing is the result of developmental arrest followed by V(D)J recombination and selection (Pelanda et al. 1997). Thus receptor editing is important in contributing to shaping the antibody repertoire.

1.1.5 Immature B cells

Immature B cells are the first B lineage cells to express surface BCRs, they display surface IgM but little or no IgD. B cells remain in the immature compartment for an average of 3.5 days (Osmond 1993) and it is in this compartment that self-reactive B cells that fail to edit their receptors are deleted or anergized (Sandel and Monroe 1999). Immature B cells differ from mature B cells in that they are particularly susceptible to BCR-induced apoptosis (Sandel and Monroe 1999). These cells therefore conform to the idea that tolerance is established by deletion of self-reactive B cells during development (Meffre et al. 2000). That tolerance is an active process and signaled through the BCR was initially suggested by experiments in which mice were treated with anti-Igs from birth and found to be B cell depleted (Lawton et al. 1972). The assumption linking these experiments to tolerance was that antibodies to IgM would mimic autoantigens by cross-liking the BCR. This assumption was subsequently corroborated in transgenic mice (Nemazee and Burki 1989; Okamoto et al. 1992; Chen et al. 1995). B cells bearing self-reactive antibodies are effectively eliminated in large numbers (Nemazee and Burki 1989) and the degree of B cell elimination is dependent on the strength of receptor cross-linking (Okamoto et al. 1992).

Anergy is a state of nonresponsiveness to antigen. It is the second mechanism by which self-tolerance is induced in immature B cells. This phenomenon was originally observed in culture of developing B cells exposed to different concentrations of anti-μ antibodies (Pike et al. 1982). B cells cultured in high concentrations of anti-μ antibodies did not develop and underwent deletion. In contrast, intermediate levels of BCR cross-linking by lower concentrations of anti-μ allowed B cell development but abrogated normal B cell function, as determined by decreased proliferation and antibody production upon mitogen exposure (Pike

et al. 1982). This hyperresponsive or anergic state has been studied extensively in mice carrying antibodies reactive to lysozyme (Goodnow et al. 1988) or single stranded DNA (Erikson et al. 1991; Tsao et al. 1993). Anergic B cells are short lived and have difficulty developing from immature to the long-lived mature B cell compartment in the spleen (Fulcher and Basten 1994). Chronic exposure to antigen in anergic cells is also associated with decreased BCR expression, possibly due to a selective block in transport of the BCR from the endoplasmic reticulum (Bell and Goodnow 1994).

The BCR is an essential regulator of immature B cell development. Mutations in the cytoplasmic domains of Igα have dramatic effects on immature B cell differentiation (Torres et al. 1996). In the absence of the cytoplasmic domain of Igα, BCR with a single functional Igβ cytoplasmic domain produces few pre-B cells and immature B cells and most of these fail to progress to the mature B cell stage (Torres et al. 1996). Igα and Igβ regulate immature B cell development in part through Lyn (Hibbs et al. 1995), Btk (Hardy et al. 1983; Khan et al. 1995) and SLP65 (also called BLNK or BASH) (Hayashi et al. 2003; Hayashi et al. 2004) in conjunction with BCR modulating coreceptors such as CD45, CD19 and CD22 as the disruption of these genes interferes with receptor editing, establishment of anergy and immature B cell development (Kishihara et al. 1993; Engel et al. 1995; Sato et al. 1996).

1.1.6 Transitional B cells and primary repertoire selection

Immature B cells that emigrate from bone marrow to the periphery are referred to as transitional B cells. Transitional B cells can be distinguished from mature B cells by a series of cell surface markers (Carsetti et al. 1995; Rolink et al. 1998; Carsetti et al. 2004) and because they express low levels of RAG mRNA (Yu et al. 1999; Meffre et al. 2000) (Fig. 1). Transitional B cells are short-lived and only 10-30% of these cells enter the long-lived mature peripheral B cell compartment (Forster and Rajewsky 1990; Osmond 1991; Rolink et al. 1998). The size of transitional B cell compartment is variable, whereas the size of the mature B cell compartment appears to be stable (Forster and Rajewsky 1990; Meffre et al. 2000). It is thought that homeostasis is maintained by altering the number of transitional B cells that enter the mature B cell compartment, possibly by altering the thresholds for negative or positive selection (Gu et al. 1991; Sandel and Monroe 1999; Levine et al. 2000). BCR signaling is an important determinant in the transition of newly generated transitional B cells to the long-lived mature B cell compartment. Loss of BCR expression by conditional IgM ablation aborts all further development from the transitional to mature B cell stages (Lam et al. 1997). In addition, many of the mutations that affect BCR signaling pathways also interfere with

transitional B cell maturation (Loder et al. 1999; Martin and Kearney 2000). In the absence of functional Igα (Torres et al. 1996), Syk (Turner et al. 1997), Btk (Martin and Kearney 2000), SLP65 (Jumaa et al. 1999), Vav (Tarakhovsky et al. 1995), Lyn (Wang et al. 1996), CD45 (Meffre et al. 2000) or CD19 (Martin and Kearney 2000) molecules, BCR signaling is inefficient to induce transitional B cells to differentiate into mature B cells.

In addition to antigen-mediated cellular selection, receptor selection and editing may also occur in transitional B cells in the periphery and is referred to as receptor revision (Meffre et al. 1998) (Fig. 1). However, transitional B cells express low levels of RAG genes and receptor revision is therefore likely to have only limited role in shaping the normal peripheral B cell repertoire (Meffre et al. 2000).

1.2 Transcription factors and B cell development

Historically, B lymphocytes have been distinguished from other hematopoietic lineages by the rearrangement of the Ig genes that is necessary to produce functional receptor. However, other genes have been identified, such as CD19, that exhibit a B lymphoid-restricted expression pattern, indicating that an intricate program of transcriptional regulation must exist (Stamenkovic and Seed 1988). In order for differentiation to occur, hematopoietic progenitors must initiate the expression of B-lineage-restricted transcription factors and repress transcription of genes specific for alternative lineages. Additonally, the expression of genes required for continued differentiation, such as the interleukin-7 receptor (IL-7Rα), must be activated (Peschon et al. 1994). Gene targeting has revealed the importance of specific transcription factors in the earliest stages of B cell commitment and differentiation in vivo (Fig. 2). The Ets family member PU.1 is required for the development of both the lymphoid and myeloid lineages (Scott et al. 1994) by regulating the cytokine-dependent proliferation and differentiation of precursor cells (DeKoter et al. 1998).

Low levels of PU.1 expression specifically induces development of common lymphoid progenitors (CLP), whereas high PU.1 levels suppress B cell fate and promote instead common myeloid progenitor (CMP) differentiation (DeKoter and Singh 2000). At the molecular level, low PU.1 concentrations activate IL-7Rα gene in contrast to high PU.1 levels, which prevent IL-7Rα expression (DeKoter et al. 2002) (Fig. 2). Similarly, Ikaros has been shown to be important for the development of multiple cell types, including B and T lymphocytes (Georgopoulos et al. 1994).

The initiation of B-lymphopoiesis in the bone marrow critically depends on two transcription

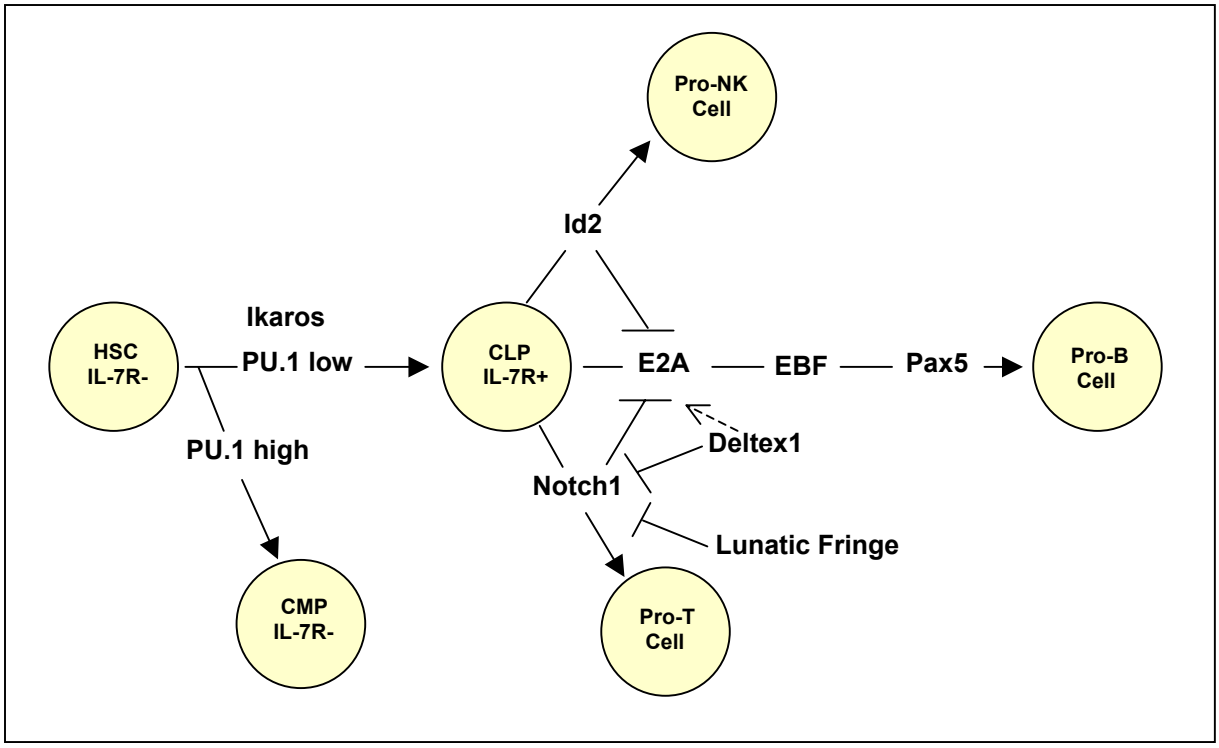


Figure 2. Transcriptional control of lineage commitment in early lymphopoiesis. Abbreviations: HSC, hematopoietic stem cells; CMP, common myeloid progenitors; CLP, common lymphoid progenitors; Pro-T cell, progenitor T cells; Pro-NK cell, progenitor natural killer cells and Pro-B cell, stands for progenitor B cells. (Figure adapted from Schebesta M. et al., 2002/ Current Opinion in Immunology).

factors: the basic helix-loop-helix (bHLH) protein E2A and the early B-cell factor (EBF). In the absence of either transcription factors, B cell development is arrested at the earliest stage, even before D_H-J_H rearrangement of the IgH-chain gene (Bain et al. 1994; Lin and Grosschedl 1995) (Fig. 2). Early T-cell development in the thymus critically depends on Notch1 signaling, which is activated by ligands of the Jagged or Delta families (Pui et al. 1999; Radtke et al. 1999). An important function of Notch1 in T-cell specification is the suppression of B-lymphopoiesis in the thymus (Wilson et al. 2001). Although employing different mechanisms, both Lunatic Fringe (Koch et al. 2001) and Deltex1 (Izon et al. 2002) inhibit Notch1 function and consequently promote B-lymphopoiesis at the expense of T-cell development in the thymus. At the molecular level, Notch1 signaling selectively interferes with the activity of E2A (Pui et al. 1999), which in turn is required for early B cell development to occur (Fig. 2).

The development of the third lymphoid lineage giving rise to NK cells critically depends on the transcription factor Id2 (Yokota et al. 1999). The HLH protein Id2 lacks the basic region required for the DNA-binding and thus functions as a negative regulator by sequestering the E2A protein into inactive heterodimers (Benezra et al. 1990) (Fig. 2). Id2 is specifically

expressed in NK cell progenitors, where it controls commitment to the NK cell lineage by suppressing other lympgoid options (Ikawa et al. 2001).

Another transcription factor, B-cell-specific activator protein (BSAP), encoded by Pax5 gene, appears to be required specifically in B lymphocyte differentiation (Urbanek et al. 1994) (Fig. 2). The activation of the B-cell-specific gene expression program and V(D)J recombination by E2A and EBF is not sufficient to commit B cell progenitors to the B lymphoid lineage in the absence of Pax5. B cell development is arrested at an early pro-B cell stage in the bone marrow of $Pax5^{-/-}$ mice (Urbanek et al. 1994). All of the above mentioned

Table 1. Transcription factors in early B lymphocyte differentiation.

Transcription Factor	DNA-binding motif	DNA-binding site	Target genes
PU.1	ets	5'GGA(A/T)	IL-7Rα, EBF, RAG-1, mb-1, B29, λ5, CD20, CD72
Ikaros	zinc finger	5'TGGGAA	RAG-1, TdT, (λ5, CD19, CD2, CD4, CD8α, CD45)
E2A	bHLH	5'CANNTG	EBF, Pax5, IL-7Rα, RAG-1/2, TdT, mb-1, λ5, V-pre-B
EBF	zinc coordination	5'GGGAAT	Pax5, RAG-1/2, B29, mb-1, blk, λ5, V-pre-B
BSAP (Pax5)	paired domain	5'GNGCANTGA AGCGTGAC	CD19, mb-1, λ5, V-pre-B, blk, N-myc, LEF-1, RAG-2, (PD-1, MPO, M-CSF-R)

Putative target genes of the transcription factors are listed. Targets of repression are shown in parenthesis. (Table is taken from O'Riordan and Grosschedl, 2000/Immunological Reviews).

transcription factors have functional binding sites within the promoters of B-cell-specific target genes, giving an indication of their contribution to B cell development (Table 1). These transcription factors function by transcriptionally activating or suppressing the transcription of appropriate genes in certain developmental stages.

1.3 Adaptor proteins in B cell development and activation

In order to exert their effector functions, lymphocytes need to be activated. This process requires at least two stimuli, a primary stimulus, which is mediated via an immunoreceptor (such as BCR, TCR or the Fc receptors (FcRs)), and a costimulatory signal, which is mediated

via so-called accessory receptors (for example, CD19 on B cells and CD28 on T cells). These two types of signals, as well many others that are generated by triggering of regulatory receptors on the lymphocyte surface, are transmitted to the intracellular compartment, where they initiate cascades of biochemical events that finally produce a cellular response. An intricate molecular network superintends these complicated processes. Many proteins with enzymatic activity, such as kinases, phosphatases and phospholipases are major components of this network. Additionally, a group of proteins that possess primarily modular protein-protein interaction domains is necessary for the proper transmission of the messages received at the plasma membrane. These proteins are called adaptor proteins.

By definition, adaptor proteins lack both enzymatic and transcriptional activities but control lymphocyte activation by mediating constitutive or inducible protein-protein or protein-lipid interactions via modular interaction domains. Such domains include Src homology 2 (SH2), Src homology 3 (SH3), phosphotyrosine-binding (PTB) and pleckstrin-homology (PH) domains as well as tyrosine-based signaling motifs (TBMSs) (Leo et al. 2002). TBMSs comprise short peptide sequences containing a core tyrosine residue, which upon phosphorylation mediates a high-affinity interaction with SH2 and PTB domains. The specificity of this interaction is determined by the amino acids flanking the tyrosine residues (Leo et al. 2002). Two groups of adaptor proteins can be defined: adaptor proteins that represent integral membrane proteins (transmembrane adaptor proteins, or TRAPs) and adaptor proteins that are preferentially located in the cytoplasm (cytosolic adaptor proteins, or CAPs) (Leo et al. 2002; Simeoni et al. 2004). All seven identified TRAPs: LAT (linker for activation of T cells), NTAL/LAB (non-T-cell activation linker; also known as linker for activation of B cells, LAB), TRIM (TCR-interacting molecule), PAG /CBP (protein associated with GEMs; also known as carboxy (C)-terminal SRC kinase (CSK)-binding protein, CBP), LIME (LCK-interacting membrane protein), SIT (SH2-domain-containing protein tyrosine phosphatase (SHP2)-interacting TRAP) and LAX (linker for activation of X cells, where X denotes an as yet unidentified cell) have short extracellular domains, which do not bind specific external ligands (Horejsi et al. 2004). In total, 18 CAP - adaptor proteins have been identified and characterized (Leo et al. 2002; Simeoni et al. 2004). In contrast to CAPs, most of which possess both modular binding domains and TBSMs, the cytoplasmic domains of TRAPs lack SH2 domains, SH3 domains and other modular protein-protein interaction domains, but contain multiple (up to 10) TBSMs (Leo et al. 2002; Horejsi et al. 2004). These sequences become phosphorylated by Src and/ or Syk protein tyrosine kinases (PTKs) after triggering of antigen receptors. After phosphorylation, TRAPs serve as anchors

for the SH2 domains of intracellular signaling and effector molecules (e.g., phospholipases, lipid kinases, protein tyrosine kinases, protein tyrosine phosphatases or CAPs), thus targeting the latter to the plasma membrane. This allows the formation and nucleation of membrane-associated signaling scaffolds, which are required for the propagation of receptor-mediated signals to the intracellular environment.

1.4 SLP65 and B cell development

1.4.1 SLP65 is a central adaptor protein in B cells

One of the CAPs, a 65 kDa protein was identified as one of the earliest substrates of PTKs upon BCR-stimulation in B cells (Wienands et al. 1998). By sequence comparison, this protein contains a C-terminal Src homology 2 (SH2) domain, which shares high similarity to SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), an adaptor protein and a PTK substrate in T lymphocytes, NK-cells (Jackman et al. 1995) as well as in pre-B cells (Su and Jumaa 2003). Due to the overall similarity (33% identical residues) to SLP76, the protein was named SLP65 for SH2 domain-containing leukocyte protein of 65 kDa (Wienands et al. 1998). Independently, SLP65 was purified from human B cells as Grb-2 binding phospho-protein and called BLNK for B cell linker of activated B cells (Fu et al. 1998). The cDNA for chicken SLP65 was cloned by another group and the protein was named BASH for B cell adaptor containing an SH2 domain (Goitsuka et al. 1998). SLP65 encodes a 456-amino acid polypeptide with basic, acidic, proline-rich and carboxy-terminal SH2 domains. The murine SLP65 protein shares 82% identity with human SLP65/BLNK. An alternatively splice variant of SLP65, which lacks basepairs 760 to 828 within the proline-rich domain, is only present in humans and it is called BLNK-S (Fu et al. 1998). SLP65 contains an N-terminal region with five tyrosines in YxxP context (Jumaa et al. 1999; Wollscheid et al. 1999). The transmembrane protein BNAS-2 (BASH N terminus-associated protein 2) has been characterized as an interaction partner with the N terminus of SLP65 (Imamura et al. 2004). Recent data demonstrate that highly conserved N terminus of SLP65 comprises a leucine zipper domain for constitutive membrane association which is essential for SLP65 function (Kohler et al. 2005).

The role of SLP65 in B cell maturation and function has been studied in mice deficient for SLP65 (Jumaa et al. 1999). SLP65-deficient mice are viable, but B cell development is affected at several stages. SLP65-deficient mice show increased proportions of pre-B cells in the bone marrow and immature B cells in peripheral lymphoid organs. B1 B cells are lacking in these mice. The mice show lower IgM and IgG_3 serum titers and poor IgM but normal IgG

immune responses. Mutant B cells show reduced Ca^{2+} mobilization and reduced proliferative responses to B cell mitogens (Jumaa et al. 1999). $SLP65^{-/-}$ mice develop spontaneous pre-B cell tumors and pre-B cell lymphomas, indicating that adaptor protein SLP65 acts as a tumor suppressor that limits pre-B cell expansion (Flemming et al. 2003).

1.4.2 SLP65 and pre-BCR signaling

1.4.2.1 Pre-BCR signaling pathways

Activation of the pre-BCR involves phosphorylation of the Igα and Igβ cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) (Fig. 3) and the formation of a

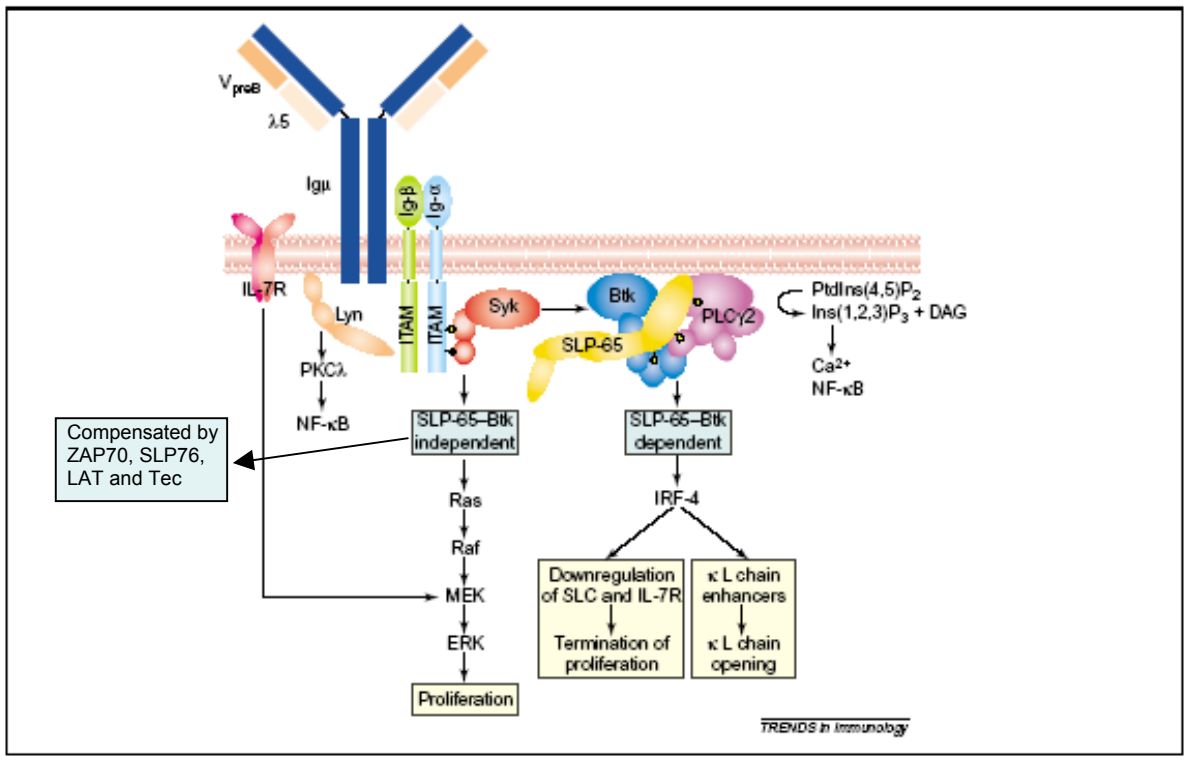


Figure 3. Pre-BCR signalling model. Activation of the pre-BCR involves phosphorylation of ITAMs in the Ig-α and Ig-β scaffold proteins, thereby recruiting and activating Syk tyrosine kinase. Proliferation is induced in a SLP-65–Btk-independent pathway involving Erk MAP kinase. A prominent substrate of Syk is the adaptor protein SLP-65. Phosphorylated SLP-65 provides binding sites for Btk (which is activated by Syk-mediated phosphorylation of Tyr551 in its kinase domain) and PLCγ2 SH2 domains. This SLP-65–Btk-dependent pathway is essential to signal termination of proliferation by downregulation of SLC and IL-7R expression and k L-chain opening. IRF-4 might regulate the large-to-small pre-B transition partly by downregulating SLC gene expression and partly by direct induction of enhancer-dependent k L-chain locus opening. In an alternative pathway, PLCγ2 can be activated by the homologous proteins ZAP-70, SLP-76, LAT and Tec. Abbreviations: BCR, B-cell receptor; Btk, Bruton's tyrosine kinase; DAG, diacylglycerol; IL-7R, interleukin 7 receptor; Ins(1,4,5)P3, inositol (1,4,5)-trisphosphate; IRF-4, interferon egulatory factor; ITAM, immunoreceptor tyrosine-based activation motif; L, light; MAP, mitogen-activated protein; PKCl, protein kinase C l; PLCγ2, phospholipase Cγ2; PtdIns(4,5)P2, phosphatidylinositol (4,5)-bisphosphate; SH2, Src-homology 2; SLC, surrogate light chain. (Figure adapted from (Hendriks and Middendorp 2004)/ TRENDS in Immunology).

lipid-raft-associated calcium signaling module. This complex contains the phosphorylated tyrosine kinases Lyn, Syk and Btk as well as other phosphorylated proteins including the SLP65 adaptor molecule, phosphoinositide 3-kinase (PI3K), Vav – guanine nucleotide exchange factor and phospholipase Cγ2 (PLCγ2) (Guo et al. 2000; Kouro et al. 2001). Syk is strongly activated by binding to the phosphorylated ITAM tyrosines of Igα and Igβ and acts as a positive allosteric enzyme, thereby initiating a positive feedback loop at the pre-BCR (Rolli et al. 2002). One important substrate of Syk is SLP65. Phosphorylation of SLP65 provides docking sites for Btk and PLCγ2 SH2 domains (Kurosaki 2002). Activated Btk then phosphorylates PLCγ2, which leads to its full activation. In this pathway, Btk can partially function as an adaptor molecule, independent of its kinase activity (Middendorp et al. 2003). Activation of PLCγ2 induces inositol (1,4,5)-triphosphate [Ins(1,4,5)P_3], diacylglycerol (DAG), calcium signaling and NF-kB activation (Fig. 3). However, NF-kB induction, which was recently shown to be independent on Src-like family PTKs and the atypical protein kinase Cλ (PKCλ) (Saijo et al. 2003), might not be essential for pre-BCR checkpoint function, as B cell development is unimpaired in mice deficient for various NF-kB sununits (Tretter et al., 2003). By contrast the downstream Ras-Raf-MEK-MAP kinase pathway (Fig.3) is a key regulatory signaling cascade, as its activation can induce proliferation and cellular maturation of pre-B cells, as well as Ig L-chain gene rearrangement (Iritani et al. 1999).

1.4.2.2 SLP65 and Btk limit proliferation of pre-B cells

Mice deficient for SLP65 or Btk show a partial block at the large, cycling pre-B cell stage, whereas an almost complete arrest is observed in SLP65-Btk double mutant mice (Jumaa et al. 1999; Jumaa et al. 2001; Kersseboom et al. 2003). Consistent with direct downregulation of SLC expression by pre-BCR signaling (Grawunder et al. 1995), pre-B cells that lack Btk or SLP65 manifest elevated levels of SLC in the cytoplasm and on the membrane

(Middendorp et al. 2002; Flemming et al. 2003). Therefore, it seems likely that pre-B cells lose their proliferative capacity because they have used up the previously synthesized SLCs for surface pre-BCR formation. Furthermore, the transition of large, cycling into small, resting pre-B cells is accompanied by downregulation of IL-7 receptor (IL-7R) expression. The finding that SLP65 is involved in the transcriptional repression of the gene encoding IL-7Rα upon pre-BCR signaling (Schebesta et al. 2002; Hayashi et al. 2003) indicates that IL-7Rα expression is controlled by the pre-BCR. It is therefore possible that, in addition to downregulating pre-BCR expression, Btk and SLP65 might partly prevent excessive cell

division of Igμ^{+} pre-B cells by regulating IL-7 responsiveness (Hendriks and Middendorp 2004).

Importantly, SLP65$^{-/-}$ mice spontaneously develop pre-B cell lymphomas expressing large amounts of pre-BCR on their cell surface (Flemming et al. 2003). It was hypothesized that, in SLP65$^{-/-}$ mice, large cycling pre-B cells are trapped in a positive-feedback loop as they are arrested in development and cannot efficiently downregulate pre-BCR or IL-7 expression, which signal proliferation (Flemming et al. 2003; Hayashi et al. 2003; Kersseboom et al. 2003). Although Btk-deficient mice do not develop pre-B cell tumors, it recently became clear that Btk cooperates with SLP65 as a tumor suppressor (Kersseboom et al. 2003). SLP65-Btk double mutant mice have a dramatically increased incidence of pre-B cell tumors, as compared with SLP65-deficient mice. Conversely, transgenic low level expression of a constitutively active form of Btk prevented tumor formation in SLP65-Btk double mutant mice, indicating that constitutively active Btk can substitute for SLP65 as tumor suppressor (Kersseboom et al. 2003).

As SLP65- or Btk-mutant pre-B cells manifest significantly increased proliferative expansion, it can be concluded that the pre-BCR mediated signaling pathway (that is the Ras-Raf-MEK-MAP kinase cascade), essential for proliferation, should be independent of SLP65 or Btk. In agreement with this, pre-BCR engagement induced Erk activation in the absence of SLP65 or Btk, and inhibition of Erk activation prevented survival of SLP65$^{-/-}$ pre-B cells (Kouro et al. 2001; Flemming et al. 2003). Therefore, two separate signaling pathways exist downstream of the pre-BCR: one proliferative pathway that is independent of SLP65 and Btk and results in Erk activation, and a second anti-proliferative pathway that involves SLP65 and Btk (Fig.3) (Hendriks and Middendorp 2004).

The finding of defective SLP65 expression in approximately 50% of childhood pre-B acute lymphoblastic leukemias (pre-B ALL) further demonstrated that SLP65 acts as a tumor suppressor in pre-B cells and indicates that loss of SLP65 and accompanying pre-B cell differentiation arrest might be one of the primary causes of pre-B ALL (Jumaa et al. 2003). The loss of SLP65 protein was caused by the incorporation of alternative exons into SLP65 transcripts, leading to premature stop codons. Likewise, in another study about 50% of childhood pre-B ALL cases manifested aberrant Btk transcripts predicted to encode Btk proteins with a substantial kinase domain deletion (Goodman et al. 2003). It is important to note that, in contrast to the mouse, deficiency of SLP65 or Btk in agammaglobulinaemia patients is not associated with increased pre-B cell expansion but rather with the lack of pre-B cell expansion (Minegishi et al. 1999; Nomura et al. 2000). This makes it difficult to envisage

how human pre-B ALL can originate from SLP65- or Btk-deficient B-cell precursors, which do not proliferate *in vivo*. Moreover, it can be concluded that the function of SLP65 and Btk in the regulation of pre-B cell proliferation in humans and mice are significantly different, which might be related to the different role of IL-7R signaling in two species (Hendriks and Middendorp 2004).

The incomplete block in SLP65-deficient mice prompted the investigation of alternative adaptor proteins that could partially replace SLP65 in pre-BCR signaling. SLP76 (a CAP adaptor protein) and LAT (a TRAP adaptor protein) which are essential in T-cell development, can complement the function of SLP65 in pre-B cells (Ishiai et al. 2000; Wong et al. 2000). Remarkably, SLP76 and LAT are also expressed in pre-B cells, where they are recruited to the pre-BCR, associate with Igα and become phosphorylated upon pre-BCR stimulation (Oya et al. 2003; Su and Jumaa 2003). Concomitant absence of LAT enhances the pre-B cell defects in SLP65-deficient mice, whereas reconstitution of LAT expression in SLP65-LAT double mutant pre-B cells restored their ability to mobilize calcium, downregulate pre-BCR expression and initiate Igκ L-chain expression (Su and Jumaa 2003). Likewise, the Btk homologue Tec has the ability to compensate for defective Btk function in pre-B cells *in vivo* (Ellmeier et al. 2000) and ZAP70 can compensate for Syk (Schweighoffer et al. 2003). Taken together, these data suggest that PLCγ2 can be activated in pre-B cells by two parallel pathways: one involving Syk, SLP65 and Btk; and another one independent of SLP65-Btk, involving ZAP70, SLP76, LAT and Tec (Fig. 3).

1.4.3 SLP65 and developmental progression of pre-B cells

Recently, gene expression profiling studies have indicated that a major change in the gene expression takes place at the transition from large cycling to small resting pre-B cells, as proliferation ceases and new differentiation programs are initiated (Hoffmann et al. 2002). A crucial role for SLP65 and Btk at this stage is inferred from the phenotype of SLP65- and Btk-deficient mice, which fail to modulate efficiently the expression of SLC, metallopeptidase BP-1, the adhesion molecule CD2, the IL-2R alpha chain (IL-2Rα/CD25), MHC class II and the membrane sialoglycoprotein CD43 (Middendorp et al. 2002; Hayashi et al. 2003; Kersseboom et al. 2003). Btk-deficient mice exhibit a specific developmental delay of about 3 hours within the small pre-B cell compartment and have reduced Igλ L-chain usage, implicating Btk in the regulation of the activation of L-chain loci for V(D)J recombination (Dingjan et al. 2001; Middendorp et al. 2002). In addition SLP65 reconstitution experiments in $Ig\mu^{+}$ $Pax5^{-/-}$ pre-B cells indicated that SLP65 is involved in the transcriptional repression of

RAG-1, RAG-2, terminal deoxynucleotidyl transferase (TdT) and IL-7Rα and in the post-translational downregulation of cell surface c-kit expression (Schebesta et al. 2002).
Interestingly, targeted inactivation of the transcription factors, interferon regulatory factor 4 (IRF-4) and IRF-8 is associated with a phenotype that is remarkably similar to that of SLP65-deficient mice: a developmental block at the pre-B cell stage, whereby IRF-4,8$^{-/-}$ pre-B cells are cycling and fail to downregulate the pre-BCR (Lu et al. 2003). As IRF-4,8$^{-/-}$ pre-B cells express normal levels of SLP65, it has been proposed that IRF-4,8 function as crucial downstream nuclear targets of SLP65 signaling. Consistent with this hypothesis, it was recently shown that, in Abelson-transformed pre-B cell lines, expression of IRF-4 and SpiB was sufficient to induce germline Igκ transcription (Muljo and Schlissel 2003). Importantly, the presence of the v-Abl kinase in these cell lines represses the expression of IRF-4, Spi-B and several pre-BCR signaling components including SLP65 (Muljo and Schlissel 2003). SLP65-deficient mice show defects in germline Igκ transcription and Igκ gene rearrangement in pre-B cells and Igκ transcription in immature B cells is also impaired (Xu and Lam 2002; Hayashi et al. 2003). The concomitant absence of CD19 appears to enhance the defects in transcriptional activation of the κ locus in SLP65-deficient mice. This might explain the virtually complete arrest of B cell development at the pre-B cell transition in SLP65-CD19 double-mutant mice (Hayashi et al. 2003).

1.4.4 SLP65 and BCR signaling

The BCR has two main roles. The first is to transmit signals that regulate B-cell fate decisions. The second is to mediate antigen processing, leading to the presentation of antigen to T cells, which allows full activation of B cells in the effector phase. Recent evidence indicates that these two roles are interrelated, but are under distinct genetic control (Niiro and Clark 2002). The BCR complex is made up of IgH and IgL chains associated with two signaling components Igα and Igβ. After BCR ligation by antigen both the PTK Syk and Src-family of PTK such as Lyn are activated initially. Then Lyn phosphorylates ITAMs in the cytoplasmic tails of Igα and Igβ, which in turn recruit and facilitate the activation of Syk, Btk and SLP65 (Niiro and Clark 2002). Syk is a key B cell signaling molecule, because disruption of Syk prevents most downstream BCR signaling (Takata et al. 1994; Jiang et al. 1998) and leads to a marked block in B cell development (Turner et al. 1995). It has been shown that SLP65 efficiently connects Syk and Btk with PLCγ2 (Fu et al. 1998; Hashimoto et al. 1999). Disruption of SLP65 gene leads to impaired activation of PLCγ2 in B cells (Ishiai et al. 1999; Jumaa et al. 1999). However, SLP65 also associates with Vav and Nck, other adaptor

proteins, both of which regulate cytoskeletal organization in B cells (Fu et al. 1998; Wienands et al. 1998). So, the defect in B cell development is more severe in SLP65-deficient mice than in PLCγ2-deficient mice (Pappu et al. 1999; Wang et al. 2000). Thus, SLP65 is a central adaptor protein that bridges pre-BCR and BCR-associated kinases with downstream signaling pathways and regulates biologic outcomes of B cell function and development.

1.5 Early growth response (EGR) family of transcription factors

The phenotype of a cell changes in response to variety of extracellular agents, such as growth factors, hormones, neurotransmitters, serum and other external stimuli. In these processes, genes whose induction occurs in the presence of protein synthesis inhibitors are referred to as immediate early genes (IEGs) (Herschman 1989). IEGs encode growth factors, growth factor receptors, cytoskeletal structures and transcription factors. The importance of the latter group is that they serve as nuclear signal transducers by initiating a cascade of gene-protein interactions. Moreover, these immediate early transcription factors are also induced during cellular growth, proliferation and differentiation. One family of these immediate early response genes which encodes nuclear proteins containing a leucine zipper DNA binding motif includes c-jun, jun-B, jun-D, c-fos, fos-B and fra-1 genes (Mitchell and Tjian 1989). Another gene family coding for zinc finger transcription factors includes four “early growth response” (EGR) genes, EGR1, EGR2, EGR3 and EGR4 (Sukhatme 1991). The individual cDNAs have been isolated from several sources and consequently different names are used for EGR1 (zif268, Krox24, tis8, NGFI-A and pAT 225) (Milbrandt 1987; Suggs et al. 1990; Wright et al. 1990), EGR2 (Krox20, pAT591) (Chavrier et al. 1988; Joseph et al. 1988), EGR3 (PILOT) (Patwardhan et al. 1991; Mages et al. 1993) and EGR4 (NGFI-C, pAT133) (Crosby et al. 1991; Muller et al. 1991). EGR genes are transiently and coordinately induced upon activation of peripheral T lymphocytes (Muller et al. 1991; Perez-Castillo et al. 1993). In addition EGR-genes are expressed in a wide range of cell types, including lymphoid and myeloid cells, such as thymocytes, B cells and monocytes, as well as non-lymphoid cells such as fibroblasts, kidney cells and neurons (Gashler and Sukhatme 1995; Beckmann and Wilce 1997). EGR transcripts are induced by a wide range of stimuli, such as activation and differentiation signals, tissue injury and apoptotic signals (Gashler and Sukhatme 1995; Beckmann and Wilce 1997).

The four EGR proteins have highly related DNA-binding zinc finger domains but their divergent flanking regions indicate specific functions of individual proteins (Decker et al. 2003). Binding of all four recombinant EGR proteins to the same consensus sequence GCG

G/TGG GCG is in agreement with the high conservation of their zinc finger domains (Decker et al. 2003). EGR-binding elements, indicative of EGR target genes, have been identified in a large panel of gene promoters, including immune receptor genes, such as the cytokine IL-2 (Skerka et al. 1995), the pro-inflammatory immune mediator TNFα (Kramer et al. 1994), and cell surface molecules such as the IL-2R beta chain (Lin and Leonard 1997), Fas/CD95 (Dinkel et al. 1997), ICAM-1 (Maltzman et al. 1996) as well as FasL and TRAIL (Li-Weber et al. 1999; Droin et al. 2003). In addition, EGR1 binding elements are reported within the promoter domains of genes encoding for transcription factors, such as EGR1, EGR4, junD and nur77 (Christy and Nathans 1989; Wang and Deuel 1992; Cao et al. 1993; Zipfel et al. 1997), growth factors such as insulin like growth factor II, basic fibroblast growth factor, epidermal growth factor receptor, platelet-derived growth factor (PDGF), tissue growth factor (TGF) (Hu and Levin 1994; Gashler and Sukhatme 1995; Biesiada et al. 1996; Cui et al. 1996; Khachigian et al. 1996; Liu et al. 2001), hormones, like the luteinizing hormone β (LH-β) (Tremblay and Drouin 1999), cell cycle regulators, including the retinoblastoma susceptibility gene Rb (Tremblay and Drouin 1999), $p21^{CIP1}$ (Ragione et al. 2003) as well as cyclin D1 and cyclin D2 (Philipp et al. 1994; Virolle et al. 2003).

A selective and specific role of individual EGR proteins in gene transcription is confirmed by the distinct phenotype of EGR knock-out animals: EGR1 knock-out mice show infertility of female animals, due to the lack of luteinizing hormone β (LH-β) gene expression (Lee et al. 1996), EGR2 knock-out mice have defective nerve development (Schneider-Maunoury et al. 1993), EGR3 knock-out mice lack formation of muscle spindles (Tourtellotte and Milbrandt 1998) and EGR4 knock-out mice show infertility of male animals due to the arrest in spermatogenesis (Tourtellotte et al. 1999).

Interestingly, a recent report indicated the role of EGR2 and EGR3 as negative regulators of T cell activation (Safford et al. 2005). T cell receptor (TCR) engagement in the absence of proper accessory signals leads to T cell anergy (Schwartz 2003). E3 ligases are involved in maintaining the anergic state (Jeon et al. 2004). Using microarray analysis EGR2 and EGR3 have been identified as being associated with the induction of anergy. Indeed, overexpression of EGR2 and EGR3 in T cells is associated with an increase in the E3 ubiquitin ligase Cbl-b and inhibition of T cell activation. Conversely, T cells from $EGR3^{-/-}$ mice reveal lower expression levels of Cbl-b and are resistant to in vivo inhibition of T cell activation (Safford et al. 2005). Together these data support the idea that EGR2 and EGR3 are involved in promoting a TCR-induced negative regulatory genetic program.

1.6 Early growth response 1 (EGR1)

EGR1 protein is a transcriptional regulator containing three zinc finger structural domains of the Cys_2-His_2 type (Cao et al. 1990) and through these zinc fingers EGR1 binds specifically to the major groove of DNA sequences which indicated above. Inducible EGR1 gene expression is mediated through different subgroups of MAPKs, including the extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 pathways (Silverman and Collins 1999). The EGR1 promoter contains several serum response elements (SREs) and Ets binding sites that mediate induction (McMahon and Monroe 1995; Watson et al. 1997). Serum response factor (SRF) and ternary complex factors (TCFs) form a ternary complex at these SRE and Ets sites. The TCF proteins are members of the Ets family, which include Elk1, Sap1a and Fli1 (Watson et al. 1997). These proteins are phosphorylated and activated by upstream MAPKs in a cell type- and stimulus-specific manner (Sharrocks 1995). Phosphorylation of Elk1 in its C-terminal domain induces conformational change that promotes binding of Elk1 to Ets sites via its N-terminal Ets domain and binding to SRF dimers via its B box domain and thus formation of an Elk1/SRF complex mediates induction of EGR1 gene expression (Clarkson et al. 1999).

EGR1-regulated genes are expressed in a tissue restricted manner. Thus specificity of EGR1 function may be due to interaction with other proteins, most likely transcription factors that bind to adjacent sites in a given promoter context, or by factors of the basic transcription network (Decker et al. 2003). Synergistic function of EGR1 with other nuclear factors has been reported, for example, with the homeobox protein Ptx1 and the steroidogenic factor 1 (Sf1) for regulation of the pituitary luteinizing hormone β (LH-β) (Lee et al. 1996; Tremblay and Drouin 1999), with RelA in the regulation of NF-kB1 (p50) transcription (Cogswell et al. 1997), with the cAMP response element-binding protein (CBP)/p300 in transcription of the lipoxygenase gene (Silverman et al. 1998), with NFATc and NFATp in IL-2 gene and TNFα gene expression (Skerka et al. 1995; Decker et al. 2003). In addition, functional interaction of EGR1 with Sp1 is reported for macrophage colony-stimulating factor (M-CSF) gene expression (Srivastava et al. 1998). In contrast complexes of EGR1 with NAB1 or NAB2 are described as transcriptional inhibitors (Russo et al. 1995; Svaren et al. 1996).

EGR1 is rapidly induced by growth factors to transduce the proliferative signal. The induction of EGR1 by external stimuli is generally transient but appears to be sustained in some prostate tumor cell lines and tumors (Abdulkadir et al. 2001; Virolle et al. 2003), suggesting that EGR1 stimulates tumor cell growth. These reports support the growth enhancer role of EGR1 observed in other cellular systems such as vascular smooth muscle and rat kidney tumor cells

(Scharnhorst et al. 2000; Fahmy and Khachigian 2002). In contradiction, in breast, lung and brain tumors, EGR1 expression is often absent or reduced and when re-expressed, results in growth suppression. Re-expression of EGR1 in tumor cells also leads to anti-apoptotic activity, which would encourage tumor cell survival (Huang et al. 1997; Calogero et al. 2001; Adamson and Mercola 2002). EGR1 is also required for, or stimulates, the differentiation of several cell types. Another contradiction is that after stress stimuli to some cell types, EGR1 is required for programmed cell death or apoptosis in both normal and tumor cells. EGR1 also plays a role in tumor progression, through the hypoxic signal generated in growing tumors. EGR1 is highly induced under these conditions and its activities stimulate angiogenesis and improved survival of tumor cells (Adamson and Mercola 2002).

1.7 EGR1 in lymphocyte development

The role EGR1 in T cell development has been studied both in EGR1 transgenic and EGR1-deficient mice. In transgenic mice overexpressing EGR1 in a RAG-gene deficient background, thymocytes bypassed the block at the $CD25^+$ $CD44^-$ DN stage and matured to the immature CD8 single-positive (ISP) cell stage, but not further to the CD4/CD8 double-positive (DP) cell stage. When these mice were irradiated, thymocytes did develop to the DP stage, suggesting transcriptional induction of additional genes by irradiation that are required to promote thymocyte development from ISP to DP stage (Miyazaki 1997). These results provided genetic evidence for two distinct steps during early thymocyte development from the $CD25^+$ $CD44^-$ DN to the DP stage and the first step, from $CD25^+$ $CD44^-$ DN to the ISP stage can be entirely promoted by overexpression of EGR1. Later, studies on EGR1xTCR double transgenic mice suggested that expression of EGR1 and/or its target genes may directly influence the thresholds required for thymocyte selection.

However, in EGR1-deficient mice, the DN to DP transition appeared normal, suggesting that EGR1 is not essential for this developmental transition (Bettini et al. 2002). The possible explanation of these two contradicting data might be that the phenotype observed after overexpression of EGR1 does not occur with normal levels of EGR1, or that in the EGR1-deficient mice there is compensation by other EGR family members or other unkown factors.

Nevertheless, it is clear that EGR1 does have some role to play in early thymocyte development, since the thymuses in EGR1-deficient mice contain more cells than their littermate controls (Bettini et al. 2002). It has been concluded that induction of EGR1 leads to increased levels of Id3 and Bcl2, resulting in efficient differentiation and survival of thymocytes and EGR1 plays a role in limiting the number of T cell precursors.

In mature B lymphocytes, EGR1 is one of the many immediate early genes induced upon BCR engagement. However, its role during early stages of B lymphopoiesis remained unclear. By analyzing EGR1 expression in bone marrow-derived B lymphocyte subsets and by testing EGR1 expression in cultivated, fetal liver-derived pre-B cells, it has been shown that EGR1 is also expressed in pre-B cells lacking surface IgM (sIgM) as well as in immature $sIgM^+$ B cells in the absence of sIgM-induced signals (Dinkel et al. 1998). These observations suggested that EGR1 might also have a regulatory function in pre-B cell development. By studying transgenic mice overexpressing EGR1 from the pre-B stage on, higher proportions of mature B cells and fewer immature B cells were found in transgenic animals compared to the control littermates (Dinkel et al. 1998). To identify if early stages of B lymphopoiesis are sensitive to EGR1 activity, in EGR1 trangenic mice B cell development was arrested at the stage of early pro-B cells by backcrossing EGR1 transgenic mice with RAG2-deficient mice. Since the null muatations in the RAG2 gene prevents rearrangement of Ig genes, B cells are arrested in these mice at the early stages of development. Phenotypic comparison of bone marrow cells from transgenic and control mice revealed that the population of $BP1^+$ pre-B cells were three to four fold increased in EGR1 transgenic mice (Dinkel et al. 1998). These data provided evidence that EGR1 supports at least two distinct steps of B cell maturation, the progression into the pre-B and into the mature B cell stage (Dinkel et al. 1998). Thus, EGR1 plays an important role in differentiation and maturation of B lymphocytes.

Specific aims

Background

SLP65 is a central adaptor protein that bridges pre-BCR and BCR-associated kinases with downstream signaling pathways and regulates biologic outcomes of B cell function and development. SLP65-deficient mice reveal severe, but not complete, blocks in B cell development at the pre-B and immature B cell stages (Jumaa et al. 1999; Pappu et al. 1999; Xu et al. 2000). SLP65$^{-/-}$ mice develop spontaneous pre-B cell tumors and pre-B cell acute lymphoblastic lymphomas (Flemming et al. 2003). The transcription factor EGR1 supports distinct stages of B cell maturation: the progression into the pre-B and the mature B cell stages (Dinkel et al. 1998).

Hypothesis

The main hypothesis of this study is that EGR1 expression is regulated through a SLP65-dependent signaling chain and therefore, EGR1 overexpression in SLP65$^{-/-}$ mice could restore or at least partially rescue developmental blocks caused by the absence of SLP65 in B cells.

Objectives

In order to test this possibility and to further study the role of EGR1 and its target genes in B cells, the following approach was exploited:

1. Phenotypic comparison by FACS to analyze the effects of EGR1 transgene activity on SLP65$^{-/-}$ B lineage cells from bone marrow and peripheral lymphoid organs.
2. Gene expression profiling experiments for B cells in certain developmental stages from SLP65$^{-/-}$ and EGR1 transgenic x SLP65$^{-/-}$ (EGRxSLP65$^{-/-}$) mice.
3. Verification of the microarray results for potential candidate EGR1 target genes by the means of independent methods.

Additionally, we have compared pre-B ALL cases between the SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice in order to study the role of EGR1 in pre-B ALL formation and tumor progression.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were purchased at Sigma-Aldrich, Merck AG, Fluka, Roche and Roth unless otherwise specified.

Agarose, Electrophoresis grade	Life Technologies, Eggenstein
β-Mercaptoethanol	Koch-Light Ltd, Suffolk, GB
EDTA	Serva Feinbiochemika GmbH, Heidelberg
Fetal Calf Serum (FCS)	Linaris, Wertheim-Bettingen
Iscove's medium	Gibco BRL, Neu Isenburg
Tris	USB, Braunschweig
Triton X100	Gibco BRL, Neu Isenburg

2.1.2 Reagents and Kits

BCA Protein Reagent	Pierce, Out Beijerland, the Netherlands
Complete® proteinase-inhibitor mix	Böhringer, Ingelheim
Gel Blotting Paper	Schleicher&Schuell, Dassel
Hyperfilm ECL	Amersham Biosciences Europe GmbH, Freiburg
Immobilon P Transfer Membrane	Millipore GmbH, Eschborn
Super Signal® West Pico (ECL)	Pierce, Out Beijerland, the Netherlands
Super Signal® West Femto (ECL)	Pierce, Out Beijerland, the Netherlands
Anti-mouse CD19 MACS Micro Beads	Mylteni Biotec GmbH, Gladbach
Anti-mouse B220 MACS Micro Beads	Mylteni Biotec GmbH, Gladbach

2.1.3 Solutions and Buffers

2.1.3.1 Common Buffers

PBS (pH 7.4)	4mM KH_2PO_4, 16mM Na_2HPO_4, 115 mM NaCl
PBS/Tween-20	PBS + 0.1% Tween-20
TAE	40 mM Tris, 20mM acetic acid, 1mM EDTA
TBE	50 mM Tris, 50 mM borate, 0.5 mM EDTA
TE	10 mM Tris/HCl pH 8.0, 1 mM EDTA

2.1.3.2 Flow cytometric analysis (FACS)

FACS buffer (standard)	PBS, 3% FCS, 0.1% NaN_3
PI solution	0.1 mM propidium iodide in PBS

2.1.3.3 Immunoblotting

NP40-lysis buffer	1% NP40, 150 mM NaCl, 50 mM Tris pH 8.8, 2 mg/ml Complete® proteinase-inhibitor mix
Sample buffer (1 X)	20% v/v glycerine, 62.5 mM Tris/HCl pH 6.8, 2% SDS,10 mg/ml bromophenol blue, 0.7% v/v β-mercaptoethanol
Running buffer	25 mM Tris, 190 mM glycine, 0.1 mM SDS
Transfer buffer	25 mM Tris, 190 mM glycine, 20% methanol, 0.01% SDS

2.1.4 Markers and standards

1 kb DNA ladder	Life Technologies, Eggenstein
Benchmark (Protein) ladder	GIBCO BRL, Neu Isenburg

2.1.5 Eukaryotic cell lines

OP9 cells	Mouse bone marrow stromal cell line
NIH 3T3 cells	Mouse embryonic fibroblast cells
697* cells	Human pre-B ALL cell line
BV173* cells	Human pre-B ALL cell line
NALM-6 cells	Human pre-B ALL cell line
SEM* cells	Human ALL cell line
L428* cells	Hodgkin's lymphoma cell line
JUM2* cells	Human mantle cell lymphoma cell line

* - these cell lines were generously provided by Dr. Hassan Jumaa (Max-Planck Institute for Immunobiology, Freiburg).

2.1.6 Cell culture medium

Fetal Calf Serum (FCS)	inactivated at 56° C for 1 hour
Iscoves‘ modified DMEM (IMDM)	IMDM - Powder medium (Invitrogen) 36 mM $NaHCO_3$ 1% (v/v) Pen-Strep (5000 U/ml PenicillinG-Sodium, 5000 U/ml Streptomycin-Sulfate in 0,85% Saline, Invitrogen),10 µM β-Mercapto-ethanol (Invitrogen)
Trypsin	Invitrogen
Cell culture flasks	Nunc, Greiner
6-, 12- and 24-well cell culture plates	Nunc, Greiner

2.1.7 Antibodies

2.1.7.1 Anti-Mouse Antibodies for Flow Cytometry

Specificity	Labeling	Clone	Catalogue number, Company	Dilution
CD117/c-kit-R	FITC		01904D, PharMingen	1:40
CD127/IL-7Rα	Biotin	B12-1	555288, BD PharMingen	1:25
CD19	PE		09655B, PharMingen	1:640
CD21/CR2	FITC		09784D, PharMingen	1:100
CD23/FcεRII	Biotin	B3B4	553137, BD PharMingen	1:40
CD23/FcεRII	FITC	B3B4	553138, BD PharMingen	1:100
CD23/FcεR	PE	B3B4	01235B, PharMingen	1:320
CD24/HAS	FITC	M1-69	PharMingen	1:100
CD25/IL-2R	Biotin	7D4	553070, BD PharMingen	1:200
CD43	Biotin		01602D, PharMingen	1:40
CD45R/B220	PE	RA3-6B2	553090, BD PharMingen	1:250
CD45R/B220	Biotin	RA3-6B2	553086, BD PharMingen	1:250
CD45R/B220	FITC	RA3-6B2	01124D, BD PharMingen	1:320
CD45R/B220	PE-Cy7	RA3-6B2	552772, BD PharMingen	1:80
CD45R/B220	APC	RA3-6B2	553092, BD PharMingen	1:80

Specificity	Labeling	Clone	Catalogue number, Company	Dilution
CD72	FITC	K10.6	550966, BD	1:160
CD74	PE	ln-1	sc-19627, Santa Cruz Biotech	
CD100	Biotin		Provided by Dr. A.Kumanogoh	1:160
IgM	FITC	II/41	553457, BD PharMingen	1:30
IgM	Biotin		1020-08, Southern Biotech	1:640
IgM	PE	R6-60.2	553409, BD PharMingen	1:100
IgD	Biotin	11-26	1120-08, Southern Biotech	1:640
IgD	FITC	SBA1	1120-02, Southern Biotech	1:640
Igκ	FITC		1050-02, Southern Biotech	1:80
Ly-6A/E (Sca-1)	FITC	D7	557405, BD PharMingen	1:160
Ly-6D/ThB	Biotin		01182D, PharMingen	1:320
Ly-6E/Sca-2	FITC	MTS35	557367, BD PharMingen	1:80
pB493	FITC		Provided by Dr. A.Rolink	1:40
pB493	PE		Provided by Dr. A.Rolink	1:20
pB493	Biotin		550434, BD PharMingen	1:40
pre-BCR	Biotin	SL156	551863, BD PharMingen	1:20
TACI	Biotin	166010	MAB1041, R&D Systems	1:20

Biotin-conjugated antibodies were detected by incubation with the following Streptavidin-conjugated fluorochromes: SA-FITC (Dako, 1:100), SA-PE (BD Pharmingen, 1:200) and SA-APC (Pharmingen, 1:400) at the indicated dilutions.

2.1.7.2 Antibodies for Western Blotting

Specificity	Clone(s)	Species	Catalogue number, Company	Dilution
Akt2	D-17	Goat	sc-7127, Santa Cruz Biotech	1:100
β-actin	AC-15	Mouse	A1978, Sigma-Aldrich	1:16000
EGR1	C-19	Rabbit	sc-189, Santa Cruz Biotech	1:500

Specificity	Clone(s)	Species	Catalogue number, Company	Dilution
Igκ-HRP		Goat	1050-05, Southern Biotech	1:10000
IgM-HRP		Goat	1020-05, Southern Biotech	1:8000
IκB-α	C-21	Rabbit	sc-371, Santa Cruz Biotech	1:200
IRF1	M-20	Rabbit	sc-640, Santa Cruz Biotech	1:200
IRF1	20	Mouse	I33220-050, BD Transduction	1:500
GAPDH		Mouse	MAB374, Chemicon Int.	1:4000
LEF1		Mouse	Provided by Dr. R.Grosschedl	1:2000
Notch1	H-131	Rabbit	sc-9170, Santa Cruz Biotech	1:200
Oct2	C-20	Rabbit	sc-233, Santa Cruz Biotech	1:200
p21	SX118	Mouse	556430, BD PharMingen	1:500
p21	F-5	Mouse	sc-6246, Santa Cruz Biotech	1:100
relB	C-19	Rabbit	sc-226, Santa Cruz Biotech	1:200
TAF10	1H8, 2B11, 2F4, 4G2	Mouse	Provided by Dr. L.Tora	1:1000 (each)
Goat IgG-HRP		Rabbit	305-035-047, Jackson Immunores.	1:20000
Mouse IgG-HRP		Goat	1858413, Pierce	1:5000
Rabbit IgG-HRP		Goat	111-035-047, Jackson Immunores.	1:10000

2.1.8 Primer sequences

2.1.8.1 Primers for genotyping of EGRxSLP65$^{-/-}$ mice

Name of the Primer	Sequence of the Primer
EGR 10-Forward	5'-CTT CCC CAA ATA GCC TTG CCA CAT GAC-3'
EGR 10-Reverse	5'-GAG ACA TCA ATT GCA TCT CGG CCT TGG-3'

2.1.8.2 Primers for semiquantitative RT-PCR

Name of the Primer	Sequence of the Primer
RAG2-Forward	5'-CAC ATC CAC AAG CAG GAA GTA CAC-3'
RAG2-Reverse	5'-TCC CTC GAC TAT ACA CCA CGT CAA-3'

Name of the Primer	Sequence of the Primer
IRF4-Forward	5’-CAG AGG GAG CCA AAA AAG-3’
IRF4-Reverse	5’-TCA CGA GGA TGT CCC GGT AA-3’
SpiB-Forward	5’-CGT CAA GCC CTT CAG TTA C-3’
SpiB-Reverse	5’-TAG GAG CAA CCC CAG CAA GA-3’
mCD19-Forward	5’-GTG GGT TTG GGG GTC TCT TCT GCT-3’
mCD19-Reverse	5’-CCC TCC TCG CTG TCT GGC TCT TC-3’

RAG2, IRF4 and SpiB primers were kindly provided by Dr. Hassan Jumaa (Max-Planck Institute for Immunobiology, Freiburg).

2.1.8.3 Primers for Real-time RT-PCR

Name of the Primer	Sequence of the Primer
mRPL5-Forward	5’-GCT GCT ATC CGA GAG AAT CCA G-3’
mRPL5-Reverse	5’-TTC AGC AGC CCT TTC CTG AG-3’
mTACI-Forward	5’-GGG ATC TGA GCA TGG TCC AG-3’
mTACI-Reverse	5’-AGA GTT TGC TTG TGA CCC ACG-3’
mSLFN1-Forward	5’-TAC TTC CTC TGG CCC GCA-3’
mSLFN1-Reverse	5’-CGG TGA TGT TCA TTT TCC AGC-3’
mSLFN2-Forward	5’-CTC CAG ATG AAT GCC CCA TC-3’
mSLFN2-Reverse	5’-TGC TCT TAG CCA CTG AGC CAT-3’
mCCR2-Forward	5’-CCA TGC AAG TTC AGC TGC C-3’
mCCR2-Reverse	5’-TGT TGA TAG TAT GCC TGT GAT GA-3’
mCalA-Forward	5’-AAT CAC CAT GCC CTC TAC AAG AA-3’
mCalA-Reverse	5’-CAC CAT CGC AAG GAA CTC CT-3’
mBlr1-Forward	5’-TCG GCT TCT GAC CAA GCT G-3’
mBlr1-Reverse	5’-TCC GCC ACC ACT TTA TCC TC-3’
mBcl3-Forward	5’-CGA GAG CAG CAG TCG TCT CA-3’
mBcl3-Reverse	5’-GAA GGG CAG GAA GGC AGG-3’
mOct1-Forward	5’-GCA ACA GTA CAC TGG CAA CGA T-3’
mOct1-Reverse	5’-AGG TTC TGA GGG TTC AGG AAC A-3’
mCyclin D1-Forward	5’-TGG TGG CTG CGA TGC A-3’
mCyclin D1-Reverse	5’-GAA GGG CTT CAA TCT GTT CCT G-3’
mCyclin D2-Forward	5’-CTC ACG TGT GAT GCC CTG AC-3’

Name of the Primer	Sequence of the Primer
mCyclin D2-Reverse	5'-CAG ACT TGG ATC CGG CGT-3'
mCyclin D3-Forward	5'-CTA TGT CTG CGG ATG AGC TCA C-3'
mCyclin D3-Reverse	5'-GCT TTG GGC ACT GGG CT-3'
mCyclin E1-Forward	5'-AGT TCC AAG CTC AAG CAC TTC C-3'
mCyclin E1-Reverse	5'-CCA CAC TCG GAG GAG GAG AA-3'
mCyclin E2-Forward	5'-TGT TGT AAA AAG TGT GAG TCC AGT GA-3'
mCyclin E2-Reverse	5'-TGA CAG CTG CCC TCC TTT TC-3'

2.1.9 Mice strains

The detailed descriptions of the generation of SLP-65$^{-/-}$ (Jumaa et al., 1999) and EGR1-transgenic mice (Dinkel et al., 1999) have been published. To generate EGR1 transgenic x SLP-65$^{-/-}$ (EGRxSLP-65$^{-/-}$) mice, EGR1-transgenic and SLP65-deficient mice were interbred. Animals were genotyped by PCR for the EGR1 transgene using genomic DNA and a transgene-specific "EGR 10" primer pair. Both transgenic and mutant mice as well as BALB/c (WT) littermates were bred and kept under specific pathogen-free conditions at the animal facilities of the Max-Planck Institute for Immunobiology, Freiburg. Animal experiments were done in compliance with the guidelines of German Law and the University of Freiburg.

2.2 Methods

2.2.1 Extraction of genomic DNA for genotyping of mice

Mouse tails were treated in 300 µl lysis buffer (200 mM NaCl, 100 mM Tris HCl, pH 8.5, 5 mM EDTA, 0.2% SDS) containing 100 µg/ml proteinase K for 7 hours to overnight (ON) at 56°C with agitation. The samples were centrifuged at 14 000 rpm for 5 min at room temperature. The supernatant was transferred into a new Eppendorf tube containing 300 µl of Isopropanol and swirled until a precipitate was visible. DNA was recovered by lifting the aggregated precipitate from the solution using disposable tips. Excess liquid was dabbed off and the genomic DNA was dispersed in a new Eppendorf tube containing 100 µl of TE buffer, pH 8.0. After complete dissolution of DNA (a few hours to overnight) at room temperature, the samples were used as template for PCR assays.

2.2.2 Polymerase Chain Reaction (PCR)

DNA was PCR amplified using *Taq*-DNA Polymerase (Invitrogen). PCR reaction was performed in 20 µl volume containing 1 µl of genomic DNA extracts or 200 ng of cDNA, 0.5 µM of each of forward and reverse primers, 250 µM of dNTP mixture (Eppendorf), PCR buffer provided together with *Taq*-DNA Polymerase (Invitrogen), 2.5 mM $MgCl_2$ and 1 unit of the enzyme per reaction. During the PCR reaction the following standard protocol was applied: denaturing of DNA at 94°C for 3 min, followed by 30 cycles of DNA amplification of 94°C for 45 seconds (sec), annealing of primers for 45 sec and elongation step at 72°C for 1 min. To complete the synthesis of DNA fragments additional elongation step at 72°C for 10 min. The annealing temperature for EGR1 specific "EGR 10" primer pair was 62°C.

2.2.3 Total RNA extraction

Total RNA was extracted either from freshly isolated and purified $B220^+$ splenic B cells or *in vitro*-cultured pre-B cells using TRIzol Reagent (GIBCO). Cells were pelleted by centrifugation at 1200 rpm for 7 min, the supernatant was carefully removed and TRIzol reagent (1 ml per 1 x 10^7 cells) was added to lyse the cells by repetitive pipetting. Samples were left for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. 200 µl of chloroform ($CHCl_3$) per 1 ml of TRIzol was added to samples and the tubes were vigorously shaked by hand for 15-20 seconds and incubated at RT for 5 min. Phase separation was performed by centrifugation at 12000 x g for 15 min at 4°C. The aqueous phase was carefully removed into a new Eppendorf (RNase free) tube and 1 volume of Isopropanol was added to precipitate RNA. Total RNA was precipitated in the presence of 10 µg LPA (linear polyacrylamide, Ambion) and 50 ng of Yeast tRNA (Invitrogen) at -20°C overnight. Total RNA was pelleted by centrifugation at 14000 rpm for 30 min at 4°C and washed two times with 70% Ethanol with the same centrifugation conditions for 15 min. At the final step RNA was purified using QIA RNeasy Cleanup Kit according to manufacturer's instructions and redissolved in DEPC-treated water. The integrity and purity of total RNA was assessed running an aliquot on an agarose gel stained with SYBR GOLD (Molecular Probes). Total RNA samples were stored at -20°C until ready to use.

2.2.4 Reverse Transcription Reaction (RT)

Reverse transcription reaction was performed in 30 µl volume containing 6 µg of DNase I-treated total RNA, Oligo-dT-Primers (100 ng per 1 µg of total RNA), 1X first strand buffer and 10mM DTT (both from Invitrogen), 0.5 mM dNTPs (Eppendorf), 60 Units of RNase

Inhibitor (Promega) and 300 Units of Superscript II Reverse Transcriptase (Invitrogen). In terms of the order of adding reaction components, total RNA and oligo-dT primers were mixed first, heated to 70°C for 10 min and kept at 4°C until addition of the remaining reaction components. The reaction was incubated at 42°C for 1.5 hours and terminated by heat inactivation at 65°C for 15 min. The cDNA product was treated with 2.5 Units of RNase H (USB) for 30 min at 37°C, followed by heat inactivation of the enzyme at 70°C for 15 min. An identical reaction without total RNA was performed to be used as a negative control – "No-RT control". The cDNA was stored at -20°C until ready to use.

2.2.5 Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed in two separate steps: cDNA synthesis – RT and amplification of cDNA – PCR steps as described in the Reverse Transcription Reaction and PCR sections, respectively.

Annealing temperatures for primer pairs were as follows:

PCR	Annealing Temperature	Primers
RAG2	58°C	RAG2-Forward and RAG2-Reverse
IRF4	56°C	IRF4-Forward and IRF4-Reverse
SpiB	60°C	SpiB-Forward and SpiB-Reverse
CD19	60.1°C	mCD19-Forward and mCD19-Reverse

2.2.6 Flow Cytometric Analysis (FACS) of cell surface markers

The phenotypic comparison of cell surface marker expression was made by FACS analysis. We used approximately 2.5-5 x 10^5 cells per sample, which were seeded into each well of 96-well plate. In the first round of centrifugation (4 min, 1200 rpm (428 g), 4°C) cells were pelleted and supernatant was immediately removed. For the first staining step the cell sample was incubated with 20 µl of an appropriate PBS-antibody-dye conjugate mix at 4°C for 20 minutes. Cells were washed with 200 µl of chilled FACS buffer (PBS, 0.1 mM NaN_3, 3% FCS), then centrifuged (4 min, 1200 rpm (428 g), 4°C) and finally supernatant was removed from the cell pellet. This washing procedure was repeated twice. A second antibody conjugate was also left on cells for 20 minutes before cells were washed again. Optional third and fourth staining rounds were carried out as described above. To discriminate living from alive cells, propidium iodide was added to a final concentration of 25 µg/ml. Measurement was done with

a FACScan Flow Cytometer and the data were analyzed using CellQuest software (both from Becton Dickinson).

2.2.7 Purification and Stimulation of B Cells for Immunoblotting

Splenic B cells were purified using either directly anti-mouse CD45R/B220 MACS MicroBeads or MACS Streptavidin MicroBeads after labelling of cells with biotinylated anti-IgD antibodies according to manufaturer's instructions. $B220^+$ splenic B cells were stimulated with anti IgM $F(ab')_2$ antibodies at the concentration of 1 μg/ml for various times at 37°C. In the immunoblotting experiments with pre-B cells, the cells from BALB/c, $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ mice were stimulated with LPS (10 μg/ml) and alternatively pre-B cells from $SLP65^{-/-}$ mice were stimulated with anti-pre-BCR (SL156) antibodies at the concentration of 1 μg/ml at 37°C.

2.2.8 Cell lysis and Protein Extraction

Cell lysates were prepared either from freshly isolated and purified $B220^+$ or IgD^+ splenic B cells. Cells were washed two times with ice cold PBS and lysed in approximately 100 μl of NP40-lysis buffer per $1x10^6$ cells. After 15 min of incubation on ice the insoluble material (nuclei, cytoskeletal components and insoluble membranes) was removed by centrifugation at 14000 rpm for 15 min at 4°C. The supernatant is the total cell lysate and the nuclear proteins were extracted from pellets using high salt extraction buffer (0.4 M NaCl, 20mM HEPES pH 7.9, 1mM EDTA, 1mM EGTA, 1mM DTT, 2 mg/ml Complete® proteinase-inhibitor mix). Protein amount was measured by colorimetric method using BCA Protein Assay Reagent (Pierce). 100 μg of protein was loaded per lane on a gel in immunoblotting experiments. Prior to load samples the protein extracts were mixed with 4X Sample buffer, containing 0.7% v/v β-mercaptoethanol and shortly boiled (denatured) at 95°C for 5 min.

2.2.9 SDS-PAGE and Western Blotting

Electrophoretic separation of proteins was carried out by the SDS-PolyacrylAmide Gel Electrophoresis (SDS-PAGE) method of Laemmli (Laemmli 1970). After separation of proteins by their charge and size in appropriate (10% or 12%) polyacrylamide gel by SDS-PAGE, gels were shortly equilibrated in Transfer buffer and electro-transferred onto PVDF - Immobilon P Transfer Membrane at 300 mA for 1.5 hours using Tank blotting system (BioRad). Membranes were blocked in Blocking buffer (5% skimmed milk in PBS) for 1 hour and the primary antibodies were incubated in Blocking buffer for 1 hour at RT (or ON at

4°C). Washing of membranes was done four times for 5 min with PBS/Tween-20 buffer. If necessary, a secondary antibody coupled to HRP (horse-radish peroxidase) was incubated and washed with the same conditions. The ECL/Hyperfilm ECL system of Amersham was used for final detection. PBS/Tween-20 buffer with 1% NaN_3 was used to destroy the HRP-enzyme of an already-bound antibody. Alternatively, blots were stripped in a standard stripping buffer (2% w/v SDS, 62.5 mM Tris HCl pH 6.8, 100 mM β-Mercaptoethanol) for 30 min at 56°C with a subsequent blocking step.

2.2.10 Microarray analysis

2.2.10.1 B-cell purification and stimulation for microarray experiments

After depletion of erythrocytes by treatment with lysis solution consisting of 0.15M NH_4Cl, biotin-conjugated rat anti-mouse IgD antibodies (Southern Biotechnology Associates Inc., Birmingham, USA) at the concentration of 1 μg/ml in PBS, 1%BSA, 2 mM EDTA buffer were used to label the cells on ice for 15 min. Cells were washed twice and then splitted into 3 samples. IgD^+ B cells were purified either directly in the case of unstimulated cells using streptavidin-conjugated MACS MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions or after 45 min and 6 hours stimulations at 37°C. Stimulated IgD^+ B cells were purified using Streptavidin Dynabeads M-280 (Dynal Biotech, Oslo, Norway) as directed by the manufacturer. The purity of B cell populations were assessed by FACS staining of the cells for PE-conjugated anti-mouse CD45R/B220 and FITC-labeled anti-mouse IgM (II/41) (both from BD PharMingen, Heidelberg, Germany) antibodies after MACS Microbead purifications. A cell purity of ≥ 94 % was routinely achieved. The cells were used to prepare total RNA for microarray experiments.

2.2.10.2 RNA amplification procedure for microarray experiments

Total RNA was extracted from IgD^+ B cells using Trizol Reagent (Invitrogen) followed by a subsequent clean-up step using RNAeasy kit (Qiagen). The integrity and purity of total RNA was assessed running an aliquot on an agarose gel stained with SYBR GOLD (Molecular Probes). 1 μg of total RNA was used as a starting material to prepare biotinylated probe sets for hybridization experiments. RNA amplification was performed by two cycles of cDNA synthesis combined with *in vitro* transcription (IVT) for target amplification. The first cycle provided initial amplification of total RNA, resulting in unlabeled cRNA. In the second cycle of IVT synthesis, biotin ribonucleotides were incorporated to produce labeled antisense cRNA target.

First cycle of amplification

First cycle, first strand of cDNA synthesis

100 ng of [5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG$(T)_{24}$] - oligo dT-T7 primer was mixed with 1 µg of RNA and incubated at 70°C for 10 min and kept at 4°C until addition of the first strand cDNA synthesis mixture consisting of 1X 1st Strand Buffer (GIBCO), 10 mM DTT (Invitrogen), 500 µM dNTPs (Eppendorf), 20 U of RNase Inhibitor (Promega), 100 U of Superscript II RT (Invitrogen) in 10 µl of total volume. The reaction was incubated at 42°C for 1 hour followed by inactivation step at 65°C for 15 min.

First cycle, second strand cDNA synthesis

The first strand reaction was spun down and cooled on ice. The second strand cDNA synthesis mixture consisting of 1X E. coli ligase buffer, 200 µM dNTPs, 20 U of DNA polymerase I (Roche), 5 U of E. coli ligase (Invitrogen), and 1 U of RNase H (USB) in total volume of 65 µl was added. The reaction was gently mixed and incubated at 16°C for 2 hours. To fill in the ends of the double-stranded cDNA, 10 U of T4 DNA Polymerase was added to the above reaction and incubated for an additional 15 min followed by inactivation step by incubation for 15 min at 70°C.

First cycle, double stranded cDNA clean-up

Double stranded cDNA was purified by phenol-chloroform extraction and use of Phase Lock Gel (Eppendorf). cDNA was precipitated with Ethanol in the presence of 5 µg of LPA and 1/10 vol. of 5 M NH_4OAc solution overnight at -20°C. The samples were centrifuged at 14000 rpm for 30 min at 4°C, washed two times with 70% of Ethanol and air dried for 5 min.

First cycle, IVT for cRNA amplification

The following reaction mixture: 7.5 mM rNTPs (Promega), 10 mM DTT (Epicentre), 60 U of RNase inhibitor (Promega),1X T7 transcription buffer and 160 U of T7 RNA polymerase (Promega) was added in total volume of 40 µl to the air-dried double stranded cDNA pellets and gently resuspended by repetitive pipetting. The reaction was incubated at 37°C for 8 hours. The RNeasy Mini column (Qiagen GmbH) was used for cRNA purification following the protocol for RNA Clean-up handbook from Qiagen. The cRNA was stored at -20°C at this point before proceeding to the next step.

Second Cycle of Amplification

Second cycle, first strand of cDNA synthesis

The first strand cDNA synthesis was performed in the same way as described above with the exception that 500 ng of Random Hexamers ($pd(N)_6$, Amersham Pharmica Biotech) were used instead of oligo-dT-T7 primers. After completion of sDNA synthesis 2 U of RNase H was added to destroy cRNA in the reaction followed by heat-inactivation of RNase H and separation of DNA/RNA hybrids at 95°C for 5 min. The reaction was then chilled on ice.

Second cycle, second strand of cDNA synthesis and clean-up

The second strand of cDNA synthesis was primed with oligo-dT-T7 primers (100 ng per reaction). The reaction was performed and double stranded cDNA was cleaned up as described in the first cycle of amplification.

Second cycle, IVT for cRNA amplification and labeling

The following reaction mixture: 1X T7 transcription buffer, 60 U of RNase Inhibitor, 1X Biotinylated rNTP mix (ENZO kit) and 160 U of T7 RNA polymerase was added in 40 µl of total volume and incubated at 37°C for 8 hours. Biotinylated cRNA was purified on an affinity RNeasy column (Qiagen GmbH) and stored at -20°C until ready to use.

2.2.10.3 Sample preparation and array hybridization

Biotinylated cRNA probes were fragmented to an average size of 50–200 bp, by incubation for 35 min at 94°C in 40 mM Tris-acetate at pH 8.1, 100 mM potassium acetate and 30 mM magnesium acetate. Samples (20 µg each) were diluted in the hybridization solution (1 M NaCl, 100 mM MES at pH 6.7, 20 mM EDTA, 0.01% Tween 20, 0.1 mg/ml of herring sperm DNA, BioB, BioC, BioD and cre control cRNAs at concentrations of 1.5, 5, 25 and 100 pM, respectively) at a final concentration of 0.05 µg/ml and heated to 99 °C for 5 min. Analysis of the samples was done by hybridizing the fragmented cRNAs to the Affymetrix MG U74Av2 chips, which contains probes for more than 9000 mouse genes and approximately 3000 expressed sequence tags (ESTs). Probe array hybridizations were carried out by placing the samples in the hybridization cartridge at a final volume of 200 µl/chip. Hybridizations were done under rotation at 45°C for 16 hours. After hybridization, the chips were washed and stained by incubation with 10 µg/ml of phycoerythrin-streptavidin (Molecular Probes) and 2 mg/ml of acetylated bovine serum albumin (Sigma) according to the *Genechip Expression*

Analysis Technical Manual (Affymetrix). Washing-staining procedures were performed in a fluidics station (model 400, Affymetrix). The arrays were scanned with a confocal scanner (GeneArray Scanner, Hewlett Packard) and primary image analysis was performed with the MicroArray Suite 5.0 Gene Expression analysis program (Affymetrix). Expression values for all chips were scaled to a target intensity value of 500. Samples were evaluated for quality by comparison of percentage present values as well as 5' to 3' ratios of glyceraldehyde-3-phosphate dehydrogenase and actin. Each sample was profiled in three independent replicates, with cRNA prepared from separate mouse. Hybridization experiments were performed at the Core Facility of Max-Planck Institute for Immunobiology, Freiburg.

2.2.10.4 Data analysis

Data analysis was done using dChip software (Li and Wong, 2001) and GeneSight v3.0 as well as ImaGene software (both from BioDiscovery, USA). Changes in the expression patterns were compared between BALB/c, SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice as well as between BALB/c and SLP65$^{-/-}$ animals to compare changes in the gene expression profiles in SLP65-deficient IgD^{+} B lymphocytes upon BCR stimulation.

2.2.11 Real-time RT-PCR

Real-time RT-PCR was conducted in two separate steps, cDNA was generated from DNase treated total RNA, as described above in the Reverse Transcription Reaction section, followed by real-time PCR step performed in ABI Prism 7700 system (Perkin-Elmer-Applied Biosystems, USA). Separation of the RT and PCR steps has the advantage of generating a stable cDNA pool that can be stored virtually indefinitely. To maximize the number of genes that can be analyzed from the same sample of RNA we used oligo-dT priming approach in RT reaction. Except primers for single exon genes, gene specific primers were designed from intron/exon boundaries close to the 3'-end of the sequences of genes to avoid false positive results arising from amplification of possible contaminating genomic DNA. Primers were designed using Primer Express® Software v 1.5 from Applied Biosystems. Primer selection was based on estimated annealing temperature of 55°C and amplicon size of 150-180 bp for all genes tested, since we run PCR for a few genes together in one plate at a time. ABsolute™ QPCR SYBR® Green Master mix (ABgene) with ROX – reference dye was used to run PCR reactions. PCR reactions were performed in total volume of 25 µl containing 1X QPCR

SYBR Green master mix with Thermo-Start® DNA polymerase, optimal balance of $MgCl_2$, SYBR Green I and dNTPs as well as 50 nM of each gene specific Forward and Reverse primers and 1 µl of cDNA solution. The following thermal cycle conditions in two stages were applied:

Stage 1:

Enzyme activation	-	95°C for 15 min

Stage 2: (40 cycles of amplification)

Denaturation	-	95°C for 20 sec
Annealing of Primers	-	55°C for 30 sec
Elongation	-	72°C for 30 sec

Relative standard curves were generated in each separate PCR reactions (for each separate PCR plate) using RPL5 gene as a calibrator. To this end, a 10 fold dilution series with arbitrary units were created. Arbitrary units were taken from the amount of input total RNA used in the RT step. PCR reactions for the genes of interest were performed from 25ng of total RNA and in triplicate format from each sample for each gene tested. The data were analyzed using Sequence Detection System (SDS) Software version 2.1 (Applied Biosystem).

3. Results

3.1 Development of bone marrow B cells

3.1.1 Analysis of B cell precursors

In the bone marrow (BM) there are five distinct subpopulations of B cells: pro-B, pre-B, IgM^+ immature B cells and IgM^+IgD^+ mature B cells expressing lower levels of CD45R/B220 ($B220^{low}$) and recirculating B cells expressing higher levels of CD45R/B220 ($B220^{hi}$). During B cell development, early stages of B cells can be distinguished by c-kit (Palacios and Nishikawa 1992) and CD43 expression (Hardy et al., 1991). As previously reported (Jumaa et al., 1999), the number of $B220^+CD43^+$ cells is increased in SLP65-mutant mice. In order to compare pro-B and pre-B cell subpopulations in the BM of $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ mice, we analyzed by FACS freshly-isolated single cell suspensions for the expression of B cell surface markers: CD45R/B220, CD117/c-kit and CD43 (Fig. 4a). The analysis of $B220^{low}$ subpopulation in the BM showed no significant differences in the $c\text{-}kit^+$ $CD43^-$ early pro-B cell subset between $EGRxSLP65^{-/-}$ (n=4; 3.70% ± 0.64%), $SLP65^{-/-}$ (n=6; 4.02% ± 2.58%) and control (BALB/c) (n=6; 4.76% ± 1.03%) mice (Fig. 4b). Likewise there were no differences in the $c\text{-}kit^+$ $CD43^+$ late pro-B cell subpopulation between $EGRxSLP65^{-/-}$ (n=4; 2.30% ± 1.10%), $SLP65^{-/-}$ (n=6; 2.65% ± 1.98%) and control (n=6; 1.41% ± 0.54%) mice in the BM (Fig 4c). No significant changes in the number of $c\text{-}kit^-$ $CD43^+$ pre-B I cells were found between $EGRxSLP65^{-/-}$ and $SLP65^{-/-}$ mice. The percentage of pre-B I cells in the BM of EGRxSLP65-/- is 46.04% ± 3.79% (n=4) and 47.26% ± 8.61% (n=6) in $SLP65^{-/-}$ mice, whereas in the control mice the percentage of the pre-B I cell subset only reaches 13.52% ± 5.07% (n=6) (Fig. 4d). The differences in the frequency of pre-B I cells between $EGRxSLP65^{-/-}$ and control mice ($P<0.001$) as well as between $SLP65^{-/-}$ and control mice ($P<0.001$) were highly significant.

After successful immunoglobulin heavy chain V-D-J rearrangement, pre-B I cells express pre-B cell receptor (pre-BCR) complexes that are composed of membrane Igμ and surrogate light chains (made up of V-preB and λ5 polypeptides) and which transmit signals through Igα-Igβ molecules (Martensson et al., 2002). An important checkpoint of B cell development is the expression of a pre-BCR, which is essential for the selection and expansion of pre-B cells (Karasuyama et al., 1997; Conley et al., 2000; Martensson and Ceredig 2000). While the pre-BCR is transiently expressed on the cell surface (ten Boekel et al., 1998) and pre-BCR positive cells can barely be detected in normal mice because the cells pass quickly through this stage, pre-B cells from mice lacking $SLP65^{-/-}$ have an enhanced proliferative capability. This anomaly has been shown to be due to a defect in pre-BCR downregulation

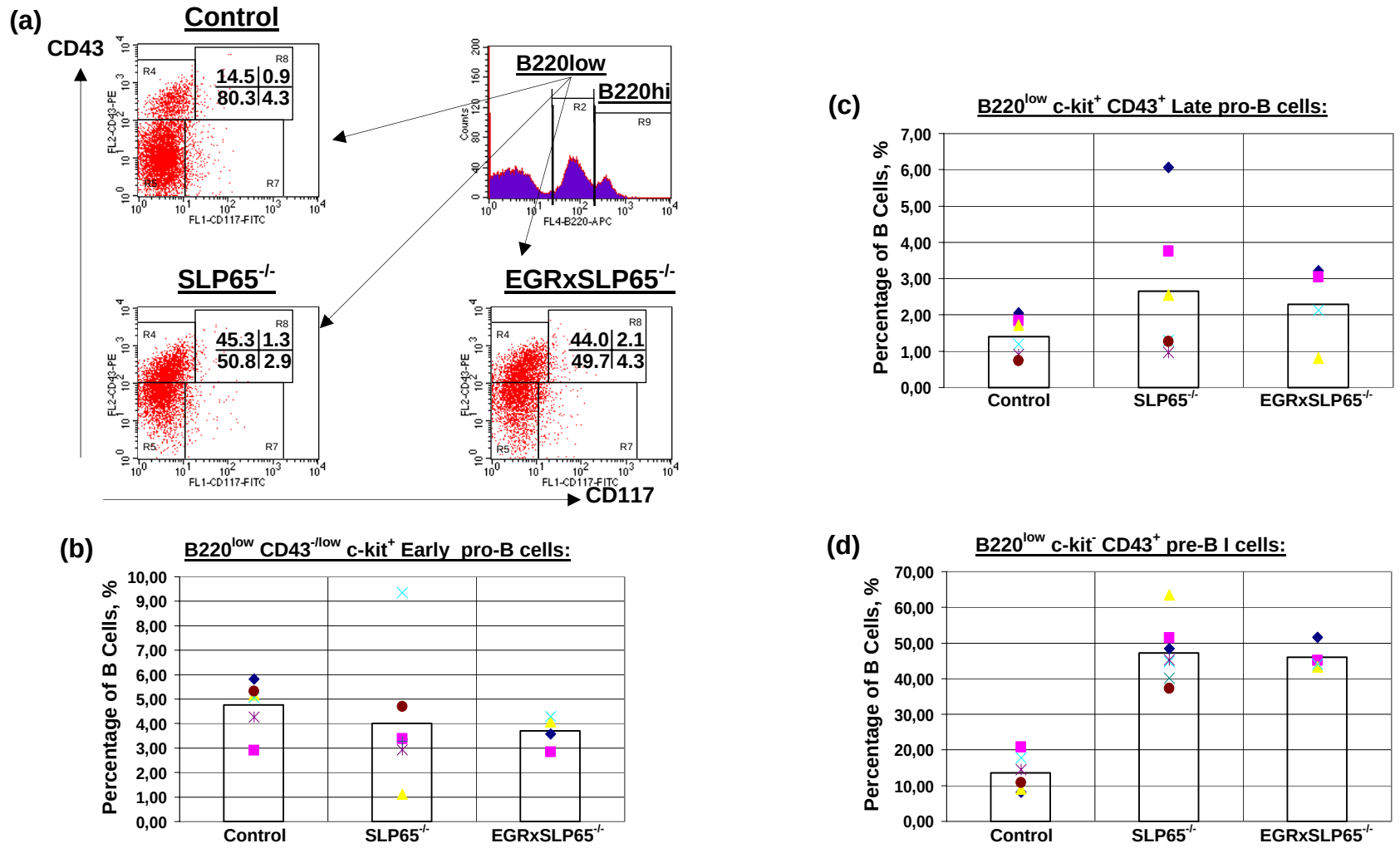


Figure 4: The distribution of pro-B and pre-B cell subpopulations in the bone marrow. (a) FACS analysis of early pro-B ($CD117^+$ $CD43^-$), late pro-B ($CD117^+$ $CD43^+$) and pre-B I ($CD117^-$ $CD43^+$) B cell subsets in the bone marrow from control (BALB/c), $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ mice. Individual examples are shown and the numbers indicate the percentage of $B220^{low}$ cells in each subset. The diagrams depict the percentages of (b) early pro-B cell, (c) late pro-B cell and (d) pre-B I cell subpopulations in the bone marrow from wild type (control), $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ mice.

(Flemming et al., 2003). Due to the block at the pre-BCR positive stage, SLP65$^{-/-}$ mice also have lower numbers of Igκ^{+} B cells in the BM (Jumaa et al., 2001).

No significant differences were found between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ mice in the numbers of pre-BCR^{+} pre-B I cells as well as Igκ^{+} immature B cells in the bone marrow (Fig. 5a). On average, 17.31% ± 3.23% (n=6) of the cells in EGRxSLP65$^{-/-}$ and 21.55% ± 5.49% (n=6) of the cells in SLP65$^{-/-}$ mice were pre-BCR^{+}, whereas in control mice this number reached only 1.97% ± 0.74% (n=6) showing the transient nature of the pre-BCR expression in normal B cells (Fig 5b). The numbers of pre-BCR^{+} pre-B I cells in both EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ mice were significantly higher than in the control mice with p-values of $P<0.0001$ and $P<0.001$, respectively. The frequency of Igκ^{+} immature B cells was relatively lower in these mice in comparison to wild type littermates (Fig 5c). On average, 22.48% ± 4.21% (n=6; P=0.005) of total B220^{low} cells in EGRxSLP65$^{-/-}$ mice and 24.38% ± 1.88% (n=6; P=0.01) of total B220^{low} cells in SLP65$^{-/-}$ mice were Igκ^{+} immature B cells. In control mice the percentage of Igκ^{+} immature B cells was 31.82% ± 3.65% (n=6), on average.

In order to investigate whether the overexpression of EGR1 in SLP65-/- mice promotes the development of IgM^{+} immature B cells, freshly isolated BM cells of EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and wild type mice were analysed for the expression of B220 and IgM by FACS. Figure 6a shows that there are no significant differences in the percentage of IgM^{+} immature B cells in the bone marrow between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ mice. There were only 3.24% ± 1.24% B220^{low} IgM^{+} immature B cells in EGRxSLP65$^{-/-}$ mice (n=5) and 3.16% ± 0.90% in SLP65$^{-/-}$ mice (n=5), but 7.03% ± 2.08% (n=6) of the total cells in the BM of control mice were B220^{low} IgM^{+} immature B cells (Fig. 6b). The differences in the percentage of IgM^{+} immature B cells between EGRxSLP65$^{-/-}$ and control mice (P=0.002) as well as between SLP65$^{-/-}$ and control mice (P=0.002) were similar and highly significant. Reciprocally, lower numbers of B220^{hi} IgM^{low} recirculating mature B cells and higher numbers of B220^{hi} IgM^{hi} transitional B cells were found in the BM of both EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ mice in comparison to the control. In addition, FACS analysis of BM cells with pB493 antibody, another B cell maturation marker expressed during B cell ontogeny showed that both EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ bone marrow cells have a less mature phenotype in comparison to the control mice. On average, 80.1% ± 4.67% (n=2) of cells in EGRxSLP65$^{-/-}$ mice, 81.1% ± 2.78% (n=3) of cells in SLP65$^{-/-}$ mice and only 61.05% ± 3.73% (n=2) of the total bone marrow cells in control animals were B220^{low} CD19^{+} c-kit^{-} pB493^{+} B cells (data not shown).

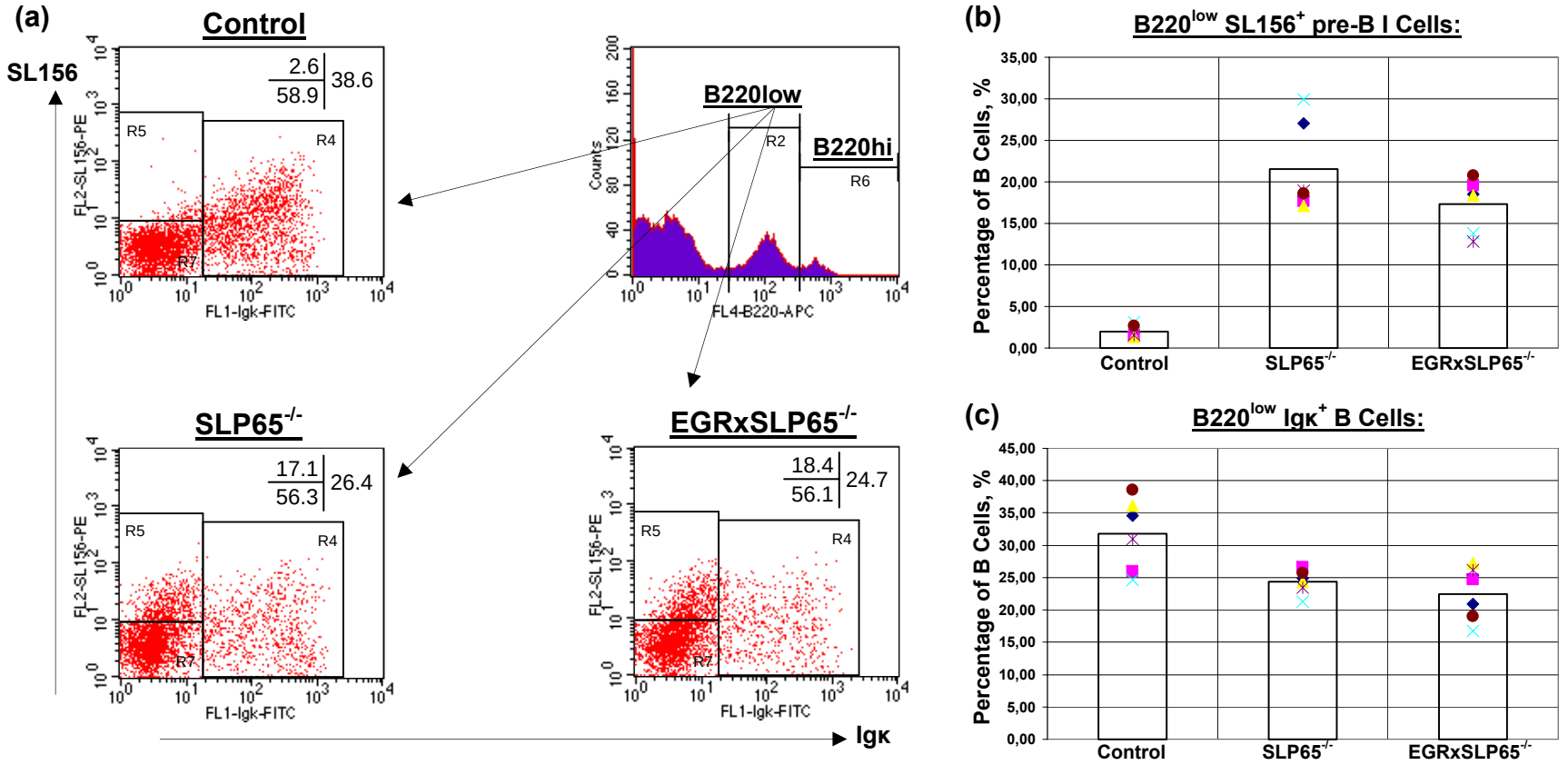


Figure 5: The distribution of pre-BCR$^+$ pre-B I cell and Igκ$^+$ immature B cell subsets in the bone marrow.
(a) FACS analysis of preBCR$^+$ (SL156$^+$) pre-B I and Igκ$^+$ immature B cell subsets in the bone marrow of control (BALB/c), SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice. Individual examples are shown and the numbers indicate the percentage of B220^{low} cells in each subset. The diagrams show the percentage of (b) B220^{low} pre-BCR$^+$ pre-B I cell and (c) B220^{low} Igκ$^+$ B cell subpopulations in the bone marrow.

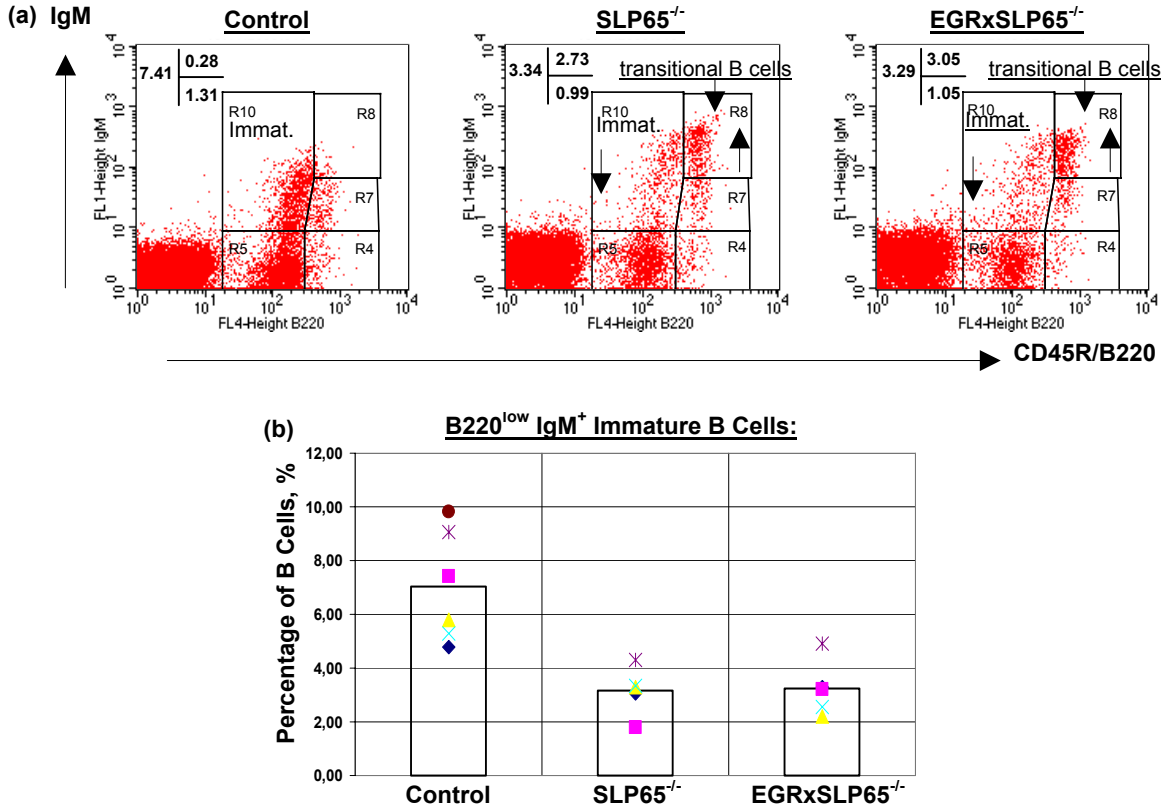


Figure 6: Comparison of immature B cell subsets in the bone marrow. (a) Immature, transitional and mature B cells in the BM from control (BALB/c), SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice. Individual examples are given and the numbers indicate the percentage of B220^{+} cells in each subset. (b) shows the percentage of B220^{low} IgM^{+} immature B cell subpopulation in the BM of wild type, SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice.

3.1.2 Expression of RAG2, IRF4 and SpiB genes in EGRxSLP65$^{-/-}$ pre-B cells

Early large pre-B cells shut down the expression of the recombination-activating genes 1 and 2 (RAG1 and RAG2), undergo several rounds of cell division and then exit the cell cycle (Grawunder et al., 1995; Hardy et al., 2000). Two important characteristics of small pre-B cells include the expression of recombination-activating gene 1 (RAG1) and RAG2 and of κ germline transcripts, both of which are essential for subsequent light chain gene rearrangement, leading to the BCR assembly (Kurosaki 2003). Activation of SpiB and IRF4, transcription factors of the Ets and IRF families, is capable of inducing κ locus transcription and possibly recombination accessibility (Muljo and Schlissel 2003). Like RAG1 and RAG2 genes, SpiB and IRF4 transcript levels are low in large pre-B cells and increase as developing B cells progress to the B220^{+} IgM^{-} CD25^{+} small pre-B cell stage (Muljo and Schlissel 2003). The genes encoding RAG-1 and RAG-2 were only downregulated in surface μ^{+} proliferating SLP65$^{-/-}$ pre-B I cells but not in surface μ^{-} SLP65$^{-/-}$ B cells (Flemming et al., 2003). Expression of the RAG2, IRF4 and SpiB genes in *in vitro*-cultivated BM-derived cells from EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control mice was analyzed by semiquantitative RT-PCR (Fig. 7).

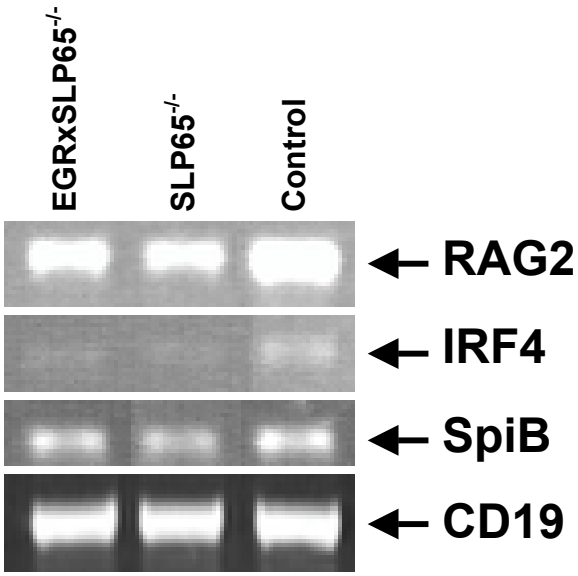


Figure 7: RT-PCR analysis of the expression of RAG2, IRF4 and SpiB transcripts in control (BALB/c), $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ *in vitro*-cultivated BM-derived B cells. 6 µg of total RNA pre-treated with DNase (RQ DNase I, Promega) was reverse transcribed using oligo-dT-T7 primers and Superscript II Reverse Transcriptase (GIBCO). The resulting cDNA was used in PCR assays to amplify the transcripts. The B cell specifc transcript CD19 was used as a control.

Similar low levels of RAG2, IRF4 and SpiB transcripts were detected in $EGRxSLP65^{-/-}$ and $SLP65^{-/-}$ pre-B cells in comparison to the control. These data once more demonstrate and confirm that there are no differences in the frequency of pre-B I and pre-B II cell subsets between $EGRxSLP65^{-/-}$ and $SLP65^{-/-}$ in the bone marrow.

3.1.3 EGR1 induced downregulation of IL-2R alpha chain in $EGRxSLP65^{-/-}$ B cells

The developmental transition from the pro-B cell to the pre-B cell stage is characterized by the downregulation of c-kit and CD43 and concomitant expression of CD2 and CD25 cell surface markers (Rolink et al., 1994). B cell development was completely blocked at the $B220^{low}$ μH^{-} $CD43^{hi}$ $CD25^{-}$ pro-B cell stage in $RAG2^{-/-}$ BM cells deficient for Ig gene rearrangements (Shinkai et al., 1992). Pro-B cells have been characterized to have a $CD43^{hi}$ CD25 (IL-2R alpha chain) negative phenotype, whereas this phenotype changes as progression towards pre-B cell stage proceeds; large pre-B cells are $CD43^{+}$ $CD25^{low}$ and small pre-B cells become $CD43^{-}$ $CD25^{+/hi}$ (Hayashi et al., 2000). To elucidate the *in vivo* function of SLP65 in the development and function of lymphoid systems the authors generated $SLP65^{-/-}$ $RAG2^{-/-}$ and $SLP65^{+/-}RAG2^{-/-}$ chimeric mice and showed abnormal accumulation of large pre-B cells with the characteristic phenotype of $CD43^{+}$ $CD25^{low}$ in the bone marrow of $SLP65^{-/-}$ $RAG2^{-/-}$ chimeras compared to the $SLP65^{+/-}RAG2^{-/-}$ chimeras and wild type (C57BL/6) mice (Hayashi et al., 2000).

FACS analysis of bone marrow cells from $SLP65^{-/-}$, $EGRxSLP65^{-/-}$, EGR1 transgenic (EGR1-tg) and control mice (Fig. 8) revealed that consistent with the above data (Hayashi et al., 2000), in $SLP65^{-/-}$ mice CD25 expression was lower both in $B220^{low}$ IgM^{-} and $B220^{low}$ IgM^{+}

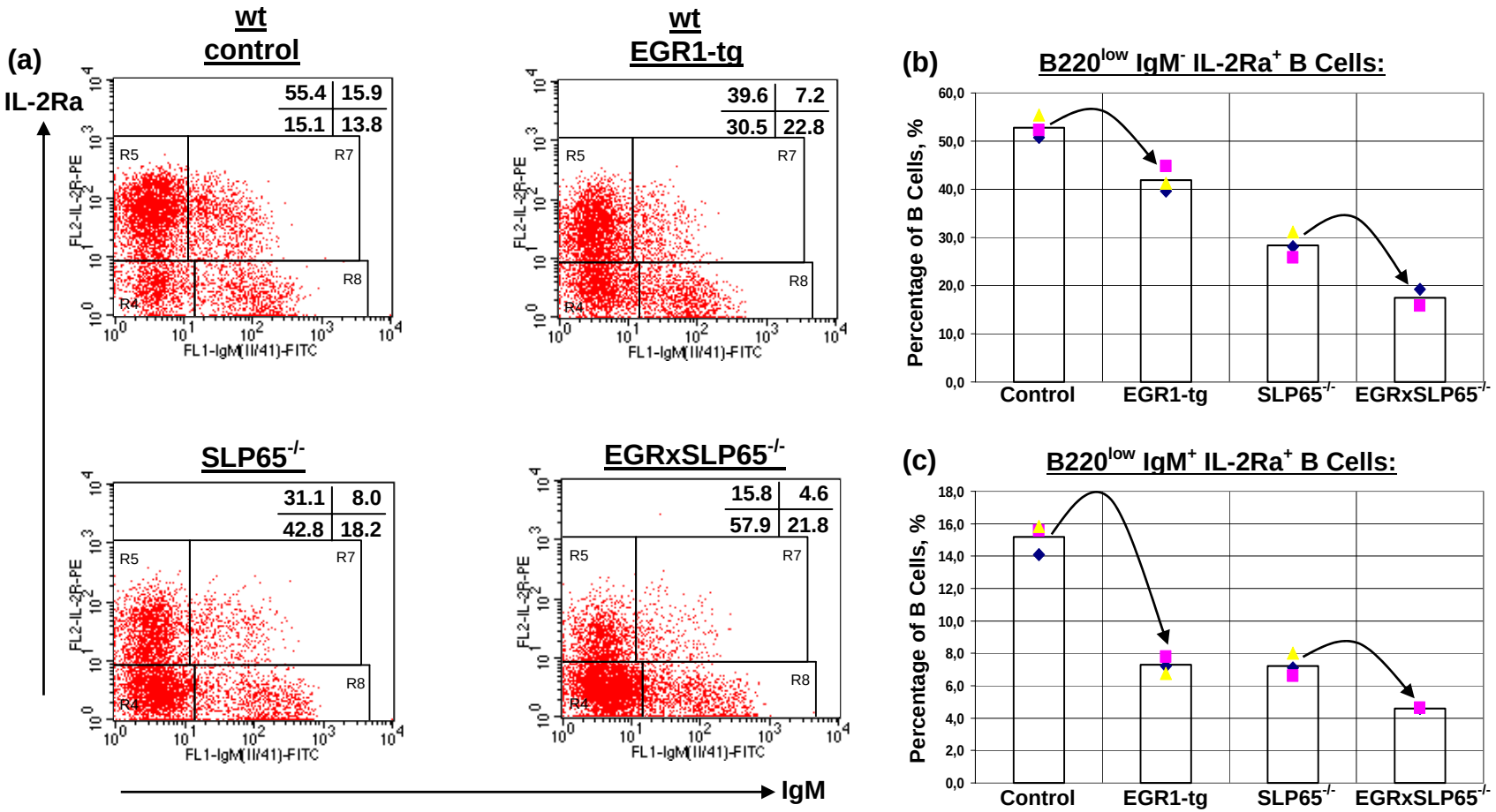


Figure 8: EGR1 induces downregulation of IL-2R alpha chain. FACS analysis of IL-2R alpha chain (IL-2Ra) expression in pre-B cells and immature B cells in the bone marrow from control (BALB/c), EGR1-tg (EGR1-transgenic), SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice. B220^{low} cells were gated for further analysis. (a) Individual examples of FACS analysis are shown and the numbers indicate the percentage of B220^{low} cells in each subset. Diagrams depict the percentages of (b) B220^{low} IgM^{-} IL-2Ra^{+} B cell and (c) B220^{low} IgM^{+} IL-2Ra^{+} B cell subpopulations in the bone marrow.

B cell subpopulations in comparison to the control mice. In SLP65$^{-/-}$ mice, 28.3% ± 2.68% (n=3; P<0.001) of B220^{low} IgM^{-} B cells and 7.2% ± 0.71% (n=3; P<0.001) of B220^{low} IgM^{+} B cells were IL-2R alpha chain positive (IL-2Rα^{+}) whereas in the control mice these numbers were higher: 52.8% ± 2.39% (n=3) of cells were B220^{low} IgM^{-} IL-2Ra^{+} B cells and 15.2% ± 0.95% (n=3) of cells in the bone marrow had a B220^{low} IgM^{+} IL-2Ra^{+} phenotype (Fig. 8a). Interestingly, IL-2Rα expression was downregulated by EGR1 overexpression both in EGRxSLP65$^{-/-}$ and EGR1-tg mice (Fig. 8, b and c). On average, there 41.9% ± 2.18% (n=3; P=0.003) of B220^{low} IgM^{-} IL-2Rα^{+} and 7.3% ± 0.51% (n=3; P=0.0005) of B220^{low} IgM^{+} IL-2Ra^{+} B cells in the BM of EGR-1 tg mice and 17.5% ± 2.40% (n=2; P=0.002) of B220^{low} IgM^{-} IL-2Ra^{+} and only 4.6% ± 0.01% (n=2; P=0.001) of B220^{low} IgM^{+} IL-2Ra^{+} B cells were found in the BM of EGRxSLP65$^{-/-}$ mice. Analysis of the IL-2Ra promoter revealed 3 EGR1 binding sites at positions -393, -1011 and -1870 relative to the transcription start. These data suggest that IL-2R alpha chain might be directly regulated by the EGR1 transcription factor in B cells.

3.2 Development of follicular B cells

3.2.1 EGR1 protein expression levels are restored in EGRxSLP65$^{-/-}$ follicular B cells

B220^{+} B cells were purified from the spleens of EGRxSLP65$^{-/-}$ (n=3), SLP65$^{-/-}$ (n=3) and BALB/c (n=3) mice using CD45R/B220 MACS microbeads. In order to increase the yield of B220^{+} B cells 3 spleens from each genotype of mice were pooled. Immunoblot analysis of the total cell lysates of B220^{+} splenic B cells from control, SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice showed diminished levels of EGR1 protein expression in SLP65$^{-/-}$ B cells, whereas the level of EGR1 protein expression is restored in EGRxSLP65$^{-/-}$ B cells (Fig. 9). This suggests that EGR1 protein expression is dependent on SLP65 in splenic B cells and therefore, we analyzed the induction of EGR1 expression in SLP65$^{-/-}$ versus wild type B cells upon BCR cross-linking.

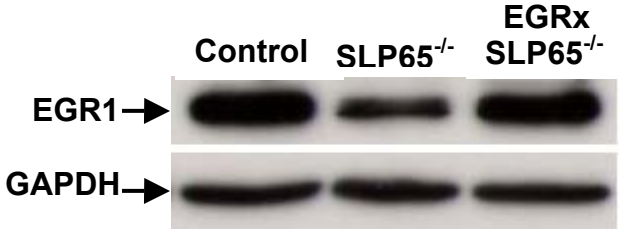


Figure 9: EGR1 protein expression levels are restored in EGRxSLP65$^{-/-}$ follicular B cells. Immunoblot analysis of B220^{+} splenic B cells from control (BALB/c), SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice. B220^{+} B cells were purified using anti-mouse CD45R(B220) MACS microbeads from spleens of mice and total cell lysates were prepared for immunoblot analysis.

3.2.2 EGR1 acts downstream of SLP65 in follicular B cells

Purified $B220^+$ splenic B cells from $SLP65^{-/-}$ and control mice were stimulated with anti-IgM $F(ab')_2$ specific antibodies for 0, 30, 60, 90, 120 and 240 minutes and total cell lysates were prepared, in the case of $EGRxSLP65^{-/-}$ mice total cell lysates were prepared only from unstimulated $B220^+$ B cells. Immunoblotting analysis revealed that EGR1 protein induction kinetics upon BCR stimulation was impaired in $SLP65^{-/-}$ B cells compared to the control (Fig. 10, a and b). EGR1, as an immediate early gene, was highly induced within the first few hours reaching its maximum levels of protein expression after 1.5 hours of BCR stimulation in normal splenic B cells, whereas in $SLP65^{-/-}$ B cells the induction of EGR1 protein was severely reduced showing 2.3 to 3.3 fold decrease compared to the control within the first hour and up to 6.5 to 7.3 fold difference within the next hours of stimulation. Impaired induction kinetics of EGR1 protein in $SLP65^{-/-}$ B cells suggested that EGR1 expression and induction is mainly dependent on the SLP65 adaptor protein and that EGR1 acts downstream of SLP65 in follicular B cells and the defects in B cell differentiation observed in $SLP65^{-/-}$ B cells might be (at least, partially) due to the impaired expression of EGR1 protein in $SLP65^{-/-}$ B cells. Since, EGR1 protein expression levels were restored in $EGRxSLP65^{-/-}$ B cells, we analyzed the spleen B cells, if the EGR1 overexpression can promote the maturation of $SLP65^{-/-}$ B cells in the spleen.

In adult mice, three major populations of splenic B cells can be identified by staining for IgM, IgD, pB493/C1qRp, CD21 and CD23 cell surface expression. The IgM^{hi} IgD^- $pB493/C1qRp^+$ $CD21^-$ $CD23^-$ B cells or transitional 1 (T1) B cells are recent immigrants from the bone marrow (Loder et al., 1999). These T1 B cells become IgM^{hi} IgD^+ $C1qRp^+$ $CD21^+$ $CD23^+$ transitional 2 (T2) B cells, some of which in turn differentiate into IgM^{lo} IgD^{hi} $C1qRp^-$ $CD21^+$ $CD23^+$ mature or recirculating B cells. Negative selection of B cells has been shown to occur in the transitional B cell stage (Carsetti et al., 1995; Norvell and Monroe 1996; Sater et al., 1998). SLP65-deficient mice show increased proportions of immature transitional B cells and reciprocally, reduced numbers of mature B cells in peripheral lymphoid organs (Jumaa et al., 1999). Phenotypic comparison of spleen B cells from $EGRxSLP65^{-/-}$ and $SLP65^{-/-}$ mice revealed that $EGRxSLP65^{-/-}$ mice have lower numbers of $B220^+$ IgM^+ IgD^- and $B220^+$ IgM^+ $pB493^+$ B cells in comparison to $SLP65^{-/-}$ mice (Fig. 11a). No significant differences were found in the expression of CD21 and CD23 between $EGRxSLP65^{-/-}$ and $SLP65^{-/-}$ B cells (data not shown). In $EGRxSLP65^{-/-}$ mice the mean number of $B220^+$ IgM^+ $pB493^+$ immature transitional B cells was 8.36% ± 0.92% (n=3; P=0.0016) and the mean number of $B220^+$ IgM^+ IgD^- T1 B cells reached only 3.42% ± 0.44% (n=3; P=0.0004),

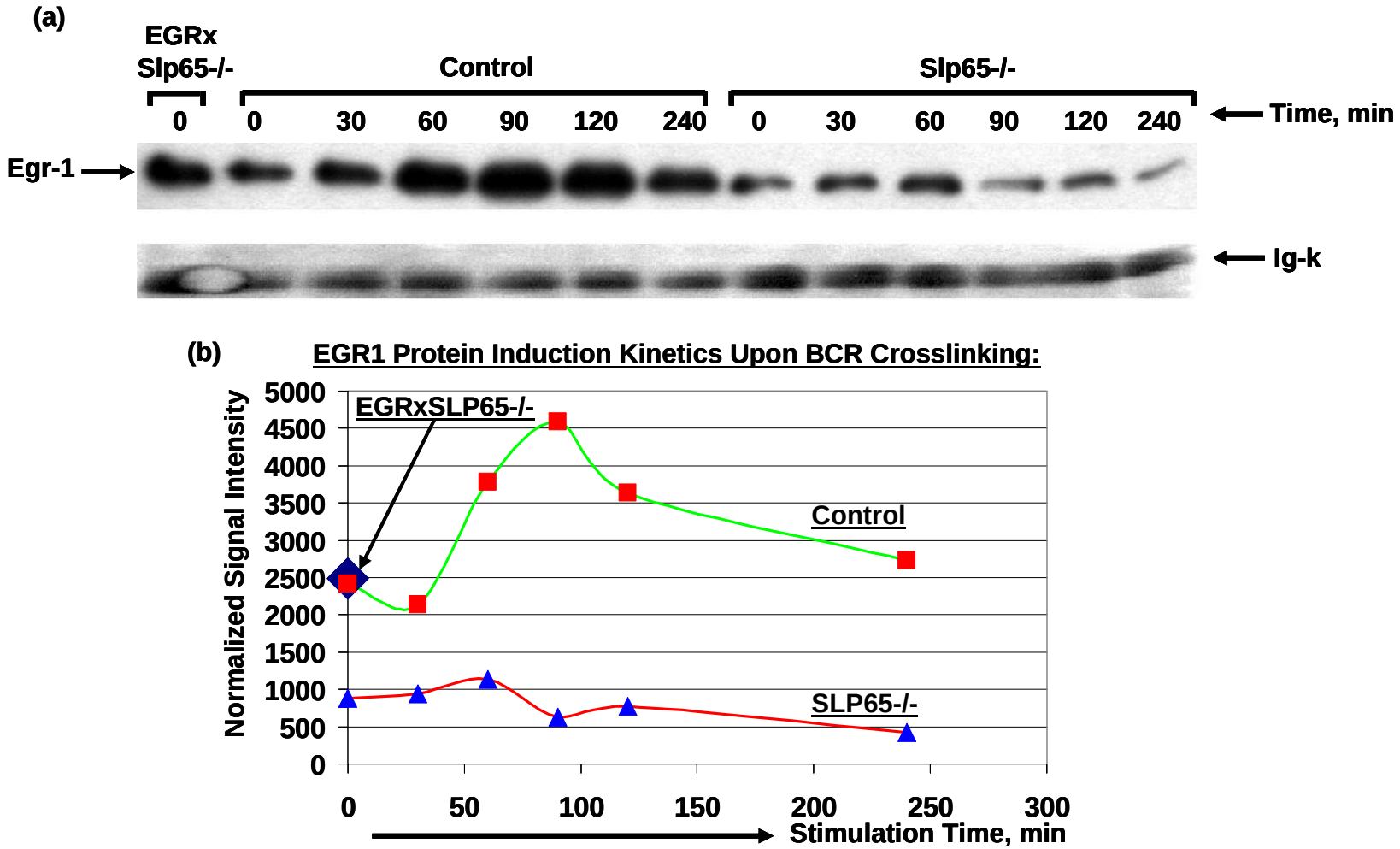


Figure 10: EGR1 acts downstream of SLP65 in follicular B cells. (a) Immunoblot analysis of $B220^{+}$ splenic B cells from $EGRxSLP65^{-/-}$, control (BALB/c) and $SLP65^{-/-}$ mice. Total cell lysates were prepared from cells purified using anti-mouse CD45R(B220) MACS microbeads directly after purification and after *in vitro* stimulation with anti-IgM $F(ab')_2$ specific antibodies for 30, 60, 90, 120 and 240 minutes. (b) shows EGR1 protein induction kinetics upon BCR engagement in control (BALB/c) and $SLP65^{-/-}$ follicular B cells.

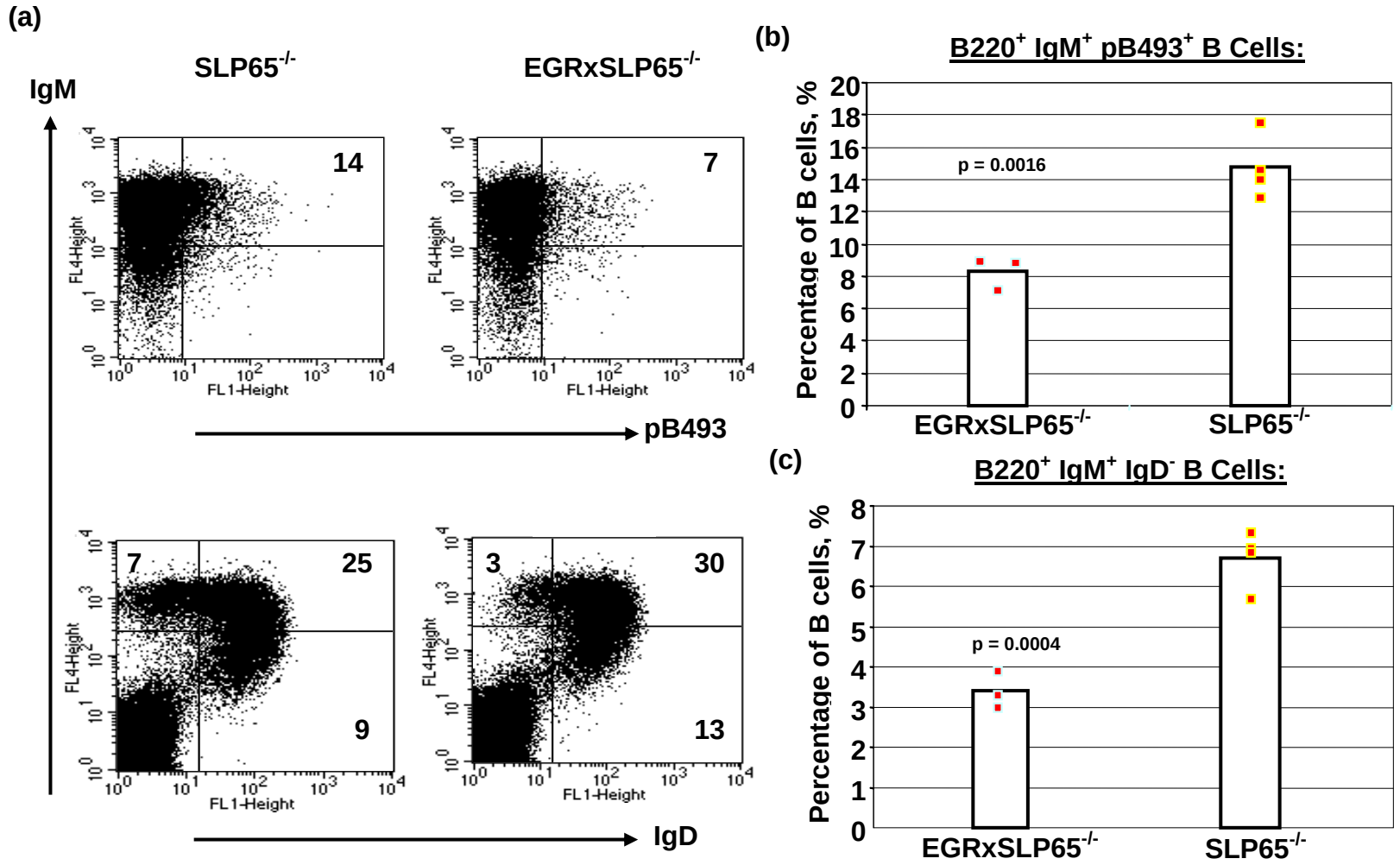


Figure 11: EGR1 induces B cell maturation in the spleen. FACS analysis of splenic B cell subsets from SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice. (a) Individual examples are shown and the numbers indicate the percentage of B220^{+} cells in certain B cell subsets. The diagrams show the percentage of (b) B220^{+} IgM^{+} pB493^{+} immature T1 and T2 B cell subpopulations and (c) B220^{+} IgM^{+} IgD^{-} immature transitional T1 B cell subset in the spleen.

whereas SLP65$^{-/-}$ mice had 14.74% ± 1.67% (n=4) of B220^{+} IgM^{+} pB493^{+} B cells and 6.76% ± 0.62% (n=4) of B220^{+} IgM^{+} IgD^{-} B cells in the spleen (Fig. 11, b and c). This suggested that EGRxSLP65$^{-/-}$ B cells have a more mature phenotype as characterised by lower expression of IgM and pB493 as well as increased IgD expression. In order to explore molecular basis of these EGR1-mediated phenotypic changes in EGRxSLP65$^{-/-}$ mice and to identify additional EGR1 target genes in B cells we decided to perform gene expression profiling experiments.

3.2.3 Gene expression profiling in IgD^{+} B cells from EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and wildtype BALB/c mice

3.2.3.1 Experimental design and data mining

To study gene expression profiles the following approach was employed (Fig. 12):

1. Comparison of gene expression profiles in purified IgD^{+} B cells from EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control (BALB/c) mice in order to identify the genes which promote EGR1-mediated changes in EGRxSLP65$^{-/-}$ B cells.

Since, EGR1 is an immediate early gene induced upon antigen (Ag) cross-linking to the BCR and taking into account that ERG1 induction is impaired in SLP65$^{-/-}$ B cells upon BCR stimulation, we have applied the following rational in order to compare the kinetics of BCR-induced genes between SLP65$^{-/-}$ and wild type B cells.

2. Comparison of gene expression profiles in IgD^{+} B cells from SLP65$^{-/-}$ and control (BALB/c) mice after 45 minutes of BCR stimulation to identify the genes which mediate the defects in primary response to antigen in SLP65$^{-/-}$ B cells.
3. Comparison of gene expression profiles in IgD^{+} B cells from SLP65$^{-/-}$ and control (BALB/c) mice after 6 hours of BCR stimulation to identify the genes which mediate the defects in secondary response to antigen in SLP65$^{-/-}$ B cells.

Total RNA was extracted either directly after purification or after appropriate stimulations from IgD^{+} B cells. mRNA was amplified by two subsequent cycles of cDNA synthesis and *in vitro* transcription. Samples were prepared from three independent biological replicates for each genotype of mice. In total 21 biotin-labeled cRNA probes were hybridized to high density Affymetrix MG U74Av2 oligonucleotide arrays each representing approximately 9000 functionally characterized genes and approximately 3000 EST cluster sequences. After hybridization and subsequent staining-washing steps the GeneChips were scanned. Data were extracted from microarray images using Microarray Suite 5.0 (MAS) software and GeneChips were globally scaled to the arbitrary value of 500. Quality control assessed by MAS 5.0

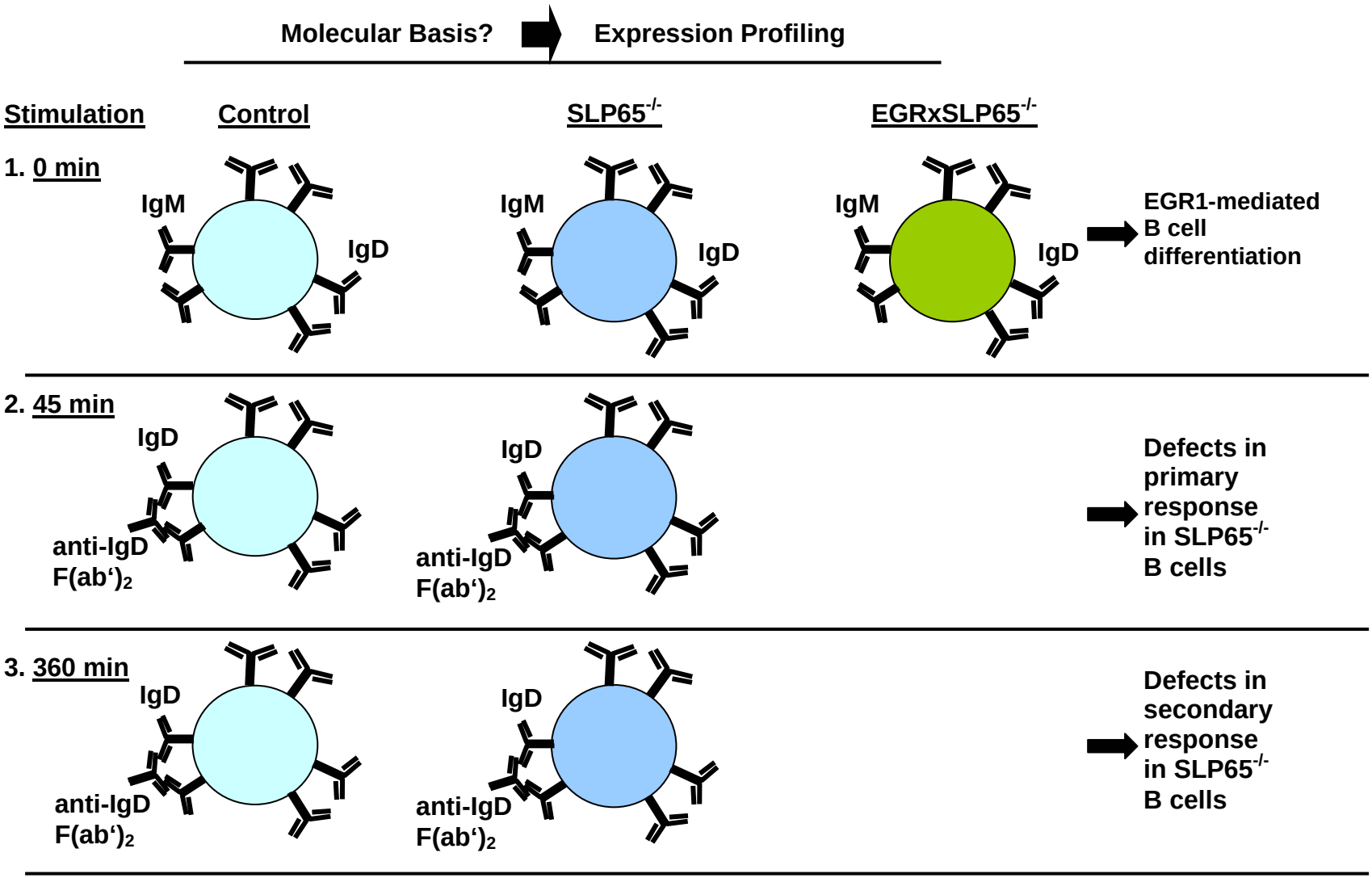
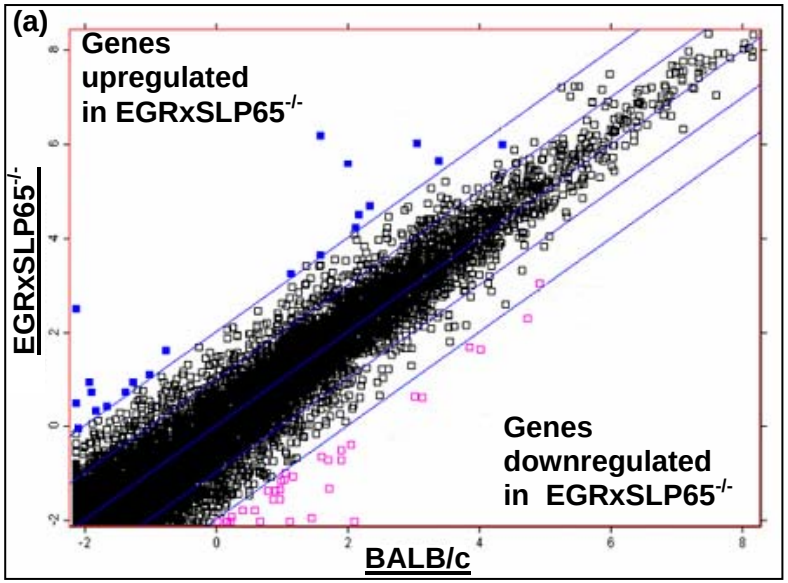


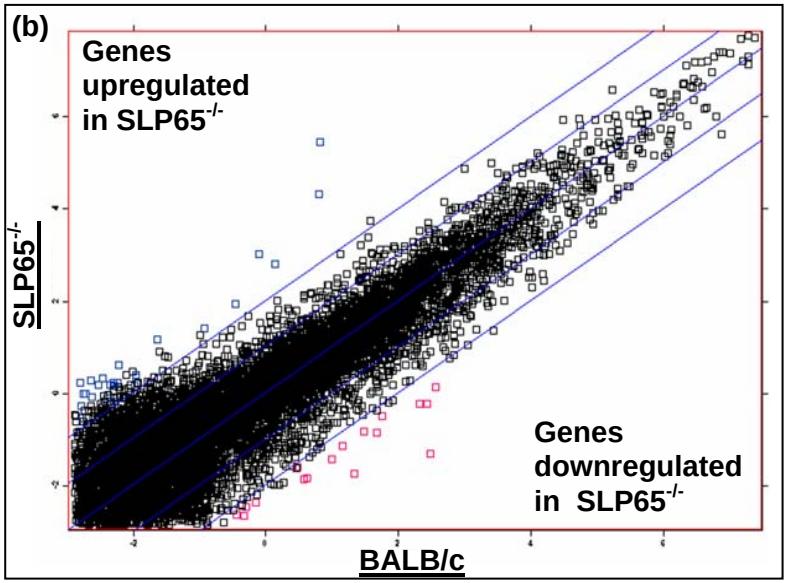
Figure 12: Experimental design for microarray analysis of gene expression profiles. IgD^+ B cells were purified from spleens of control (BALB/c), $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ mice. Probe sets were prepared from unstimulated and cells stimulated *in vitro* with anti-IgD $F(ab')_2$ antibodies for 45 minutes or 6 hours. Gene expression profiles were established using Affymetrix MG U74 microarrays.

software showed that background signal is below 100 and noise signal is below 4 arbitrary units in all arrays indicating good hybridization quality in all arrays. The 21 .CEL files were generated by MAS 5.0 and data analysis was performed using DChip software (Li and Wong 2001). Briefly, all 21 arrays were normalized together, signal intensities were log-transformed and model based expression indexes (MBEI) were calculated. Gene expression profiles were established and the changes in the expression patterns between control, SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ B cells were analyzed.

3.2.3.2 Genes differentially regulated in EGRxSLP65$^{-/-}$ B cells

In total, 243 genes (1.96% of all 12422 genes on the arrays) were found to be differentially expressed between all comparisons: SLP65$^{-/-}$ versus EGRxSLP65$^{-/-}$, control versus SLP65$^{-/-}$ and control versus EGRxSLP65$^{-/-}$ IgD^{+} B cells with the t-test p value of $P<0.05$ (Fig. 13 a, b and c). A gene is considered to be differentially expressed if its relative expression is 2-fold higher or lower compared to the baseline level. Among these 243 changes there were 170 genes with annotated function and 33 EST sequences and cDNA clones as well as 40 RIKEN cDNA sequences. In SLP65$^{-/-}$ B cells 70 genes are downregulated by at least 2-fold and 40 genes 2-fold upregulated compared to the control. Comparison of EGRxSLP65$^{-/-}$ B cells to the control revealed that 45 genes downregulated and 142 genes upregulated at least 2-fold in EGRxSLP65$^{-/-}$ B cells. In total 141 genes were found to be differentially regulated between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ B cells, 111 genes are upregulated 2-fold and the expression of 30 genes were at least 2-fold lower in EGRxSLP65$^{-/-}$ B cells compared to SLP65$^{-/-}$ B cells. First of all, we were interested in those genes which are differentially regulated between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ B cells, but whose expression levels are the same in EGRxSLP65$^{-/-}$ and control B cells. This is because we would expect that changes in the expression patterns of such genes would restore and/or correct the defects caused by the absence of SLP65 in EGRxSLP65$^{-/-}$ B cells. There are 30 such genes with characteristic expression patterns of SLP65$^{-/-}$ $\neq$ EGRxSLP65$^{-/-}$ $\approx$ BALB/c, 16 genes being upregulated and 14 genes being downregulated in EGRxSLP65$^{-/-}$ B cells compared to SLP65$^{-/-}$ B cells. A part of the genes and their possible functions are listed in the Table 2. Amoung them are a few genes and families of genes the expression of which has been previously reported to be regulated by the EGR1 transcription factor. For instance, the expression of GADD45 genes (Zhan et al., 1998; Thyss et al., 2005), complement component C1q genes (Lynch et al., 2004) or ICAM genes (Maltzman et al., 1996; Ji et al., 2003). GADD45-beta gene (2.8 fold), complement component C1q, alpha chain (2.7 fold) and two





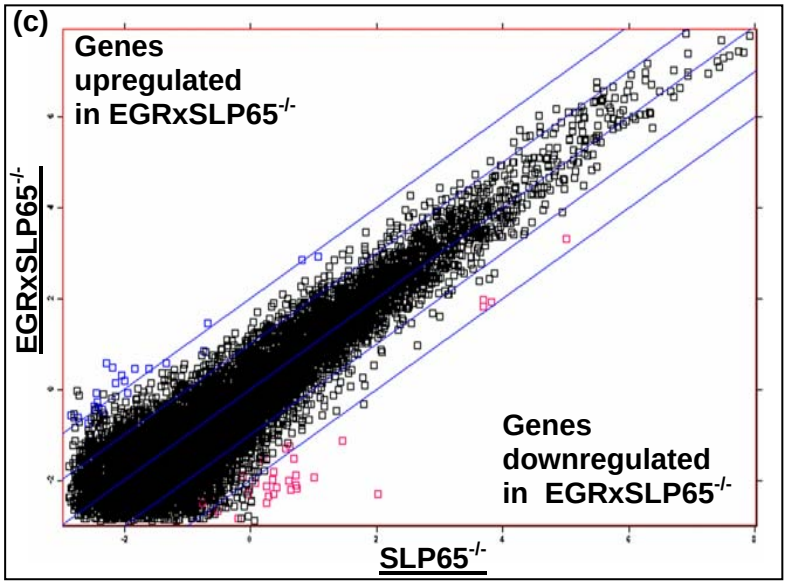


Figure 13: Microarray analysis of gene expression profiles of IgD$^+$ B cells from EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control mice. Scatter plots of comparisons (a) EGRxSLP65$^{-/-}$ versus control, (b) SLP65$^{-/-}$ versus control as well as (c) EGRxSLP65$^{-/-}$ versus SLP65$^{-/-}$ are shown. In total 243 genes (1.96% of all 12422 genes on the arrays) were found to be differentially expressed between all comparisons. A gene is considered to be differentially expressed if its relative expression is twofold higher or lower in comparison to the baseline. In total, 187 genes between EGRxSLP65$^{-/-}$ and control, 110 genes between SLP65$^{-/-}$ and control as well as 141 genes between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ B cells were found to be at least twofold differentially regulated.

Table 2: Expression profiling results: genes expressed at equal levels in EGRxSLP65$^{-/-}$ and control (BALB/c) IgD^{+} B cells.

Probe Set	Genes and Functions	Accession	Ratio of Relative Expression Levels		
			SLP65$^{-/-}$ / Control	SLP65$^{-/-}$ / EGRxSLP65$^{-/-}$	EGRxSLP65$^{-/-}$ / Control
	cell adhesion				
99053_at	intercellular adhesion molecule 2 (ICAM-2)	X65493	**-2.9**	**4.2**	**1.4**
94004_at	calponin 2	Z19543	**-8.8**	**7.1**	**-1.2**
	Apoptosis				
93836_at	BCL2/adenovirus E1B 19 kDa-interacting protein 1, NIP3	AF041054	**2.3**	**-2.4**	**-1.1**
161666_f_at	growth arrest and DNA-damage-inducible 45 beta (GADD-45b)	AV138783	**-2.3**	**2.8**	**1.2**
	humoral immune response				
98562_at	complement component 1, q subcomponent, alpha polypeptide	X58861	**-1.9**	**2.7**	**1.4**
98980_at	CD37 antigen	U18372	**-3.9**	**4.6**	**1.2**
	G1/S transition of mitotic cell cycle				
103910_at	TAF10	AJ249987	**-1.7**	**2.4**	**1.4**
	negative regulation of cell proliferation				
102264_at	schlafen 1	AF099972	**2.0**	**-2.4**	**-1.2**
	regulation of transcription				
97813_at	v-rel reticuloendotheliosis viral oncogene homolog A, relA	M61909	**-2**	**2.3**	**1.1**
103091_at	avian reticuloendotheliosis viral (v-rel) oncogene related B, relB	M83380	**-2.9**	**2.6**	**-1.1**
	serine/threonine protein kinase				
95721_at	MAP kinase-activated protein kinase 2	AW120722	**-8.5**	**6.1**	**-1.4**
101007_at	MAP kinase-interacting serine/threonine kinase 2	AI845732	**-3.1**	**2.4**	**-1.2**
160558_at	thymoma viral proto-oncogene 2, Akt2	U22445	**-2.2**	**2.5**	**1.2**
	response to external stimulus				
101870_at	immunoglobulin heavy chain 4 (serum IgG1)	V00793	**3.5**	**-2.2**	**1.6**
160836_at	CD100 - SEMA 4D	U69535	**-1.8**	**2.4**	**1.4**
	tumor infiltration				
93397_at	chemokine (C-C) receptor 2, CCR2	U56819	**1.8**	**-3.0**	**-1.7**

A gene is considered differentially expressed if its relative expression is twofold higher or lower in comparison to the baseline (P=0.05). Only 16 genes out of 30 identified genes are listed and additional 14 genes and ESTs with similar expression patterns are not shown.

other cell adhesion molecules ICAM-2 (4.2 fold) and calponin 2 (7.1 fold) were upregulated in EGRxSLP65$^{-/-}$ B cells. Transcription factors belonging to the NFkB complex, relA (2.3 fold) and relB (2.6 fold) as well as serine/threonine protein kinases Akt2/PKB-beta (2.5 fold), MAPKAPK2 (6.1 fold) and MAPK-interacting kinase 2/MKNK2 (2.4 fold) were also upregulated in EGRxSLP65$^{-/-}$ B cells. Interestingly, the expression of SLFN1 gene, negative regulator of cell proliferation (Schwarz et al., 1998), was decreased by 2.4 fold, whereas the expression of TAF10 gene, which is necessary in G1/S transition of mitotic cell cycle (Metzger et al., 1999), was by increased 2.4 fold in EGRxSLP65$^{-/-}$ B cells. The expression of CD37 antigen, a gene involved in T-cell dependent humoral immune responses (Knobeloch et al., 2000), was increased 4.6 fold and the expression of CD100 antigen, a natural ligand for CD72 (Kumanogoh et al., 2000), was increased 2.4 fold, whereas the expression of CCR2, involved in tumor infiltration (Sica et al., 2000), was decreased 3.0 fold in EGRxSLP65$^{-/-}$ B cells. All of the above mentioned genes were found to be non-differentially expressed between EGRxSLP65$^{-/-}$ and wild type IgD^{+} B cells, suggesting that EGR1 transgene activity in EGRxSLP65-/- B cells either directly or indirectly altered the expression of these genes to levels comparable with control B cells.

Among 141 differentially regulated genes, 95 genes were upregulated and 16 genes were downregulated in EGRxSLP65$^{-/-}$ in comparison to SLP65$^{-/-}$ B cells and the expression patterns of these 111 genes were found to be differential also between EGRxSLP65$^{-/-}$ and wild type B cells. A partial list of the genes and their possible functions are listed in Table 3. The expression of one gene and one cDNA clone had expression patterns characteristic of EGRxSLP65$^{-/-}$ > SLP65$^{-/-}$ > BALB/c. The expression of both the TRAF1 gene, negative regulator of TNFR2-mediated proliferation and NFkB activation (Tsitsikov et al., 2001), and a cDNA clone, MGC:6827, was 2.2- fold higher in SLP65$^{-/-}$ B cells compared to the control, while being expressed at even higher levels a further 2.0 and 2.7 fold, respectively, in EGRxSLP65$^{-/-}$ compared to SLP65$^{-/-}$ B cells. Five genes: CD72 (-7.9 fold), ApoE (-14.3 fold), LDLR-related protein 10 (-9.4 fold), Ndr1 (-8.9 fold) and epoxide hydrolase 1 (-28.8 fold) were downregulated in SLP65$^{-/-}$ mice compared to the control, whereas the expression of CD72 (3.5 fold), ApoE (4.1 fold), LDLR-related protein 10 (4.8 fold), Ndr1 (2.0 fold) and epoxide hydrolase 1(4.2 fold) increased in EGRxSLP65$^{-/-}$ mice in comparison to SLP65$^{-/-}$ littermates. While 26 genes had the expression patterns characteristic of EGRxSLP65$^{-/-}$ > BALB/c > SLP65$^{-/-}$ mice, another 62 genes were characterized as being upregulated only in EGRxSLP65$^{-/-}$ B cells, whereas the expression levels of these genes were equal in SLP65$^{-/-}$ and control mice. There are 11 genes which were downregulated in EGRxSLP65$^{-/-}$ mice, but

Table 3: Genes differentially expressed between EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control (BALB/c) IgD^{+} B cells.

Probe Set	Genes and Functions	Accession	Ratio of Relative Expression Levels: SLP65$^{-/-}$ / Control	Ratio of Relative Expression Levels: SLP65$^{-/-}$ / EGRxSLP65$^{-/-}$	Ratio of Relative Expression Levels: EGRxSLP65$^{-/-}$ / Control
	Calcium-binding proteins, S-100/ICaBP type				
103887_at	S100 calcium binding protein A9 (calgranulin B), CalB	M83219	**4.9**	**-2.2**	**2.3**
100959_at	S100 calcium binding protein A13	X99921	**1.2**	**2.8**	**3.3**
103448_at	S100 calcium binding protein A8 (calgranulin A), CalA	M83218	**6.1**	**-2.5**	**2.4**
	regulation of transcription				
97497_at	Notch gene homolog 1, (Drosophila)	Z11886	**-1.5**	**3.7**	**2.4**
99622_at	Kruppel-like factor 4 (gut)	U20344	**1.1**	**-2.2**	**-2.1**
102893_at	POU domain, class 2, transcription factor 1, Oct1	X68363	**-2.1**	**-1.3**	**-2.7**
92344_at	SWI/SNF related, actin dependent regulator of chromatin	AF010600	**-2.6**	**-1.5**	**-3.9**
	RNA processing				
95049_at	small nuclear ribonucleoprotein D2	AI837853	**1**	**2.4**	**2.4**
96188_at	adenosine deaminase, RNA-specific	AF052506	**-1.6**	**3.8**	**2.4**
	translation elongation factor				
103674_f_at	eukaryotic translation initiation factor 2, subunit 3	AJ006584	**-7.5**	**-7.7**	**-57.7**
160129_at	eukaryotic translation elongation factor 1 delta	AI839632	**-3.2**	**8.7**	**2.7**
	Metalloendopeptidase				
94186_at	Tnf receptor-associated factor 1	L35302	**2.2**	**2**	**4.3**
	protein serine/threonine phosphatase				
95631_at	protein phosphatase 4, catalytic subunit	AF088911	**-2.3**	**4.8**	**2.1**
	protein tyrosine kinase				
92668_at	Bruton agammaglobulinemia tyrosine kinase	L10627	**-3.3**	**11.5**	**3.5**
93662_s_at	zeta-chain (TCR) associated protein kinase (70kD)	AI386093	**1.9**	**2.2**	**4.1**
	1-phosphatidylinositol 3-kinase complex				
104461_at	phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	AW121773	**-1.6**	**-2**	**-3.1**
	negative regulation of cell proliferation				
92471_i_at	schlafen 2, SLFN2	AF099973	**-2.1**	**4.6**	**2.1**

Only 17 genes out 111 differentially regulated genes are listed here (P=0.05).

Table 3 (continued): Genes differentially expressed between EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control (BALB/c) IgD^{+} B cells.

Probe Set	Genes and Functions	Accession	Ratio of Relative Expression Levels: SLP65$^{-/-}$ / Control	Ratio of Relative Expression Levels: SLP65$^{-/-}$ / EGRxSLP65$^{-/-}$	Ratio of Relative Expression Levels: EGRxSLP65$^{-/-}$ / Control
	mitochondrial electron transport chain complex				
93820_at	cytochrome c oxidase, subunit VIIa 3	AF037371	**1**	**3.5**	**3.4**
96280_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 2	AI851603	**1.1**	**2.1**	**2.3**
99463_at	cytochrome P450, steroid inducible 3a13	X63023	**1.4**	**-2.8**	**-2**
	chloride transport				
94464_at	chloride channel 3	AF029347	**-3.6**	**-2.3**	**-8.3**
95654_at	chloride intracellular channel 1	AF109905	**-3**	**8.1**	**2.7**
	lipid transport				
96186_at	low-density lipoprotein receptor-related protein 10	AI839286	**-9.4**	**4.8**	**-2**
95356_at	apolipoprotein E	D00466	**-14.3**	**4.1**	**-3.5**
	small GTPase mediated signal transduction				
96059_at	RAB6, member RAS oncogene family	AA823336	**-1.1**	**2.7**	**2.5**
101113_at	ras homolog gene family, member A2	AI846668	**-1.1**	**2.4**	**2.1**
	response to external stimulus				
101487_f_at	lymphocyte antigen 6 complex, locus E, Ly-6E	U47737	**1**	**2.7**	**2.8**
101587_at	epoxide hydrolase 1, microsomal	U89491	**-28.8**	**4.2**	**-6.8**
102372_at	immunoglobulin joining chain	M90766	**-1.8**	**-2.9**	**-5.1**
101878_at	CD72 antigen	J04170	**-7.9**	**3.5**	**-2.3**
102730_at	histocompatibility 2, M region locus 3	AA163474	**1**	**-2.3**	**-2.3**
92694_at	chitinase 3-like 3	M94584	**1.5**	**-5.2**	**-3.5**
99405_at	immunoglobulin kappa chain variable 28 (V28)	U30241	**-1.7**	**4.4**	**2.5**
160553_at	lymphocyte antigen 6 complex, locus D, Ly-6D	X63782	**1.5**	**4.4**	**6.4**
101054_at	CD74	X00496	**-3.7**	**1.2**	**4.3**
93078_at	lymphocyte antigen 6 complex, locus A, Ly-6A/E	X04653	**19.7**	**1.5**	**29.0**
	apoptosis regulator				
93536_at	Bcl2-associated X protein	L22472	**1.5**	**2.8**	**4.3**

Only 20 genes out of 111 differentially regulated genes are listed here (P=0.05).

were non-differentially expressed between SLP65$^{-/-}$ and control mice. Interestingly, the expression of DEAD box popypeptide gene (-5.4 fold), translation initiation factor EIF2G gene (-7.5 fold) and chloride channel 3, CLCN3 (-3.6 fold) were downregulated in SLP65$^{-/-}$ mice compared to the control, whereas in EGRxSLP65$^{-/-}$ mice the expression of these genes: DEAD box popypeptide gene (-3.1 fold), translation initiation factor EIF2G gene (-7.7 fold) and chloride channel 3, CLCN3 (-2-3 fold) was further downregulated to even lower levels compared to SLP65$^{-/-}$ mice. Two calcium binding protein genes, calgranulin A (6.1-fold) and calgranulin B (4.9-fold) were upregulated in SLP65$^{-/-}$ mice compared to the control, while Calgranulin A (-2.5 fold) and calgranulin B (-2.2 fold) downregulated in EGRxSLP65$^{-/-}$ mice compared to SLP65$^{-/-}$ littermates.

3.2.3.3 Genes which are differentially regulated upon BCR stimulation in SLP65$^{-/-}$ B cells

The first comparison between unstimulated and stimulated samples was performed using DChip software and the criteria: present call ≥ 33% (i.e. the gene is present in at least one of three replicate arrays) and the two group t-test has a p-value of $P<0.05$. This resulted in 886 genes to be found differentially regulated in all the following 6 comparisons: BALB/c – 0 min versus BALB/c – 45 min; BALB/c – 0 min versus BALB/c – 6 hours; BALB/c – 45 min versus BALB/c – 6 hours; as well as SLP65$^{-/-}$ – 0 min versus SLP65$^{-/-}$ – 45 min; SLP65$^{-/-}$ – 0 min versus SLP65$^{-/-}$ – 6 hours and SLP65$^{-/-}$ – 45 min versus SLP65$^{-/-}$ – 6 hours. When we applied more stringent criteria: present call ≥ 66% (i.e. a gene is called present in at least two of three replicate arrays) and two group t-test has a p-value of $P<0.01$, 600 genes (4.83% of all 12422 genes on the arrays) were found to be differentially regulated in all of the above 6 comparisons. Upon BCR stimulation in BALB/c mice, the expression of 37 genes was increased twofold and that of 62 genes was decreased twofold after 45 minutes of stimulation. After 6 hours BCR stimulation, 44 genes were upregulated and 168 genes were downregulated in wild type B cells. 22 genes were found to be upregulated and 8 genes were downregulated after 6 hours of stimulation in comparison to 45 minutes of stimulation in BALB/c mice. In SLP65$^{-/-}$ mice, 44 genes are upregulated and 10 genes downregulated after 45 minutes, whereas 39 genes are upregulated and 56 genes downregulated after 6 hours of BCR stimulation. Comparison of B cells from SLP65$^{-/-}$ mice stimulated for 45 min or 6 hours identifed 21 genes that were upregulated and 184 genes that were downregulated. Among differentially regulated 600 genes, we were interested in those genes which are differentially expressed between SLP65$^{-/-}$ and wild type mice after 45 minutes and 6 hours of BCR

stimulation, because the changes in the expression patterns of those genes would reflect the defects in the primary response (within the first 45 minutes of BCR stimulation) and the secondary response (within a few hours of BCR stimulation) in SLP65$^{-/-}$ B cells. Comparison of SLP65$^{-/-}$ with BALB/c B cells after 45 min of BCR stimulation revealed that there are only 9 genes downregulated, whereas 61 genes are upregulated (Fig. 14a). Only 4 genes: simple repeat sequence-containing transcript, regulator of transcription, (-3.4 fold), DEAD-box polypeptide 6, RNA helicase gene, (-8.8 fold), PSMB1, proteosomal subunit C5, (-3.0 fold), MRPL39, mitochondrial ribosomal protein, (-2.1 fold) and 5 additional EST sequences and cDNA clones were downregulated in SLP65$^{-/-}$ B cells after 45 minutes of stimulation compared to the control (Table 4). Among 61 upregulated genes, Max protein gene (4.5 fold), L-Myc and N-Myc interacting protein (FitzGerald et al., 1999), CD63 antigen (3.0 fold), a gene associated with early stages of tumor progression (Radford et al., 1997), TRIF gene (6.0 fold), essential for TLR3 and TLR4 mediated signaling (Yamamoto et al., 2003), serum inducible kinase (2.1 fold), annexin A1 (4.8 fold), Ly-6A gene (10.2 fold), a few cytoskeleton genes and a few kappa light chain variable region genes along with other genes and EST sequences were increased in SLP65$^{-/-}$ B cells compared to the control. A partial list of the genes and their possible involvement in cellular processes are listed in the Table 5. Comparison between SLP65$^{-/-}$ and wild type B cells of genes induced after 6 hours of BCR stimulation identified 20 genes whose expression is decreased (Table 6) and 12 genes whose expression is increased (Table 7) in SLP65$^{-/-}$ B cells compared to the control (Fig. 14b). Interestingly, inhibitors of NFkB signaling, IkB-alpha (-2.3 fold), IkB-beta (-2.2 fold); a protein tyrosine phosphatase, Ptp4a3 (-2.6 fold); negative regulator of c-Myb dependent trans-activation gene, Trim28 (-3.0 fold); negative regulator of cell cycle progression, p21/Cdkn1A (-3.1 fold); inhibitor of caspase-independent cell death gene, Spi2A (-2.2 fold); MAPKAPK2 (-2.5 fold); two interferon regulatory factors, IRF1 (-3.8 fold) and IRF8 (-2.0 fold); a gene involved in MHC class II Ag processing, H2-Eb1 (-2.0 fold); two genes involved in MHC class I Ag processing, TAP1 (-3.2 fold) and TAP2 (-2.1 fold) as well as a few other genes and EST sequences were downregulated in SLP65$^{-/-}$ B cells (Table 6). The expression levels of the following genes, induced or suppressed after 6 hours of BCR stimulation, were higher in SLP65$^{-/-}$ B cells: inhibitor of phospholipase A2, annexin A1 (4.7 fold), phospholipase A2 (4.5 fold), a chemokine receptor, CXCR4 (4.0 fold), Ly-6E (2.3 fold), lactotransferrin (3.9 fold), zinc finger protein 125 gene (2.8 fold), aldehyde dehydrogenase 2 gene (3.6 fold), cathelin-like protein gene (3.8 fold) as well as 4 other EST sequences were upregulated in SLP65$^{-/-}$ B cells in comparison to the control (Table 7).

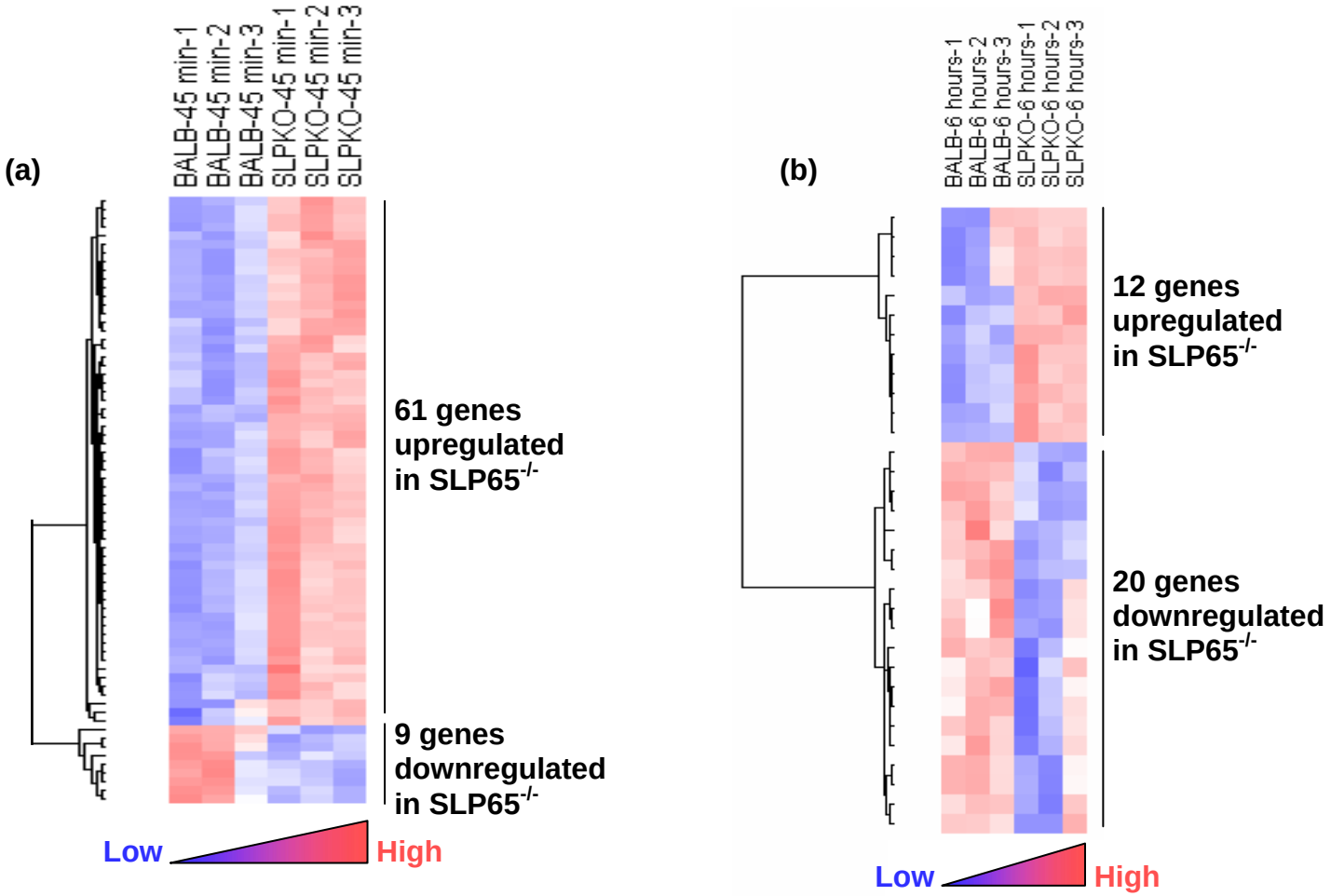


Figure 14: Genes differentially regulated in SLP65$^{-/-}$ B cells upon BCR stimulation. Hierachical clustering for comparisons between SLP65$^{-/-}$ and control after (a) 45 minutes of BCR stimulation and (b) 6 hours of stimulation. A gene is considered to be differentially expressed if its relative expression is 2 fold higher or lower in comparison to the baseline. After 45 minutes of BCR stimulation there are 9 genes downregulated and 61 genes upregulated in SLP65$^{-/-}$ B cells compared to the control. After 6 hours of BCR stimulation 20 genes were found to be downregulated and 12 genes upregulated in SLP65$^{-/-}$ B cells in comparison to the control.

Table 4: Genes mediating defects in the primary response to antigen in SLP65$^{-/-}$ B cells (downregulated) after 45 minutes of BCR stimulation.

Accession	Genes	Ratio of Relative Expression Levels					
		Control			SLP65$^{-/-}$ / Control		
		t=45´/t=0	t=6h/t=0	t=6h/t=45´	t=0	t=45´	t=6h
D50494	DEAD-box polypeptide 6, RNA helicase	**1.1**	**-1.3**	**-1.5**	**-2.6**	**-8.8**	**-10.9**
AA419684	cDNA clone, Mm.24548	**6.3**	**6.4**	**1.0**	**1.2**	**-4.1**	**-1.4**
X67863	Srst, regulation of transcription	**2.2**	**2.5**	**1.1**	**1.0**	**-3.4**	**1.1**
AF090314	PSMB1, proteasome subunit	**2.8**	**2.1**	**-1.3**	**-1.3**	**-3.0**	**-1.1**
AI847033	ESTs, similar to mitochondrial translational release factor 1	**2.7**	**3.2**	**1.2**	**1.3**	**-2.7**	**-1.2**
AW123796	cDNA clone, MGC:6827, IMAGE:2649084	**2.3**	**2.8**	**1.2**	**2.2**	**-2.7**	**1.3**
AI837905	cDNA clone, Mm.89082	**1.7**	**1.6**	**-1.1**	**-1.6**	**-2.3**	**-1.1**
AI850352	RIKEN cDNA 3110040D16 gene	**1.7**	**2.6**	**1.5**	**-1.2**	**-2.3**	**-1.4**
AW045881	mitochondrial ribosomal protein L39	**1.6**	**2.0**	**1.2**	**-1.5**	**-2.1**	**-1.1**

9 genes were downregulated in SLP65$^{-/-}$ B cells in comparison to the wild type B cells after 45 minutes of stimulation (P=0.01). Abbreviations: t=0 – unstimulated sample; t=45´ - 45 minutes and t=6h – samples stimulated for 6 hours.

Table 5: Genes mediating defects in the primary response to antigen in SLP65$^{-/-}$ B cells (upregulated) after 45 minutes of BCR stimulation.

Accession	Genes	Ratio of Relative Expression Levels					
		Control			SLP65$^{-/-}$ / Control		
		t=45´/t=0	t=6h/t=0	t=6h/t=45´	t=0	t=45´	t=6h
AI851740	actin related protein 2/3 complex, actin polymerization	**-1.1**	**-1.2**	**-1.1**	**1.0**	**2.8**	**2.1**
AI851227	actin-related protein 10 homolog	**-1.9**	**-1.5**	**1.3**	**1.1**	**3.2**	**2.3**
AV329607	aldehyde dehydrogenase 2, mitochondrial	**-1.8**	**-3.8**	**-2.2**	**1.0**	**2.6**	**3.6**
AF020313	amyloid beta (A4) precursor protein-binding 1 interacting protein	**-2.1**	**-1.8**	**1.2**	**1.6**	**3.1**	**2.1**
AV003419	annexin A1	**3.1**	**3.2**	**1.0**	**1.7**	**4.8**	**4.7**
AL009226	bromodomain-containing 2, protein serine/threonine kinase	**-1.6**	**-2.5**	**-1.6**	**-2.3**	**3.0**	**1.8**
X94353	cathelin-like protein (Camp), innate host defence	**8.8**	**14.7**	**1.7**	**2.8**	**6.1**	**3.8**
D16432	Cd63 antigen	**7.6**	**35.8**	**4.7**	**1.2**	**3.0**	**1.5**
AF109905	chloride intracellular channel 1	**-2.1**	**-1.9**	**1.1**	**-2.3**	**2.8**	**1.9**
AF061272	C-type (calcium dependent) lectin, superfamily member 8	**85.6**	**467.9**	**5.5**	**3.1**	**3.2**	**1.0**
AF037371	cytochrome c oxidase, subunit VIIa 3	**-1.4**	**-1.6**	**-1.2**	**1.0**	**2.9**	**2.6**
AF098508	dynactin 3, microtubule associated complex	**-1.3**	**-1.6**	**-1.3**	**-2.2**	**2.0**	**1.4**
D73368	enhancer of rudimentary homolog (Drosophila)	**-1.7**	**-1.7**	**1.0**	**-1.5**	**3.7**	**1.7**
AF026481	eukaryotic translation initiation factor 1A	**-1.3**	**1.0**	**1.2**	**-1.1**	**3.1**	**2.7**
X68193	expressed in non-metastatic cells 2 (diphosphate kinase)	**-1.8**	**-1.7**	**1.1**	**1.1**	**2.5**	**1.3**
X54352	f-box and WD-40 domain protein 2, Wnt receptor signaling	**-2.3**	**-2.0**	**1.1**	**-1.6**	**2.0**	**1.1**
M18194	fibronectin 1, cell adhesion	**1.5**	**-2.1**	**-3.2**	**1.9**	**4.0**	**2.4**
AB013137	glutaredoxin 1 (thioltransferase)	**3.1**	**2.9**	**1.0**	**1.3**	**2.8**	**2.1**
AI183202	heterogeneous nuclear ribonucleoprotein A1	**-1.5**	**-2.2**	**-1.5**	**2.1**	**3.1**	**1.7**
AI844131	heterogeneous nuclear ribonucleoprotein A2/B1	**-2.6**	**-2.4**	**1.1**	**1.4**	**3.2**	**2.7**
AA822174	hydroxysteroid (17-beta) dehydrogenase 11	**-1.3**	**-1.5**	**-1.2**	**1.9**	**3.8**	**2.6**

61 genes were upregulated in SLP65$^{-/-}$ B cells in comparison to the wild type B cells after 45 minutes of stimulation. Only 21 out of 61 differentilally regulated genes are listed in this table (P=0.01). Abbreviations: t=0 – unstimulated sample; t=45´ - 45 minutes and t=6h – samples stimulated for 6 hours.

Table 5 (continued): Genes mediating defects in the primary response to antigen in SLP65$^{-/-}$ B cells after 45 minutes of BCR stimulation.

Accession	Genes	Ratio of Relative Expression Levels					
		Control			SLP65$^{-/-}$ / Control		
		t=45´/t=0	t=6h/t=0	t=6h/t=45´	t=0	t=45´	t=6h
AF045024	Igk light chain variable region, Mm.89457	**-1.1**	**1.2**	**1.3**	**-1.3**	**2.5**	**1.1**
AF045026	Igk light chain variable region, Mm.89459	**-1.2**	**1.1**	**1.3**	**-1.6**	**2.6**	**1.2**
U30241	immunoglobulin kappa chain variable 28 (V28)	**-1.2**	**1.0**	**1.2**	**-1.7**	**2.5**	**1.3**
X04653	lymphocyte antigen 6 complex, locus A, (Ly-6A)	**1.1**	**14.0**	**12.6**	**18.9**	**10.2**	**-1.1**
M21050	Lysozyme	**3.4**	**2.8**	**-1.2**	**-1.5**	**2.8**	**3.3**
M63903	Max protein, interacts with L-Myc and N-Myc	**-2.5**	**-2.8**	**-1.1**	**1.1**	**4.5**	**2.3**
X88903	mRNA for variable light chain, Mm.14127	**1.0**	**1.2**	**1.2**	**-1.3**	**2.2**	**1.1**
AW121314	NTF2-related export protein 1, mRNA transport	**-1.6**	**-1.6**	**1.0**	**-1.3**	**2.1**	**1.4**
AF093857	peroxiredoxin 5, peroxidase	**1.4**	**1.6**	**1.1**	**1.0**	**3.1**	**3.9**
AF023463	phytanoyl-CoA hydroxylase, oxidoreductase	**-2.3**	**-6.8**	**-2.9**	**1.0**	**2.3**	**2.2**
X73331	ribosomal protein L37a	**-2.4**	**-3.3**	**-1.4**	**1.2**	**5.1**	**3.5**
AW045418	ribosomal protein L44	**-2.0**	**-3.9**	**-1.9**	**1.2**	**2.7**	**2.5**
AF099977	schlafen 4, SLFN4	**11.0**	**21.7**	**2.0**	**1.6**	**2.6**	**1.8**
M96163	serum-inducible kinase	**2.8**	**21.9**	**7.9**	**1.3**	**2.1**	**1.2**
M73696	solute carrier family 20, member 1, phosphate transport	**1.9**	**1.5**	**-1.3**	**-1.8**	**2.4**	**1.7**
U96810	Supt4h2 gene, Mm.87825	**-1.8**	**-2.6**	**-1.4**	**-2.4**	**3.8**	**3.2**
AJ245617	Trf (TATA binding protein-related factor)-proximal protein	**-2.6**	**-2.5**	**1.0**	**-2.0**	**2.4**	**1.2**
AW209763	Trif gene, essential for TLR-3 and TLR-4 mediated signaling	**-4.3**	**-7.9**	**-1.8**	**1.9**	**6.0**	**4.7**
AW210080	ubiquitin-conjugating enzyme E2N	**1.0**	**-1.1**	**1.0**	**1.4**	**2.2**	**1.9**
AW122465	expressed sequence AA617263	**-1.5**	**-1.8**	**-1.2**	**-1.2**	**2.1**	**1.2**
AI835041	expressed sequence AI227013	**-5.7**	**-2.7**	**2.1**	**1.3**	**4.7**	**2.7**
AW122882	expressed sequence AI551766	**-1.4**	**-2.2**	**-1.6**	**-1.1**	**2.9**	**2.3**
X67210	expressed sequence AU044919	**5.4**	**4.0**	**-1.3**	**2.3**	**9.3**	**7.8**

61 genes were upregulated in SLP65$^{-/-}$ B cells in comparison to control wild type B cells after 45 minutes of stimulation. Only 23 out of 61 differentially expressed genes are listed and additional 17 EST sequences and RIKEN cDNAs with the similar expression patterns are not shown in this table (P=0.01). Abbreviations: t=0 – unstimulated sample; t=45´ - 45 minutes and t=6h – samples stimulated for 6 hours.

Table 6: Genes mediating defects in the secondary response to antigen in SLP65$^{-/-}$ B cells (downregulated) after 6 hours of BCR stimulation.

Accession	Genes	Ratio of Relative Expression Levels					
		Control			SLP65$^{-/-}$ / Control		
		t=45´/t=0	t=6h/t=0	t=6h/t=45´	t=0	t=45´	t=6h
AW120722	MAPKAP kinase 2	**2.3**	**2.2**	**1.0**	**-4.3**	**-1.9**	**-2.5**
U57524	Nfkbia (IkB-alpha)	**3.0**	**5.9**	**2.0**	**-2.8**	**1.0**	**-2.3**
U19799	Nfkbib (IkB-beta)	**1.2**	**2.0**	**1.7**	**-1.6**	**-1.3**	**-2.2**
AF035645	Ptp4a3, protein tyrosine phosphatase	**-7.3**	**-12.9**	**-1.8**	**-8.3**	**-1.4**	**-2.6**
U09507	Cdkn1A (p21)	**7.0**	**37.0**	**5.3**	**1.8**	**-1.9**	**-3.1**
M21065	IRF1	**1.0**	**5.1**	**4.8**	**-2.2**	**1.8**	**-3.8**
M32489	IRF8	**-3.8**	**-2.7**	**1.4**	**-1.8**	**1.5**	**-2.0**
AV138783	GADD45-beta, DNA repair	**12.8**	**24.1**	**1.9**	**-2.8**	**-1.2**	**-2.2**
U60020	TAP1, MHC class I Ag processing	**-2.8**	**1.1**	**3.0**	**-2.0**	**1.0**	**-3.2**
U60091	TAP2, MHC class I Ag processing	**-1.7**	**1.5**	**2.6**	**-2.2**	**1.1**	**-2.1**
X00958	H2-Eb1, MHC class II antigen processing	**-1.2**	**-2.6**	**-2.2**	**-5.4**	**-1.7**	**-2.0**
X99644	Trim28, negative regulat. of c-Myb depend. trans-activation	**-2.6**	**-3.4**	**-1.3**	**-8.4**	**-1.2**	**-3.0**
M64085	Serpina3g (Spi2A), inhibits caspase-independent cell death	**-1.4**	**2.9**	**4.1**	**1.4**	**2.3**	**-2.2**
AV374868	cytokine inducible SH2-containing protein 3	**5.5**	**31.1**	**5.7**	**1.8**	**1.6**	**-2.2**
D50494	DEAD-box polypeptide 6, RNA helicase	**1.1**	**-1.3**	**-1.5**	**-2.6**	**-8.8**	**-10.9**
AW120557	U6 snRNA-associat. SM-like prot. 4, spliceosome complex	**-1.7**	**-2.6**	**-1.5**	**-3.7**	**-1.1**	**-2.1**
AI839286	low-density lipoprotein receptor-related protein 10	**-2.5**	**-2.5**	**1.0**	**-5.4**	**-1.6**	**-2.4**
AW121862	RIKEN cDNA 2310044H10 gene	**-1.9**	**-3.0**	**-1.6**	**-3.6**	**-1.3**	**-3.2**
AW049776	RIKEN cDNA 2410015A15 gene	**-3.3**	**-4.0**	**-1.2**	**-5.7**	**1.0**	**-2.6**
AW125010	RIKEN cDNA 2810477H02 gene	**-4.3**	**-5.2**	**-1.2**	**-7.8**	**1.0**	**-2.2**

20 genes were downregulated in SLP65$^{-/-}$ B cells in comparison to the wild type B cells after 6 hours of stimulation (P=0.01).
Abbreviations: t=0 – unstimulated sample; t=45´ - 45 minutes and t=6h – samples stimulated for 6 hours.

Table 7: Genes mediating defects in the secondary response to antigen in SLP65$^{-/-}$ B cells (upregulated) after 6 hours of BCR stimulation.

Accession	Genes	Ratio of Relative Expression Levels					
		Control			SLP65$^{-/-}$ / Control		
		t=45´/t=0	t=6h/t=0	t=6h/t=45´	t=0	t=45´	t=6h
AV003419	annexin A1	**3.1**	**3.2**	**1**	**1.7**	**4.8**	**4.7**
U34277	phospholipase A2 group VII	**2.6**	**2.2**	**-1.2**	**1.2**	**2.7**	**4.5**
Z80112	chemokine (C-X-C) receptor 4	**2.3**	**-1.4**	**-3.1**	**1.2**	**2.4**	**4**
J03298	Lactotransferrin	**20.1**	**23.1**	**1.2**	**2.6**	**5.1**	**3.9**
X94353	cathelin-like protein	**8.8**	**14.7**	**1.7**	**2.8**	**6.1**	**3.8**
AV329607	aldehyde dehydrogenase 2, mitochondrial	**-1.8**	**-3.8**	**-2.2**	**1**	**2.6**	**3.6**
AI037577	cDNA sequence BC003993	**-3.8**	**-3.5**	**1.1**	**3.1**	**6.6**	**3.1**
AJ005350	zinc finger protein 125	**4.1**	**3.9**	**-1.1**	**5**	**1.6**	**2.8**
AW047875	ESTs	**-2.5**	**-4**	**-1.6**	**-1.2**	**3.3**	**2.5**
U47737	lymphocyte antigen 6 complex, locus E	**-1.3**	**-1.2**	**1.1**	**1**	**3.4**	**2.3**
AW122882	expressed sequence AI551766	**-1.4**	**-2.2**	**-1.6**	**-1.1**	**2.9**	**2.3**
AI842858	RIKEN cDNA 2510049I19 gene	**-2.4**	**-3.5**	**-1.4**	**1.2**	**3.2**	**2.1**

12 genes were upregulated in SLP65$^{-/-}$ B cells in comparison to the wild type B cells after 6 hours of stimulation (P=0.01). Abbreviations: t=0 – unstimulated sample; t=45´ - 45 minutes and t=6h – samples stimulated for 6 hours.

3.2.3.4 Genes selected for validation experiments

From the 141 genes differentially regulated between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ B cells and 102 genes differentially regulated between SLP65$^{-/-}$ and control B cells upon BCR stimulation, we have selected a list of genes for additional experiments to verify the microarray results. The genes were selected first of all on the availability of antibodies and in addition a few genes were selected to verify the changes at the transcriptional level by real-time RT-PCR. In total 21 genes were selected for further validation experiments. Among these 6 genes, Ly-6A/E, Ly-6D, Ly-6E, CD74, CD72 and CD100, were selected to check by FACS analysis for the differences in surface expression of these proteins between EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and wild type B cells. A further 8 genes, Akt2, Notch1, TAF10, IkB-alpha, IRF1, Oct2, relB and p21/Cdkn1A genes were selected to check by immunoblotting analysis for the differences between EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control mice as well as the differences between SLP65$^{-/-}$ and wild type B cells upon BCR stimulation. Finally 6 differentially regulated genes, CalA, CCR2, SLFN1, SLFN2, Oct1 and Blr1 were selected to run Real Time RT-PCR assays to check for the differences between EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control B cells. Bcl3, which was not differentially regulated between EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and wild type B cells whether stimulated or not, was used as a control.

3.2.3.5 Validation of differentially regulated genes by FACS

Although microarray analysis identified CD74, CD72 and CD100 genes as differentially regulated genes in comparisons, FACS analysis showed no significant differences in the cell surface expression of CD74, CD72 and CD100 antigens between EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control B cells. CD74 has been shown to be involved in MHC class I antigen processing (Henne et al., 1995). The mean number of B220^{+} CD74^{+} B cells in the spleen were 17.93% ± 1.78% (n=5) in EGRxSLP65$^{-/-}$, 16.87% ± 0.35% (n=2) in SLP65$^{-/-}$ and 15.10% ± 0.26% (n=3) in control mice (data not shown). CD72 is a B cell co-receptor that is expressed in all stages of B cell development except plasma cells. Ligation of CD72 to its ligands, e.g., to CD100, enhances B cell growth and differentiation (Wu and Bondada 2002). Almost all B220^{+} B cells in the spleen express CD72 and CD100 antigens and 97.25% ± 1.14% (n=5) of cells in EGRxSLP65$^{-/-}$, 95.54% ± 1.54% (n=7) of cells in SLP65$^{-/-}$ and 96.26% ± 1.07% of cells in control mice were B220^{+} CD72^{+} B cells (data not shown). 96-97% of all the splenic B220^{+} B cells were CD100^{+} in all mice compared (data not shown).

Although levels of Ly-6 proteins vary with stages of B cell differentiation and activation, their function largely remains unknown. Ly-6 proteins have been proposed to act by mediating

cell:cell adherence (Pflugh et al., 2002). Comparison of splenic cells from EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control mice by FACS revealed that EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ B cells express relatively high levels of all of the tested Ly-6 antigens, Ly-6A/E, Ly-6D and Ly-6E antigens, showing again the less mature phenotype of splenic B cells from EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ mice compared to the control (Fig. 15a). According to the microarray results, the expression of Ly-6A/E antigen was upregulated by 19.7 fold in SLP65$^{-/-}$ B cells and to even higher levels (29.0-fold upregulated) in EGRxSLP65$^{-/-}$ B cells compared to the control. This upregulation was confirmed by FACS data, where 27.64% ± 4.61% (n=8; P<0.001) of cells in EGRxSLP65$^{-/-}$, 20.53% ± 0.99% (n=2; P=0.02) of cells in SLP65$^{-/-}$ and only 13.28% ± 6.40% (n=6) of cells in control mice expressed Ly-6A/E antigen (Fig. 15c). Microarray results showed a 6.4 fold upregulation of Ly-6D and a 2.8 fold upregulation of Ly-6E antigens in EGRxSLP65$^{-/-}$ B cells compared to the control, but no significant differences were found between SLP65$^{-/-}$ and control B cells in the expression of the above antigens. FACS data confirmed the differences between EGRxSLP65$^{-/-}$ and control B cells but the expression of Ly-6D and Ly-6E antigens were as high in SLP65$^{-/-}$ B cells as in EGRxSLP65$^{-/-}$ B cells (Fig. 15, b and d). On average, 31.45% ± 6.36% (n=10; P<0.001) of cells in EGRxSLP65$^{-/-}$, 30.84% ± 4.48% (n=7; P<0.00004) of cells in SLP65$^{-/-}$ and 17.19% ± 4.32% (n=9) of cells in control mice were Ly-6E^{+} B cells. On average, 34.41% ± 2.31% (n=5; P<0.001) of cells in EGRxSLP65$^{-/-}$, 32.39% ± 3.15% (n=7; P<0.001) of cells in SLP65$^{-/-}$ and only 19.53% ± 3.52% (n=6) of cells from control mice expressed Ly-6D antigen on the surface. Thus, microarray results were confirmed by FACS analysis on the protein level for 3 genes out of 6 selected differentially regulated genes.

3.2.3.6 Reduced TACI expression in EGRxSLP65$^{-/-}$ spleen B cells

To further phenotypically characterize the spleen B cells from EGRxSLP65$^{-/-}$ mice we have analyzed spleen B cells from EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control mice for the expression of receptors for BAFF (B cell Activating Factor belonging to the TNF Family), since this TNF family member, BAFF/BLyS, is a fundamental survival factor for B cells. BAFF is produced by cells of the myeloid lineage, notably by dendritic cells that express CD11c at high levels and possibly also produced by some T cells (Schneider et al., 1999; Nardelli et al., 2001). BAFF binds to two receptors with different affinities: BAFF-R and TACI (Gras et al., 1995; Gross et al., 2000; Wu et al., 2000). Both receptors are type I single transmembrane receptors of TNF family and are expressed on CD19^{+} B lymphocytes, TACI is also expressed on some T cells (Laabi et al., 1994; Gras et al., 1995; Gross et al., 2000). TACI

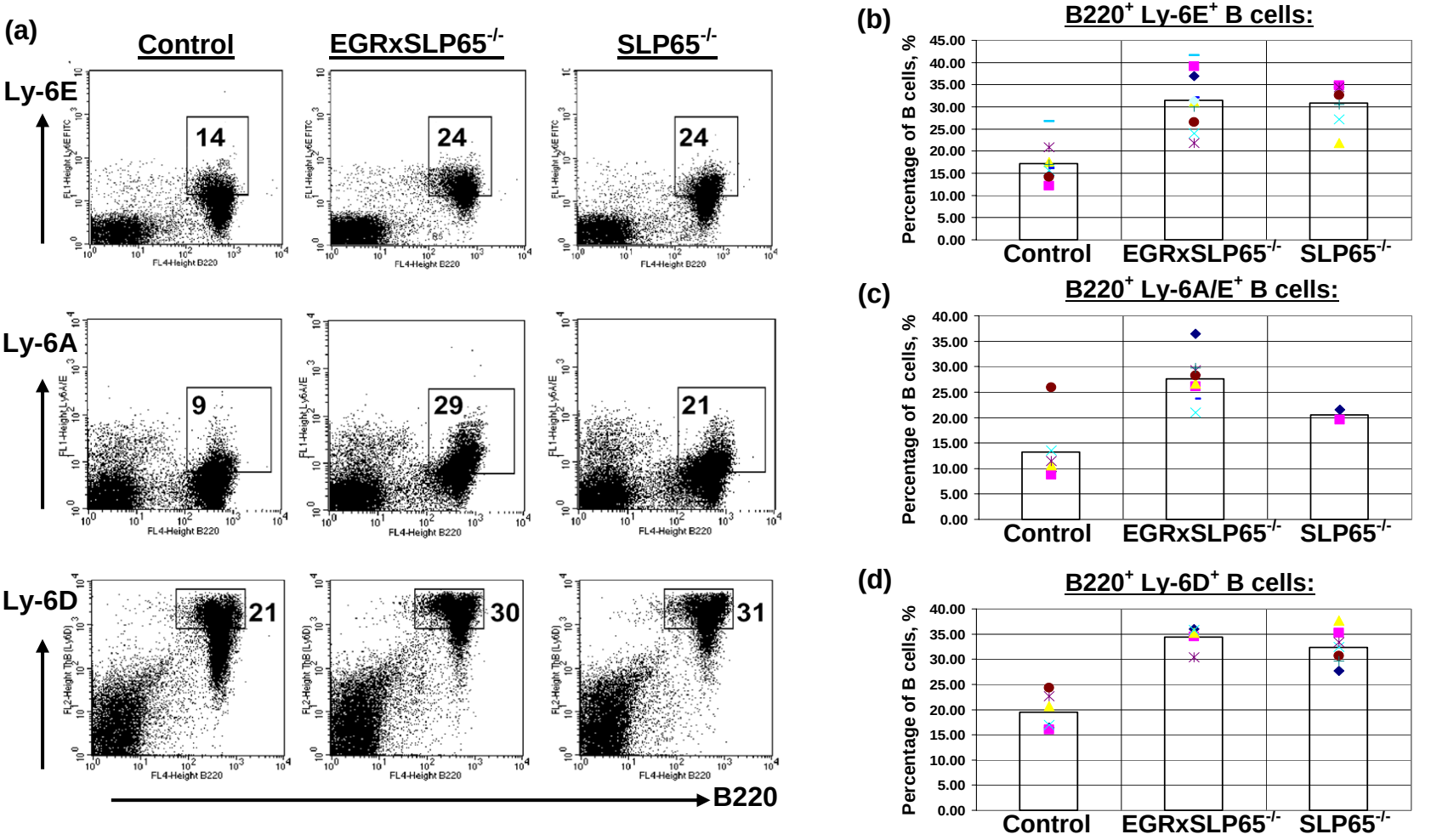


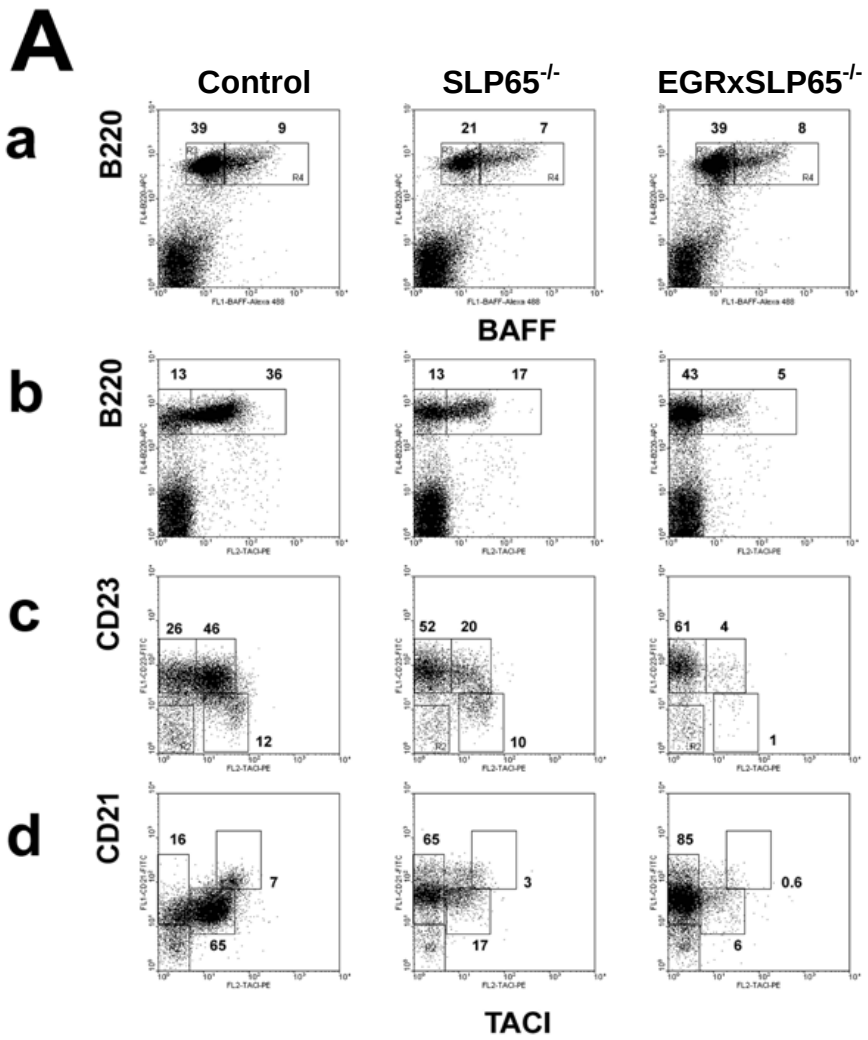
Figure 15: Immature transitional splenic B cells from EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ mice express high levels of Ly-6 antigens. Due to the block at transitional B cell stages leading to the higher frequency of immature transitional B cells in the spleen of SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice splenic B cells from both mutant animals express relatively high levels of all three Ly-6 antigens tested. (a) Individual examples are given and the numbers indicate the percentage of B220^{+} cells in a specific subset. The diagrams show the percentage of (b) B220^{+} Ly-6E^{+} B cell, (c) B220^{+} Ly-6A^{+} B cell and (d) B220^{+} Ly-6D^{+} B cell subsets in the spleen.

(Transmembrane Activator and CAML Interactor) acts as a receptor for both APRIL and BAFF ligands and plays an important role in B cell development and function. TACI may modulate BAFF responses in concert with BAFF-R, possibly by tuning down proliferation and/or maturation of immature B cells (Melchers 2003).

FACS analysis with Alexa Fluor 488- labeled recombinant BAFF protein showed that the overall expression of the receptors for BAFF is similar in splenic B cells from EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control mice (Fig. 16A, a). However, FACS analysis for the expression of TACI on splenic B cells revealed that SLP65$^{-/-}$ B cells express lower levels of TACI and the expression of TACI was drastically decreased in EGRxSLP65$^{-/-}$ B cells in comparison to SLP65$^{-/-}$ and control mice (Fig. 16A, b). To further characterize distinct B cell subpopulations in the spleen for the expression of TACI, we stained spleen cells with a combination of anti-CD21 and anti-CD23 together with TACI antibodies. The mean number of B220^{+} CD23^{+} TACI^{+} mature B cells was 45.49% ± 12.09% (n=3) in BALB/c, 18.95% ± 4.20% (n=3; P=0.02) in SLP65$^{-/-}$ and only 4.66% ± 0.24% (n=3; P=0.014) in EGRxSLP65$^{-/-}$ mice (Fig 16A, c and B). The percentage of B220^{+} CD23^{-} TACI^{hi} marginal zone B cells were as follows: 3.57% ± 0.47% (n=3) in BALB/c, 2.72% ± 0.91% (n=3; P=0.16) in SLP65$^{-/-}$ and only 0.39% ± 0.14% (n=3; P<0.004) in EGRxSLP65$^{-/-}$ mice. Characterization by CD21 and TACI expression identified 51.80% ± 9.39% (n=3) of cells in BALB/c, 13.81% ± 0.79% (n=3; P<0.01) of cells in SLP65$^{-/-}$ and 4.16% ± 0.59% (n=3; P<0.007) of cells in EGRxSLP65$^{-/-}$ mice were B220^{+} CD21^{+} TACI^{+} mature B cells. On average, 7.04% ± 1.66% (n=3) of cells in BALB/c, 2.34% ± 0.60% (n=3; P=0.01) of cells in SLP65$^{-/-}$ and 0.52% ± 0.18% (n=3; P=0.01) of cells in EGRxSLP65$^{-/-}$ mice were B220^{+} CD21^{hi} TACI^{hi} marginal zone B cells (Fig. 16A, d). Promoter sequence analyses showed that there are no putative EGR1 transcription factor binding sites in the mouse TACI promoter, whereas there are 3 binding sites at positions -100, -1066 and -1585 relative to the start of transcription in the human TACI promoter. This suggests that TACI is not a direct EGR1 target gene in mouse and downregulation of TACI cell surface expression in EGRxSLP65$^{-/-}$ mice might be due to the secondary effects mediated by EGR1 overexpression in EGRxSLP65$^{-/-}$ B cells.

3.2.3.7 Validation of differentially regulated genes by Immunoblotting assays

Total cell lysates from IgD^{+} splenic B cells of EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and BALB/c mice were analyzed by immunblotting assay for the expression of Akt2, Notch1 and TAF10 proteins. In order to increase the yield, spleens from each genotype of mice, EGRxSLP65$^{-/-}$ (n=3), SLP65$^{-/-}$ (n=3) and BALB/c (n=3) were pooled before purification of IgD^{+} B cells.



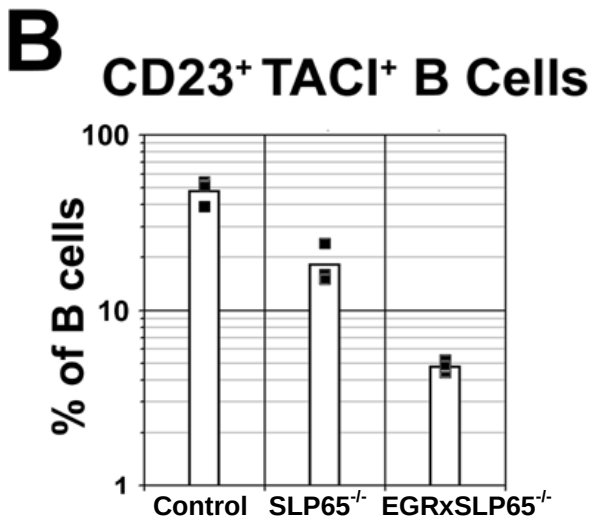


Figure 16: Reduced TACI expression in EGRxSLP65$^{-/-}$ spleen B cells. Spenic cells from control (BALB/c), SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice were stained with Alexa 488 labeled recombinant BAFF protein. (A) BAFF binding to BAFF-R and TACI (a) as well as the following stainings of spleen B cells from control (BALB/c), SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice: B220 and TACI (b), CD23 and TACI (c), CD21 and TACI (d) are shown. The diagram (B) shows the percentage of B220^{+} CD23^{+} TACI^{+} B cells in the spleen.

Although microarray data showed differential expression levels of the genes, Akt2 and Notch1 between EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control B cells, Akt2 and Notch1 proteins were expressed in IgD^{+} B cells at comparable levels in all samples by immunoblot analysis (data not shown). According to microarray data, the TAF10 gene was upregulated 2.4 fold in EGRxSLP65$^{-/-}$ B cells compared to SLP65$^{-/-}$ B cells. Western-blot analysis confirmed that the TAF10 protein levels were higher in EGRxSLP65$^{-/-}$ B cells (5.3 fold) in comparison to SLP65$^{-/-}$ B cells (Fig. 17, a, b and c). Promoter sequence analyses identified putative EGR1 transcription factor binding sites in the TAF10 promoter (Table 8). There are 3 binding sites at the positions -138, -637 and -1942 relative to the start of transcription in the mouse as well as a single binding site at the position -266 relative to the start of transcription in human TAF10 gene promoter, suggesting that TAF10 might be a direct target for EGR1 transcription factor in B cells.

Although p21 gene was not differentially expressed between unstimulated EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control B cells, according to the microarray data, after 6 hours of BCR stimulation the expression levels of p21 gene were decreased by 3.1 fold in SLP65$^{-/-}$ B cells compared to the control. In the next step, we examined p21 protein expression in unstimulated and BCR stimulated samples by western blotting. B220^{+} B cells were purified from spleens of mice and total cell lysates were prepared either directly or after 45 minutes, 4 hours and 6 hours of anti-IgM F(ab')$_2$ stimulation. p21 protein could not be detected either in total cell lysates or nuclear extracts, whereas it was detectable in NIH 3T3 mouse fibroblast cells after overnight starvation and 6 hours of serum response (data not shown). Additionally, the expression of IkB-alpha, Oct2 and relB proteins were found to be unchanged between unstimulated EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control B cells as well as between SLP65$^{-/-}$ and wild type B cells upon BCR stimulation, although the transcripts of these proteins were identified as differentially regulated by microarray analysis (data not shown).

Interestingly, the IRF1 protein induction kinetics upon BCR stimulation detected by immunblot assay were very similar to the IRF1 transcript induction kinetics measured by microarray analysis (Fig. 18, a, b and c). According to our data, IRF1 expression is slightly decreased or unchanged within the first hour of stimulation and it is increased after 6 hours of BCR stimulation in normal splenic B cells. However, IRF1 expression cannot be properly regulated in the absence of SLP65 adaptor protein within the first a few hours of stimulation and is thus decreased after 6 hours of BCR stimulation in SLP65$^{-/-}$ B cells. Thus, differences in mRNA levels of 8 selected genes were confirmed for 2 genes on the protein level by immunoblot analysis.

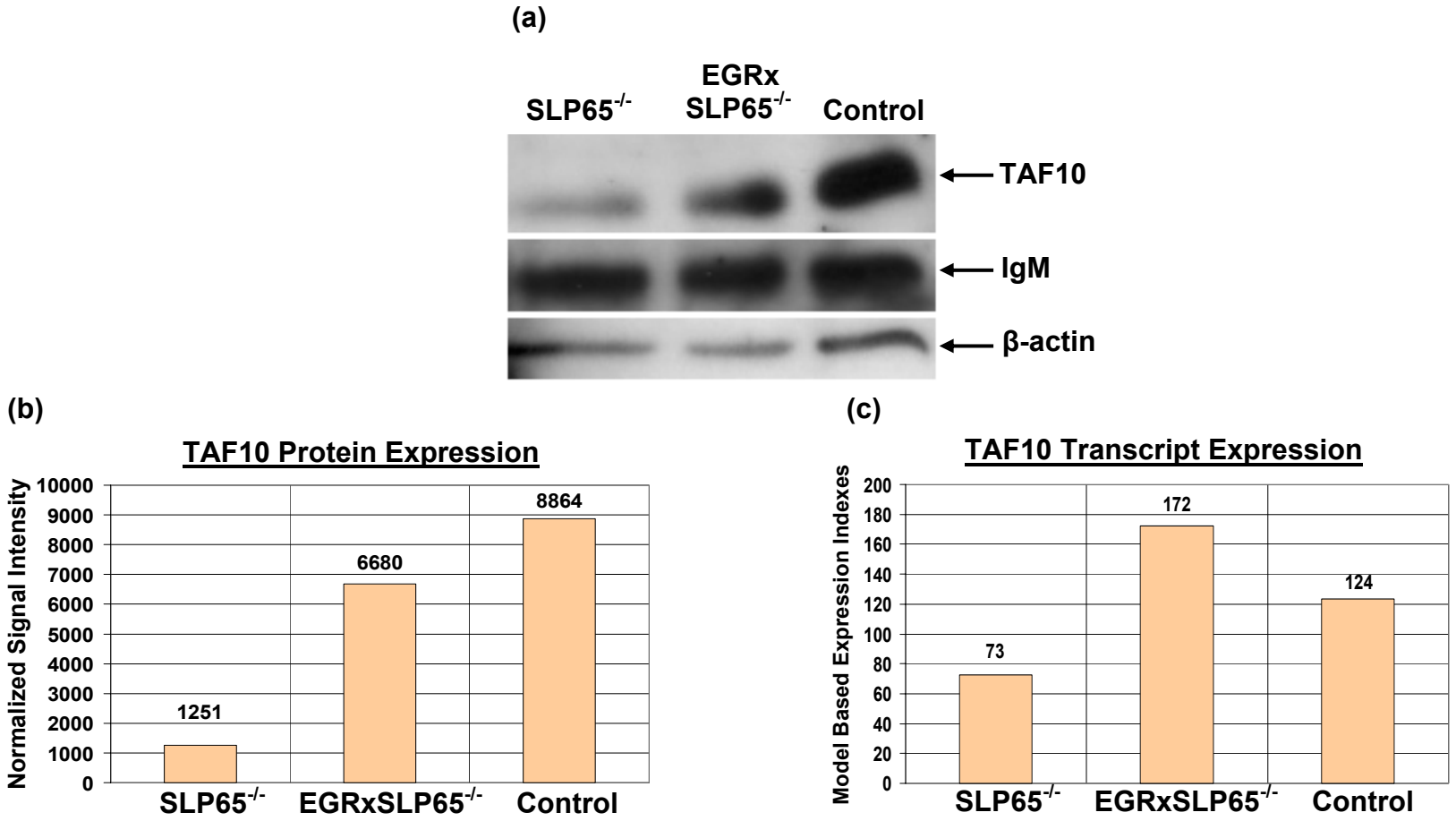


Figure 17: TAF10 protein expression levels are partially restored in EGRxSLP65$^{-/-}$ IgD^{+} splenic B cells. (a) Western-blot analysis of TAF10 protein expression in IgD^{+} splenic B cells from control (BALB/c), SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice. IgM and β-actin were used as internal controls. The diagrams depict relative levels of expression of (b) TAF10 protein and (c) TAF10 transcript measured by microarrays.

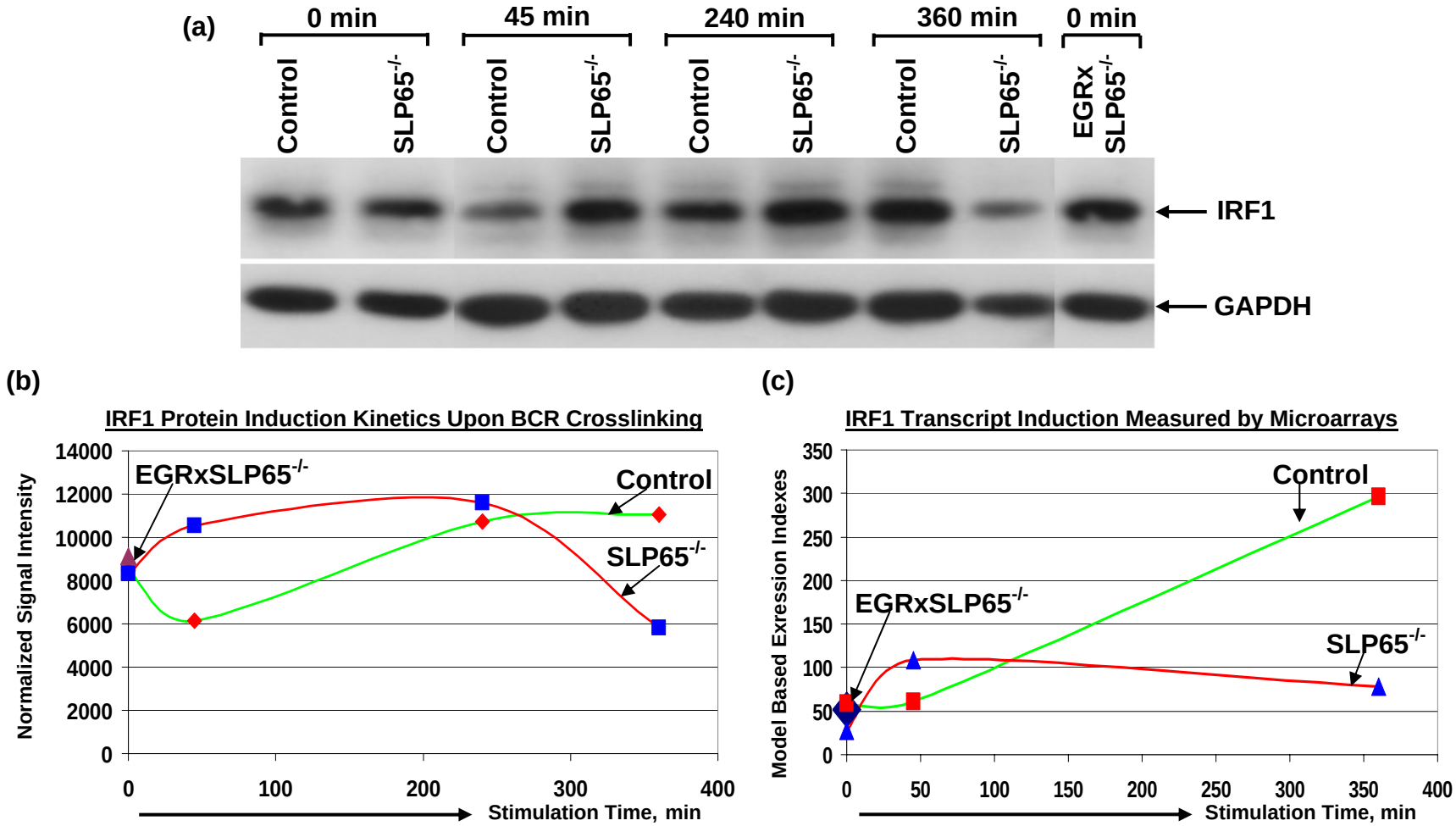


Figure 18: IRF1 protein induction kinetics upon BCR cross-linking in splenic B cells. (a) Immunoblot analysis for IRF1 protein expression in $B220^{+}$ splenic B cells from control (BALB/c), $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ mice. The cells were purified using anti-mouse CD45R(B220) MACS microbeads. Total cell lysates were prepared either directly after purification or after stimulation with anti-IgM F(ab')2 antibodies for 45, 240 and 360 minutes. GAPDH was used as an internal control. The diagrams show schematically (b) IRF1 protein induction and (c) IRF1 transcript induction kinetics upon BCR stimulation.

3.2.3.8 Validation of differentially regulated genes by Real-Time RT-PCR analysis

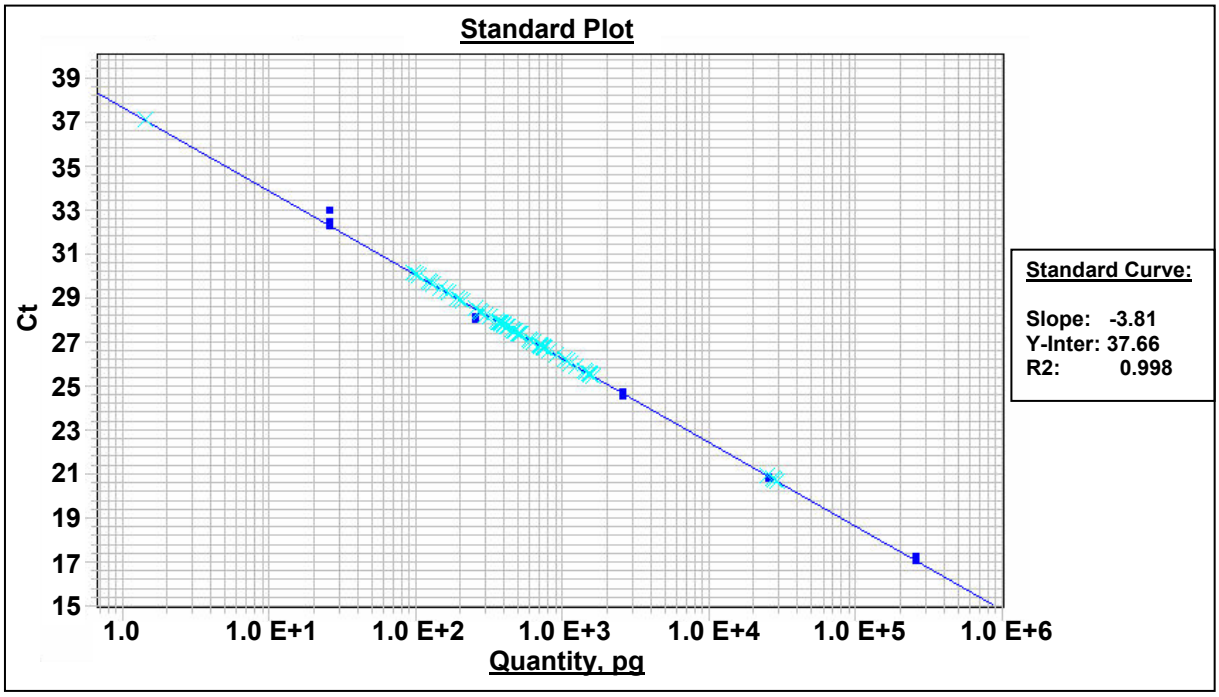
We employed real-time RT-PCR assay using SYBR Green I, dsDNA-binding dye, to verify the microarray results for 6 genes which were differentially regulated between EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control B cells. According to the expression profiles, calgranulin A (CalA), chemokine (C-C) receptor 2 (CCR2), schlafen 1 (SLFN1), schalfen 2 (SLFN2), POU domain, class 2, transcription factor 1 (POU2F1/Oct1), Burkitt lymphoma receptor 1 (Blr1/CXCR5) genes were 2- to 6- fold differentially regulated between EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control B cells. B-cell leukemia/lymphoma 3 (Bcl3) gene, which is non-differentially regulated in all samples, was taken as a negative control in the assays. Additionally, as an independent positive control we took TACI gene for analysis, because we found differential cell surface expression of TACI in EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ B cells in comparison to the control. Ribosomal protein L5 (RPL5) gene was used as an endogenous control and as a calibrator in real-time RT-PCR analysis. RT-PCR was conducted in two separate steps, cDNA synthesis from DNase treated total RNA - reverse transcription (RT) step followed by real-time PCR step performed in ABI Prism 7700 system (Perkin-Elmer-Applied Biosystems, USA). Separation of the RT and PCR steps has the advantage of generating a stable cDNA pool that can be stored virtually indefinitely. To maximize the number of genes that can be analyzed from the same sample of RNA we used an oligo-dT priming approach in the RT reaction. Except for primers for single exon genes, gene specific primers were designed from intron/exon boundaries close to the 3'-end of the sequences of genes to avoid false positive results arising from amplification of possible contaminating genomic DNA. A relative quantification method was used in the RT-PCR analysis. Relative standard curves were generated in each separate PCR reactions (for each separate PCR plate) using RPL5 gene as a calibrator (Fig. 19a). To this end, a 10- fold dilution series with arbitrary units was created (Fig. 19b). Arbitrary units were taken from the amount of input total RNA used in the RT step. The analysis showed linear amplification within the range between 250ng and 25pg of total RNA input. PCR reactions for the genes of interest were performed from 25ng of total RNA and in triplicate format from each sample for each gene tested. The slopes of the standard curves were used to determine the exponential amplification efficiency of PCR products (E) and efficiencies of PCR reactions by the following equations:

$$\text{Exponential Amplification Efficiency (E)} = 10^{(-1/\text{slope})}$$

$$\text{PCR Efficiency} = [10^{(-1/\text{slope})}] - 1$$

CalA, TACI, Bcl3, SLFN1, SLFN2 and CCR2 genes were amplified with a PCR efficiency of 86.77%. For genes Oct1 and Blr1 PCR efficiency of about 85.43% was achieved. Using delta-

(a)



(b)

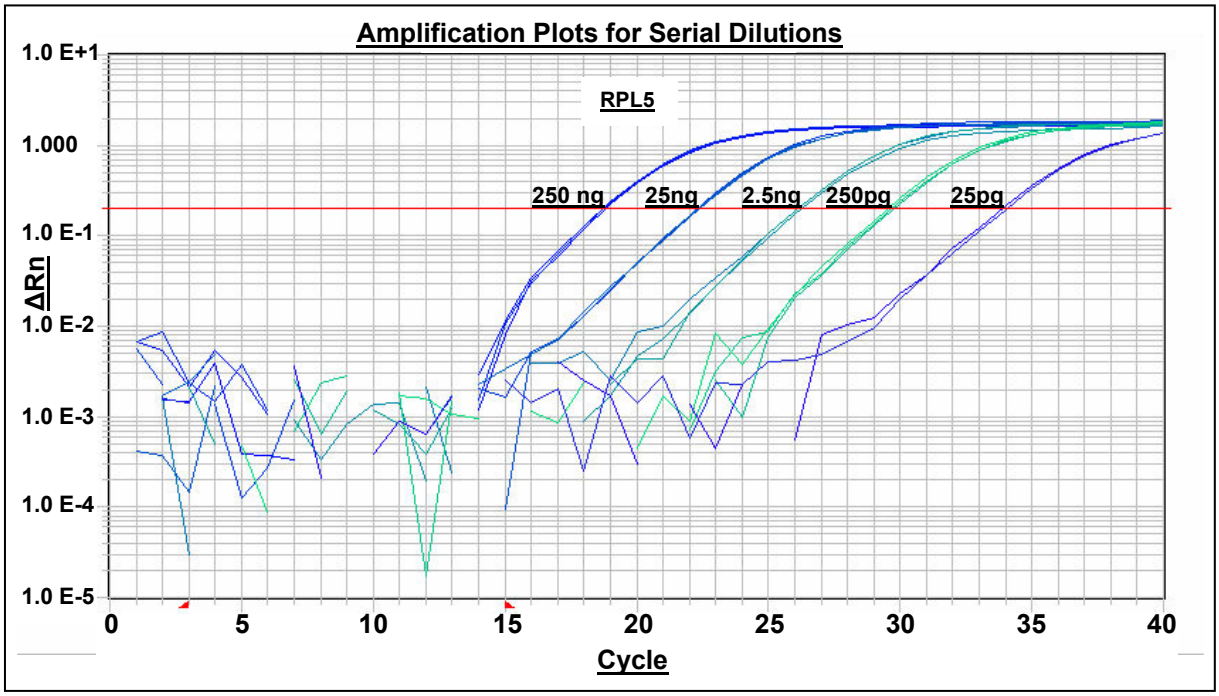


Figure 19: Relative quantification of gene expression using Real Time RT-PCR. RT-PCR was performed in two separate steps: cDNA was generated using a 30 µl reaction volume containing 6 µg of DNase-treated total RNA and PCR was performed in ABI Prism 7700 system using SYBR Green I fluorescent dye. The relative standard curve method has been used for quantification. Standard plot (a) using RPL5 (ribosomal protein, large subunit 5) gene as a calibrator and amplification plots (b) for the dilution series with arbitrary units are shown.

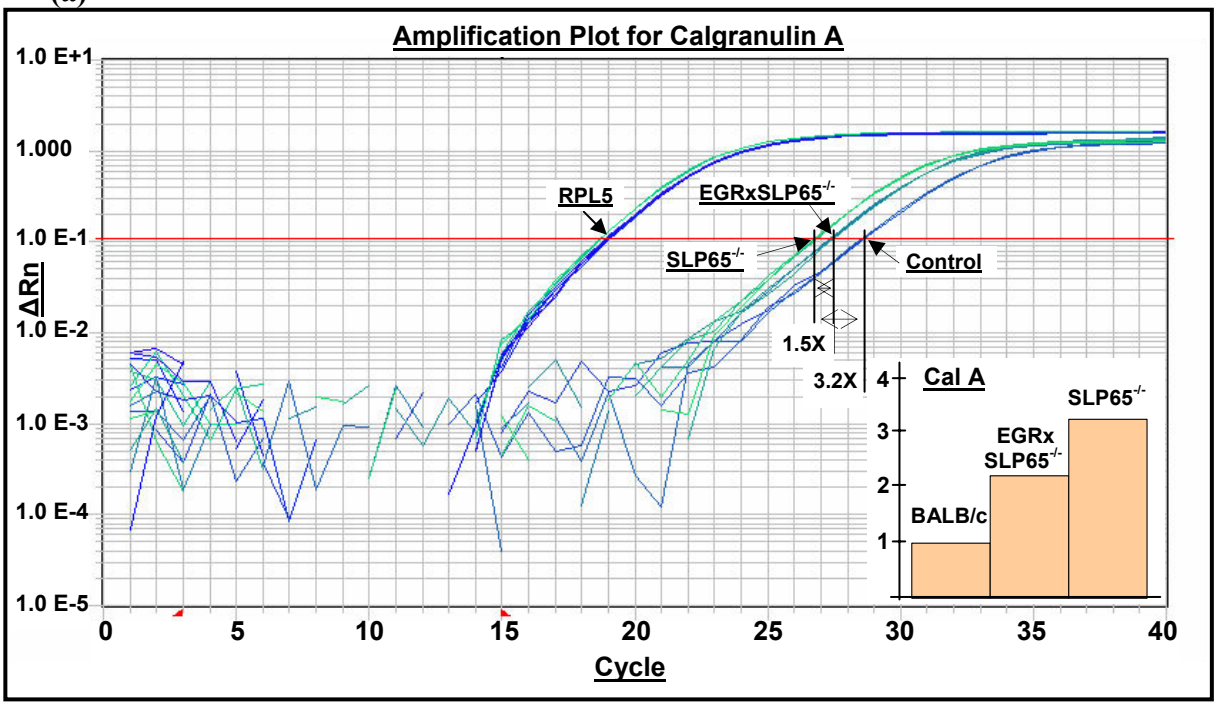
Ct (threshold cycle) method, the relative quantities (Q) of the expression levels of a gene between two different samples can be assessed by the following formula:

$$Q = E^{(\text{experiment-MEAN-Ct} - \text{baseline-MEAN-Ct})}$$

Theoretically, E (exponential amplification efficiency) is equal to 2 assuming that PCR efficiency is 100%, which makes the above formula more simple $Q = 2^{\text{deltaCt}}$ (Vandesompele et al., 2002a; Vandesompele et al., 2002b). In the analysis we used the amplification efficiency value of E = 1.87 for the genes amplified with the PCR efficiency of 86.77% and E = 1.85 for PCR efficiency of 85.43% instead of E = 2 value in the above equation. The data were analyzed manually setting the threshold value to 0.2 in all PCR reactions and in all PCR plates. Mean values and standard deviations to the Ct values for each gene were calculated.

The analysis showed that a house-keeping gene RPL5 was amplified with the Ct value differences below 0.5 between all replicates and all samples compared, suggesting equivalent amount of input RNA in all replicates and between samples from EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control mice. The results revealed that the CalA gene expression in SLP65$^{-/-}$ B cells is relatively high (3.2 fold) compared to the control, whereas in EGRxSLP65$^{-/-}$ B cells its expression is decreased by 1.5 fold (Fig. 20a). The expression of CCR2 gene was 6.4 fold higher in SLP65$^{-/-}$ than in control B cells, whereas it was reduced to control levels in EGRxSLP65$^{-/-}$ B cells (Fig. 20b). Interestingly, the SLFN1 gene was found to be downregulated (2-fold) and SLFN2 gene 1.5 fold upregulated in EGRxSLP65$^{-/-}$ B cells compared to the cells from SLP65$^{-/-}$ mice (Fig. 21, a and b). Oct1 gene expression was 6.0-fold higher in SLP65$^{-/-}$ B cells compared to the control, but was 5.1 fold lower in EGRxSLP65$^{-/-}$ B cells compared to SLP65$^{-/-}$ cells (Fig. 22a). The expression levels of B1r1 were similar in EGRxSLP65$^{-/-}$ and control, whereas it was upregulated by 1.8 fold in SLP65$^{-/-}$ B cells (Fig. 22b). As expected, the analysis of Bcl3 gene, which was shown to be non-differentially regulated in microarray data, revealed no differences in its expression between all samples (Fig. 23a). On the other hand, the expression pattern of the TACI gene, an independent positive control, was consistent with FACS data (Fig. 23b). Its expression was decreased by 1.4 fold in SLP65$^{-/-}$ B cells and by 5.4 fold in EGRxSLP65$^{-/-}$ cells compared to the control. Thus, microarray results as well as FACS data for TACI expression were verified in 81.25% of comparisons (13 out of 16 comparisons = 8 genes x 2 comparisons) by real-time RT-PCR (Table 9). The 2.5 kb 5'-flanking regions of the genes were analyzed for putative EGR1 transcription factor binding sites and the relative positions of such sites to the start of transcription are listed in the Table 8. Although, there are two EGR1 binding sites in the mouse and four binding sites in the human Blr1 gene promoter further careful analysis is

(a)



(b)

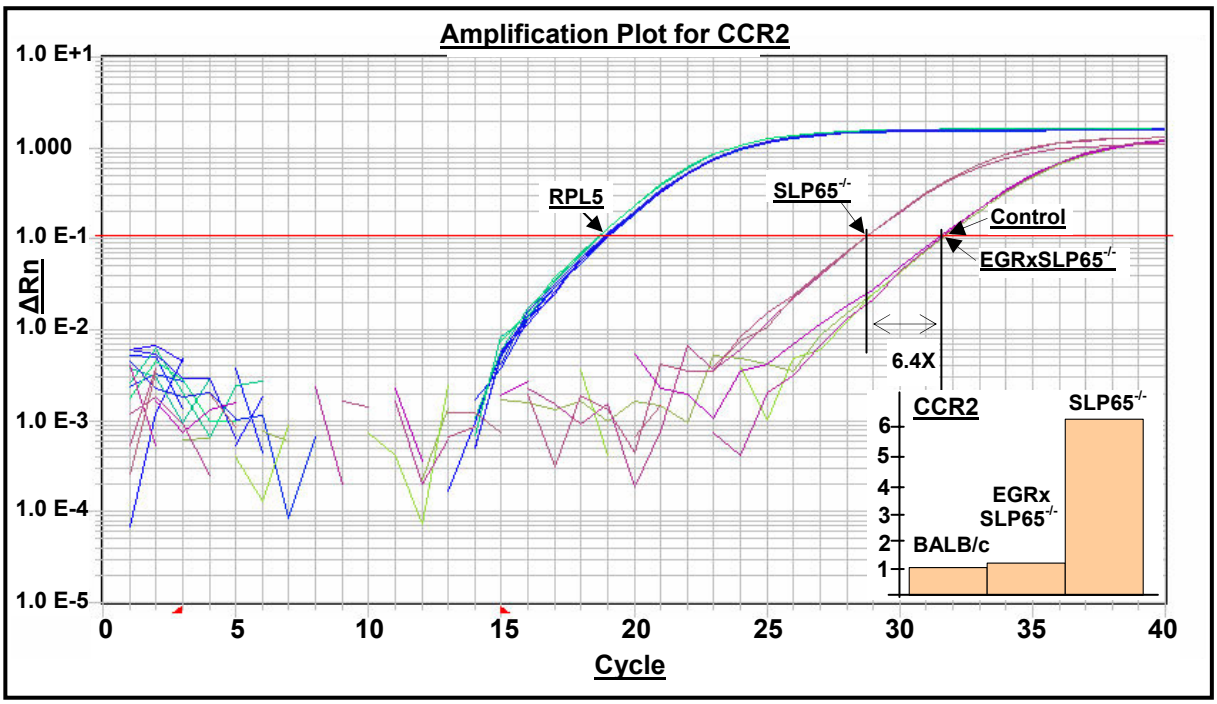
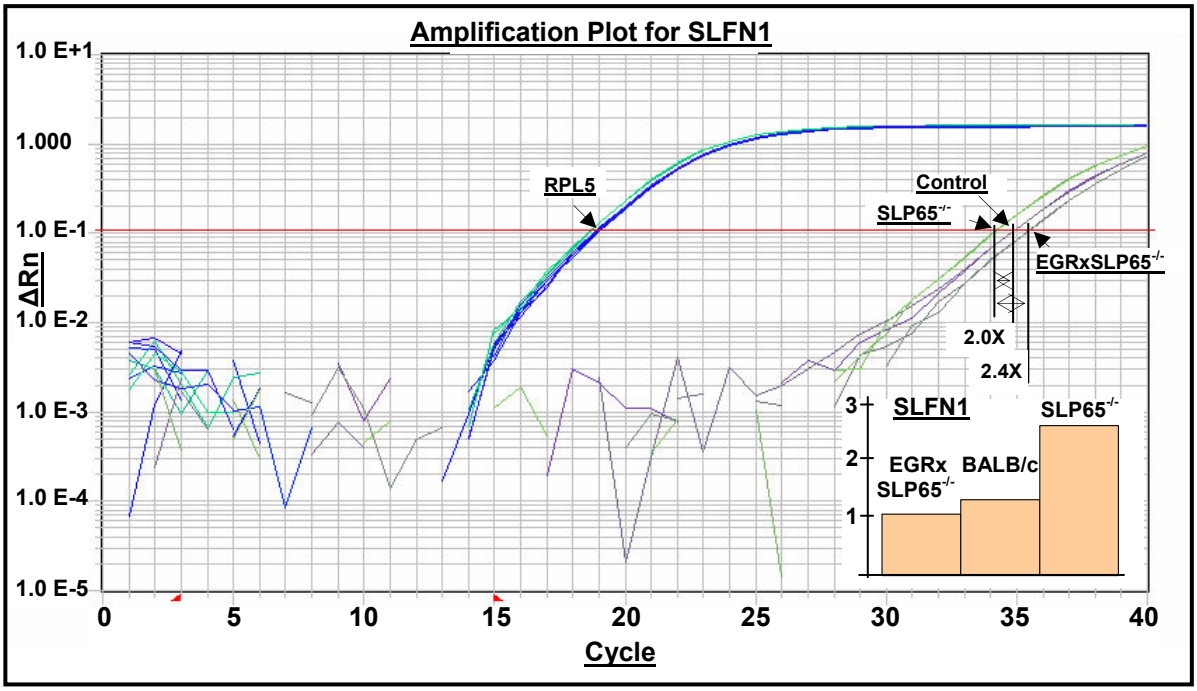


Figure 20: EGR1-mediated downregulation of calgranulin A (CalA) and chemokine (C-C) receptor 2 (CCR2) genes. Real Time RT-PCR analysis for the relative expression of CalA (a) and CCR2 (b) genes in $B220^{+}$ splenic B cells from control (BALB/c), $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ mice are shown. $SLP65^{-/-}$ splenic B cells express relatively high levels of CalA and CCR2 genes in comparison to the control, whereas EGR1 activity in $SLP65^{-/-}$ B cells downregulates both CalA and CCR2 genes.

(a)



(b)

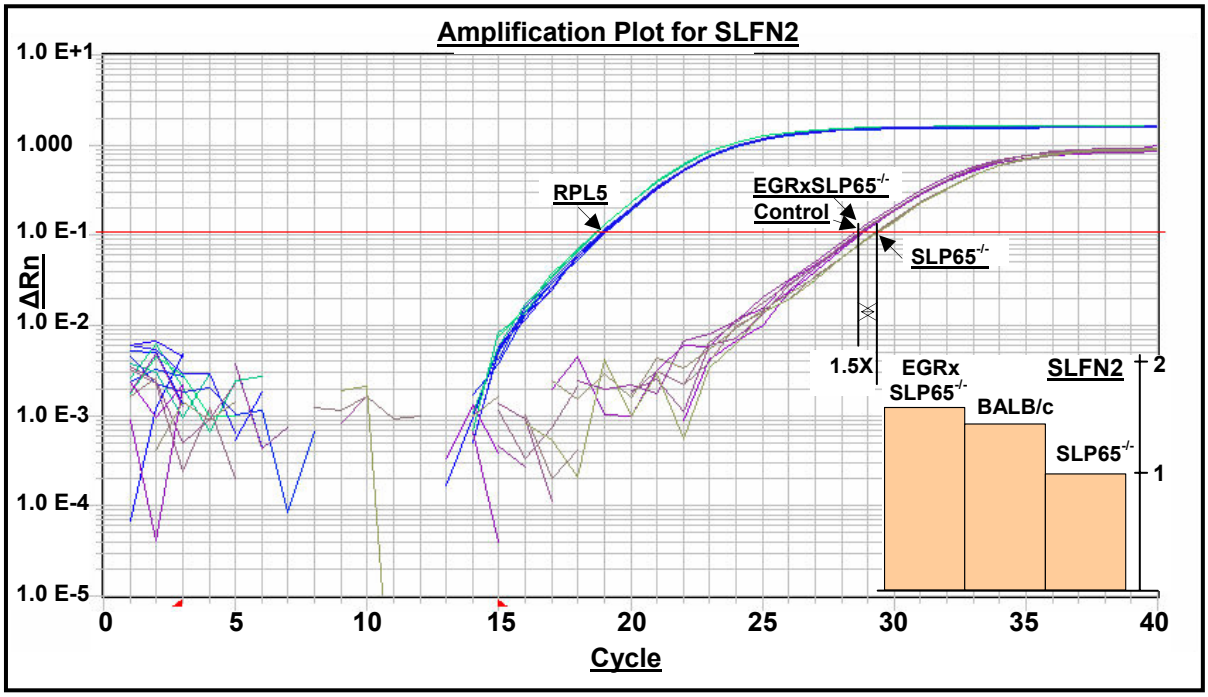


Figure 21: EGR1-mediated regulation of schlafen 1 (SLFN1) and schlafen 2 (SLFN2) genes. Real Time RT-PCR analysis for the relative expression of SLFN1 (a) and SLFN2 (b) genes in $B220^{+}$ splenic B cells from control (BALB/c), $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ mice are shown. $SLP65^{-/-}$ splenic B cells express relatively high levels of SLFN1 gene and relatively low levels of SLFN2 gene in comparison to the control, whereas EGR1 activity in $SLP65^{-/-}$ B cells downregulates SLFN1 gene and upregulates SLFN2 gene.

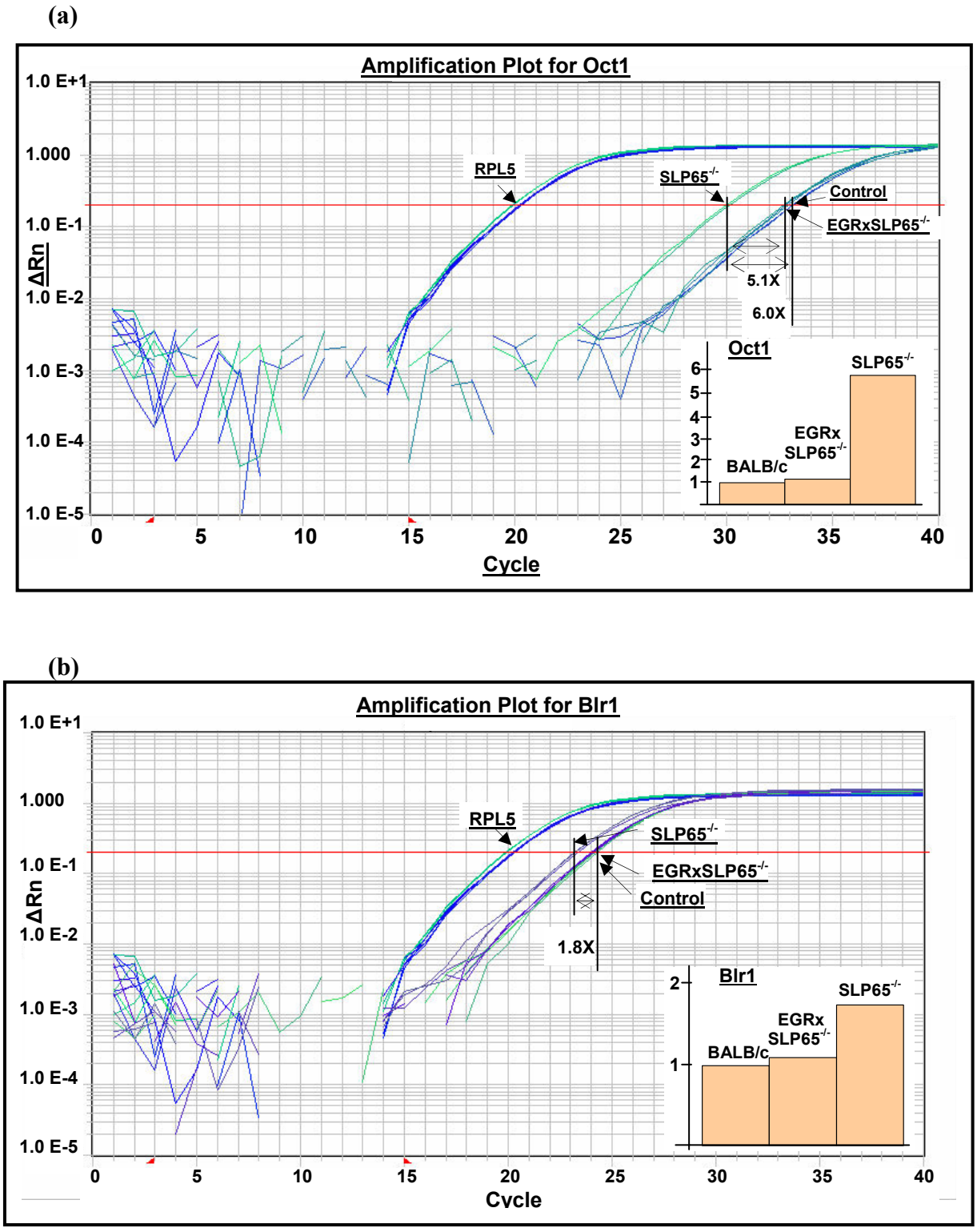


Figure 22: EGR1-mediated downregulation of Oct1 (POU2F1) and Burkitt lymphoma receptor 1 (Blr1) genes. Real Time RT-PCR analysis for the relative expression of Oct1 (a) and Blr1 (b) genes in $B220^{+}$ splenic B cells from control (BALB/c), $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ mice are shown. $SLP65^{-/-}$ splenic B cells express relatively high levels of Oct1 and Blr1 genes in comparison to the control, whereas EGR1 activity in $SLP65^{-/-}$ B cells downregulates both Oct1 and Blr1 genes.

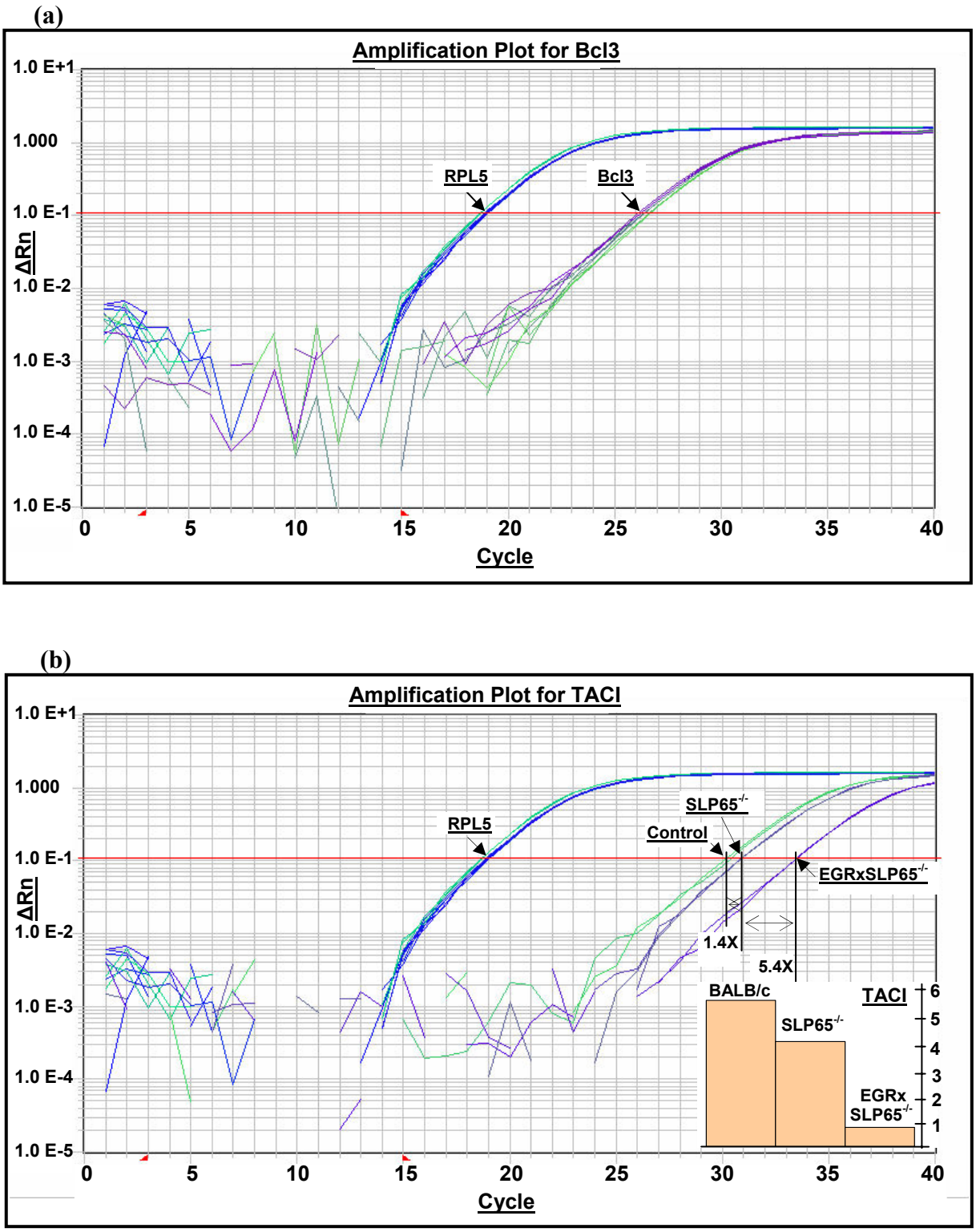


Figure 23: EGR1-mediated downregulation of TACI gene. Real Time RT-PCR analysis for the relative expression of Bcl3 (a) and TACI (b) genes in $B220^{+}$ splenic B cells from control (BALB/c), $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ mice are shown. Bcl3 gene is served as a control which is not differentially expressed from microarray experiments. $SLP65^{-/-}$ B cells express relatively lower levels of TACI gene in comparison to the control and EGR1 activity in $SLP65^{-/-}$ B cells downregulates TACI gene.

Table 8: **EGR1 consensus sequence gG C/A/T G G/T GGG C/A/T Gg binding sites within the 2.5 kb 5' flanking regions of the mouse and human genes.**

Gene	Number of EGR1 binding sites		Positions relative to the start of transcription	
	Mouse	Human	Mouse	Human
Calgranulin A	2	4	-454; -1008	-297; -627; -1614; -2211
SLFN1	3	NA*	-1316; -2189; -2371	
SLFN2	2	NA*	-1076; -1674	
CCR2	1	1	-2327	-1992
Oct1 (POU2F1)	3	2	-676; -870; -1143	-528; -665
Blr1 (CXCR5)	2	4	-419; -813	-1219; -1249; -1337; -2314
TACI	0	3		-100; -1066; -1585
TAF10	3	1	-138; -637; -1942	-266
Ly-6A/E	1	NA*	-2070	

* - Sequences are not available (NA) from GenBank® Sequence Database.

Table 9: **Validation of microarray based gene expression profiles and FACS data by Real Time RT-PCR.**

Accession	Genes	Relative Expression in SLP65$^{-/-}$ vs Control		Validation (Yes/No)	Relative Expression in EGRxSLP65$^{-/-}$ vs SLP65$^{-/-}$		Validation (Yes/No)
		Microarray Data	Real Time RT-PCR		Microarray Data	Real Time RT-PCR	
M83218	**CalA**	6.1	3.2	Y	-2.5	-1.5	Y
U56819	**CCR2**	1.8	6.4	Y	-3.0	-5.5	Y
AF099972	**SLFN1**	2.0	2.0	Y	-2.4	-2.4	Y
AF099973	**SLFN2**	-2.1	-1.4	Y	4.6	1.5	Y
X68363	**Oct1**	-2.1	6.0	N	-1.3	-5.1	Y
X71788	**CXCR5**	-3.0	1.8	N	5.0	-1.6	N
M90397	**Bcl3**	-1.1	1.1	Y	1.1	-1.2	Y
		Relative Expression in SLP65$^{-/-}$ vs Control			Relative Expression in EGRxSLP65$^{-/-}$ vs SLP65$^{-/-}$		
		FACS Data ($B220^{+}TACI^{+}$)	Real Time RT-PCR		FACS Data ($B220^{+}$ $TACI^{+}$ B cells)	Real Time RT-PCR	
	TACI	5.3% vs 28.9%	-1.4	Y	1.7% vs 5.3%	-5.4	Y

needed to check the possible regulation of Blr1 by EGR1, since the microarray data and real-time RT-PCR results are inconsistent. CalA, CCR2, SLFN1, SLFN2 and Oct1 genes might be direct target genes regulated by EGR1 transgene activity in EGRxSLP65$^{-/-}$ B cells.

3.3 EGR1 transgene activity results in a higher frequency of pre-B ALL cases in EGRxSLP65$^{-/-}$ mice

3.3.1 Higher incidence of pre-B ALL cases in EGRxSLP65$^{-/-}$ mice

Mice deficient in the adaptor protein SLP65 have reduced numbers of mature B cells, but an increased pre-B cell compartment. Compared to wild type cells, SLP65$^{-/-}$ pre-B cells have an enhanced *ex vivo* proliferative capacity. This proliferation requires interleukin 7 (IL-7) and expression of the pre-B cell receptor (pre-BCR). In addition, SLP65$^{-/-}$ mice have a high incidence of pre-B cell lymphomas. These data infer that the B-cell specific adaptor protein SLP65 acts as a tumor suppressor in pre-B cells by limiting pre-B cell expansion. Pre-B cell leukemia was observed in 5-10% of SLP65$^{-/-}$ mice (Flemming et al., 2003). It has been shown that the murine SLP65$^{-/-}$ pre-B cell leukemia resembles human childhood pre-B acute lymphoblastic leukemia (pre-B ALL) and indeed, in about 50% of all tested human childhood pre-B ALL cases a complete loss or drastic reduction of SLP65/BLNK expression was observed (Jumaa et al., 2003).

In our studies we have analyzed 59 SLP65$^{-/-}$ mice and in 4 cases (6.78%) we observed pre-B ALL leukemia (Table 10). Surprisingly, the frequency of leukemia in EGRxSLP65$^{-/-}$ mice was twice as high (13.33%), 12 out of 90 mice analyzed had pre-B ALL. The pre-B ALL leukemia, caused by pre-BCR expressing leukemic pre-B cells, was associated with splenomegaly in these mice and in 2 cases the mice developed solid tumors. After establishing a pre-B ALL cell line from a EGRxSLP65$^{-/-}$ mouse, we tested tumor formation of these cells *in vivo* by injecting the EGRxSLP65$^{-/-}$ pre-B ALL cells as well as SLP65$^{-/-}$ pre-B ALL cells (kindly provided by Dr. Hassan Jumaa) intravenously ($1x10^7$ cells per mouse) into immune-deficient RAG2/$\gamma c^{-/-}$ mice which lack any B, T and NK cells. Injection of the pre-B ALL cells from both EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ mice resulted in splenomegaly and leukemia 5 weeks after injection (Fig. 24).

It is possible that the activity of EGR1 transgene in EGRxSLP65$^{-/-}$ pre-B cells regulates (either directly or indirectly) genes which are important in pre-B cell proliferation and genes which are involved in the cell cycle control and as a consequence of these changes, EGRxSLP65$^{-/-}$ mice have higher frequency of pre-B ALL cases. To assess this possibility we have searched for potential candidate genes and EGR1 binding sites in their promoter regions.

Table 10: EGRxSLP65$^{-/-}$ mice have a higher frequency of pre-B acute lymphoblastic leukemia than SLP65$^{-/-}$ mice.

Mouse Genotype	Mice Analyzed	pre-B ALL	Percentage of mice, %
SLP65$^{-/-}$	59	4	**6,78**
EGRxSLP65$^{-/-}$	90	12	**13,33**
Total Number	149	16	

Table 11: EGR1 consensus sequence gG C/A/T G G/T GGG C/A/T Gg binding sites within the 2.5 kb 5' flanking regions of the mouse and human genes.

Gene	Number of EGR1 binding sites		Positions relative to the start of transcription	
	Mouse	Human	Mouse	Human
Pro/pre-B cell proliferation:				
LEF1	2	4	-204; -215	-320; -466; -582; -937
IL-7R alpha chain	3	1	-611; -1565; -1612	-2357
Cell cycle control:				
IRF1	2	6	-102; -282	-292; -424; -954; -1001; -1075; -1614
p21	1	4	-188	-502; -1426; -1454; -1594
Cyclin genes:				
Cyclin D1	4	8	-42; -405; -935; -2038	-55; -97; -337; -633; -1504; -1541; -2021; -2151
Cyclin D2	3	6	-828; -1866; -2320	-867; -1257; -1423; -1496; -1890; -2448
Cyclin D3	2	0	-107; -849	
Cyclin E1	2	2	-309; -740	-784; -1118
Cyclin E2	8	3	-36; -526; -814; -1022; -1142; -1202; -1570; -2493	-1312; -2128; -2481

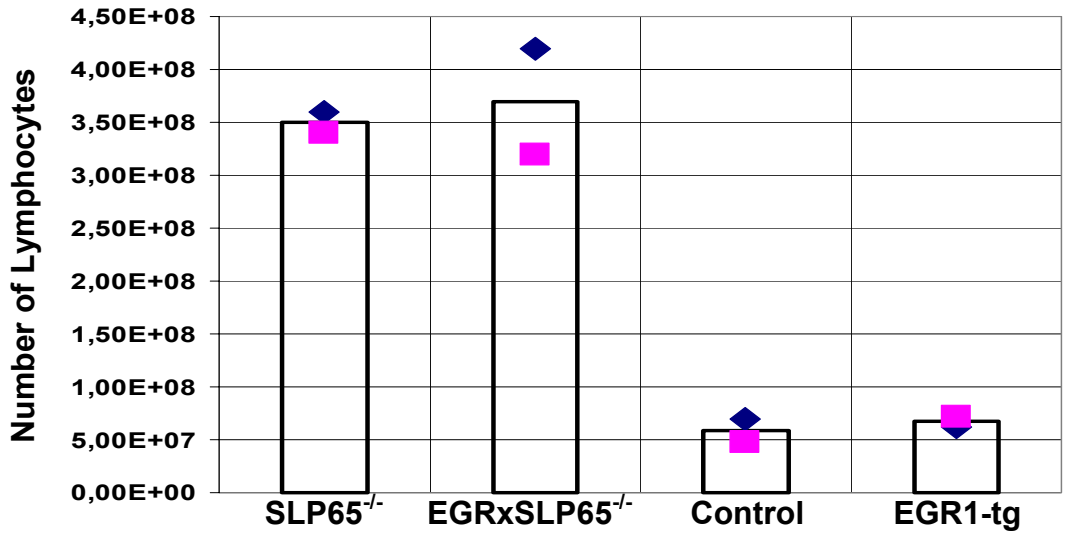


Figure 24: Reconstitution of tumor cells leads to splenomegaly in RAG2/γc$^{-/-}$ mice. Cells from SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ pre-B ALL cell lines were injected intravenously into immune-deficient RAG2/γc$^{-/-}$ mice lacking B, T and NK cells. Injection of 1x10^{7} cells per mouse resulted in splenomegaly 5 weeks after injection.

One possible candidate gene would be IL-7R alpha chain, because activation of IL-7 signaling pathway results in survival, proliferation and differentiation of pro-B and pre-B cells. IL-7 activates its receptor that consists of the IL-7R alpha chain and the common gamma chain (IL-2RG/CD132). The cytoplasmic domain of IL-7R alpha chain has been shown to be directly involved in the activation of JAK/STAT pathway in precursor B cells (van der Plas et al., 1996). Furthermore, IL-7 can be produced by some human tumor cells and is involved in tumor development and progression (Al-Rawi et al., 2003) and significantly higher levels of IL-7 and IL-7R expression were found in most aggressive tumors (Al-Rawi et al., 2004). Analysis of promoter regions revealed that there are 3 putative EGR1 binding sites in the mouse and a single binding site in human IL-7R alpha chain (Table 11). However, as demonstrated by FACS analysis, IL-7R alpha chain expression was not significantly different between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ pre-B cells, or between pre-B cells of these two mutant strains and wild type mice (data not shown).

Another interesting candidate gene is the lymphoid enhancer factor 1 (LEF1) which promotes survival and proliferation of pro-B and pre-B cells. LEF1 regulates the transcriptional response of the canonical Wnt/β-catenin pathway. Recently a link between Wnt signaling and normal B cell development has been established by the demonstration that Wnt proteins are mitogenic for pro/pre-B cells and that this effect is mediated by LEF1 (Reya et al., 2000). Interestingly, there are 2 putative EGR1 binding sites in the mouse and 4 binding sites in human LEF1 gene 5' regulatory regions (Table 11).

As a negative regulator of cell proliferation, IRF1 has been implicated in the regulation of the cyclin-dependent kinase inhibitor-1A (Cdkn1A/p21) (Tanaka et al., 1996). Moreover, we found by expression profiling that both IRF1 and p21 genes were downregulated in SLP65$^{-/-}$ B cells during BCR stimulation and IRF1 downregulation in SLP65$^{-/-}$ B cells upon BCR cross-linking was confirmed at the protein level. The analysis of the 5' regulatory regions revealed 2 putative EGR1 binding sites in the mouse and 6 binding sites in human IRF1 gene promoter as well as a single binding site in the mouse and 4 EGR1 binding sites in human p21 gene promoter sequences (Table 11). However, as with the spleen cells, we were unable to detect the p21 protein either in total cell lysates or nuclear extracts from pre-B cells with different anti-p21 antibodies from different companies (Santa Cruz Biotech and BD PharMingen antibodies) even though the p21 protein was detectable in NIH 3T3 mouse fibroblast cells after overnight starvation and 6 hours of serum response. Despite this it is still possible that, LEF1 and IRF1 are candidate genes, for differential regulation between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ pre-B cells and this might be a possible explanation for the higher frequency of pre-B ALL cases observed in EGRxSLP65$^{-/-}$ mice.

3.3.2 EGR1-regulated LEF1 and IRF1 protein expression in pre-B cells and the association of high levels of LEF1 and IRF1 protein expression with tumor formation

Total cell lysates of bone marrow-derived *in vitro*-cultivated pre-B cells from control, SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice as well as EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ pre-B ALL cell lines were analyzed for the expression of LEF1 and IRF1 proteins by immunoblot assay. The analysis revealed that EGRxSLP65$^{-/-}$ pre-B cells express significantly higher levels of both LEF1 and IRF1 proteins compared to SLP65$^{-/-}$ and wild type pre-B cells (Fig. 25a). Interestingly, both LEF1 and IRF1 protein expression levels were even higher in EGRxSLP65$^{-/-}$ pre-B ALL cells compared to normal EGRxSLP65$^{-/-}$ pre-B cells. Moreover, LEF1 and IRF1 protein expression levels were significantly elevated in SLP65$^{-/-}$ pre-B ALL cells compared to normal SLP65$^{-/-}$ pre-B cells. The relatively high levels of LEF1 and IRF1 protein expression in EGRxSLP$^{-/-}$ pre-B cells as well as in both pre-B ALL cell lines (expressed to even higher extent) suggested that high levels of LEF1 and IRF1 expression in pre-B cells might be associated with tumor formation. In order to test this possibility, we have isolated pre-B ALL cells from a EGRxSLP65$^{-/-}$ mouse which had splenomegaly. CD19^{+} B cells from the spleen were separated from the non-B cell compartment using anti-mouse CD19 MACS microbeads. Total cell lysates from *ex vivo* CD19^{+} pre-B ALL cells as well as

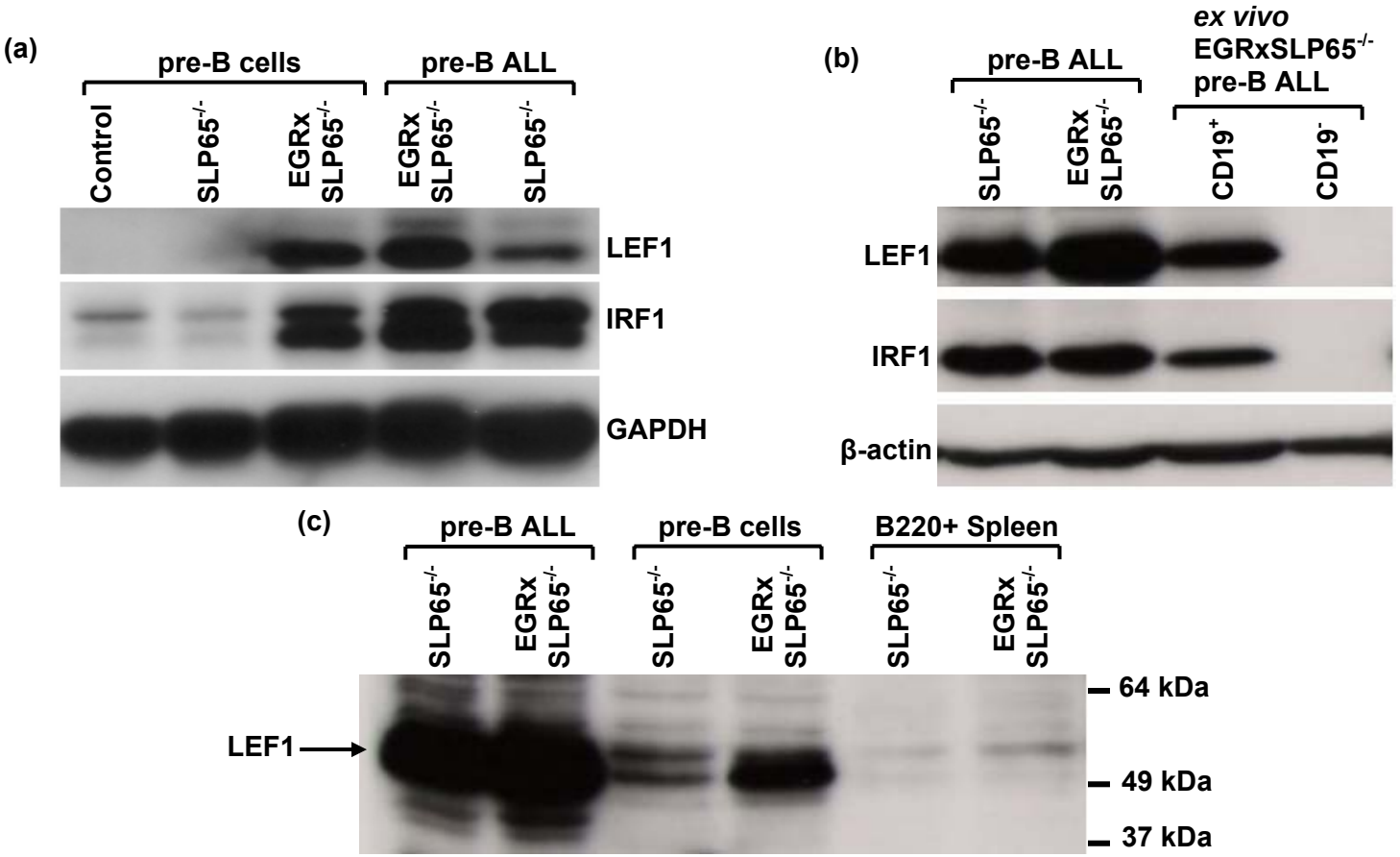


Figure 25: EGR1 regulates LEF1 and IRF1 protein expression in pre-B cells. (a) Immunoblot assay of total cell lysates for LEF1 and IRF1 expression in *in vitro*-cultivated pre-B cells from control (BALB/c), SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice as well as from SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ pre-B ALL cell lines. GAPDH was used as an internal control. (b) Immunoblot analysis of pre-B ALL cell lines versus CD19^{+} and CD19^{-} cell fractions from the spleen of a EGRxSLP65$^{-/-}$ mouse with splenomegaly. The cells were purified using anti-mouse CD19^{+} MACS microbeads. (c) Total cell lysates from pre-B ALL cell lines, *in-vitro*-cultivated pre-B cells and B220^{+} spleen B cells from SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice were analyzed for the expression of LEF1 protein.

the $CD19^-$ fraction of cells from the spleen of $EGRxSLP65^{-/-}$ mouse were compared with $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ pre-B ALL cell lines for the expression of LEF1 and IRF1 proteins by western-blot analysis (Fig. 25b). Comparable high levels of both LEF1 and IRF1 proteins were detected specifically in the $CD19^+$ pre-B ALL cell fraction. In another experiment we isolated pre-B ALL cells from the spleen of a $SLP65^{-/-}$ mouse which had developed a solid tumor and comparison of LEF1 and IRF1 protein expression in these cells with $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ pre-B ALL cells lines showed similar results (data not shown). Since normal $B220^+$ splenic B cells from both $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ mice do not express LEF1 protein (Fig. 25c), although they do express IRF1 (as shown in Fig. 18), the above data suggest that EGR1 might regulate the expression of LEF1 and IRF1 in pre-B cells and high levels of LEF1 and IRF1 might indeed be associated with the pre-B ALL formation in the mouse.

3.3.3 Human pre-B ALL cell lines express high levels of LEF1, IRF1 and EGR1 proteins

Next, we investigated the expression patterns of LEF1, IRF1 and EGR1 proteins in human lymphoma cell lines and compared them to the mouse pre-B ALL cell lines. In total, 6 human lymphoma cell lines: NALM-6 (pre-B ALL) and five other cell lines, generously provided by Dr. Hassan Jumaa (MPI, Freiburg); 697 (pre-B ALL), BV173 (pre-B ALL), SEM (ALL), L428 (Hodgkin's lymphoma) and JUM2 (mantle cell lymphoma) together with $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ pre-B ALL cell lines were tested by immunoblot assay (Fig. 26a). The results showed that LEF1 is specifically expressed only in acute lymphoblastic lymphoma cases, including both human and mouse cell lines at a comparably high levels (Fig. 26b). IRF1 was also detected in all acute lymphoblastic lymphoma cells as well as in JUM2 -mantle cell lymphoma cells at a comparable level (Fig. 26c). Neither LEF1 nor IRF1 proteins were detectable in L428 - Hodgkin's lymphoma cells. While EGR1 was undetectable in $SLP65^{-/-}$ pre-B ALL cells, all human pre-B ALL cells tested as well as L428 and JUM2 cells expressed significantly high levels of EGR1 protein compared to $EGRxSLP65^{-/-}$ pre-B ALL cells (Fig. 26d). These data confirmed our hypothesis and suggested that high levels of expression of EGR1, LEF1 and IRF1 proteins might be associated with pre-B ALL lymphoma formation both in the mouse and in humans.

3.3.4 Induction of EGR1, LEF1 and IRF1 protein expression in pre-B cells

As an immediate early gene, transient EGR1 expression is rapidly induced upon stimulation by the B cell antigen receptor (BCR) cross-linking (McMahon and Monroe 1995).

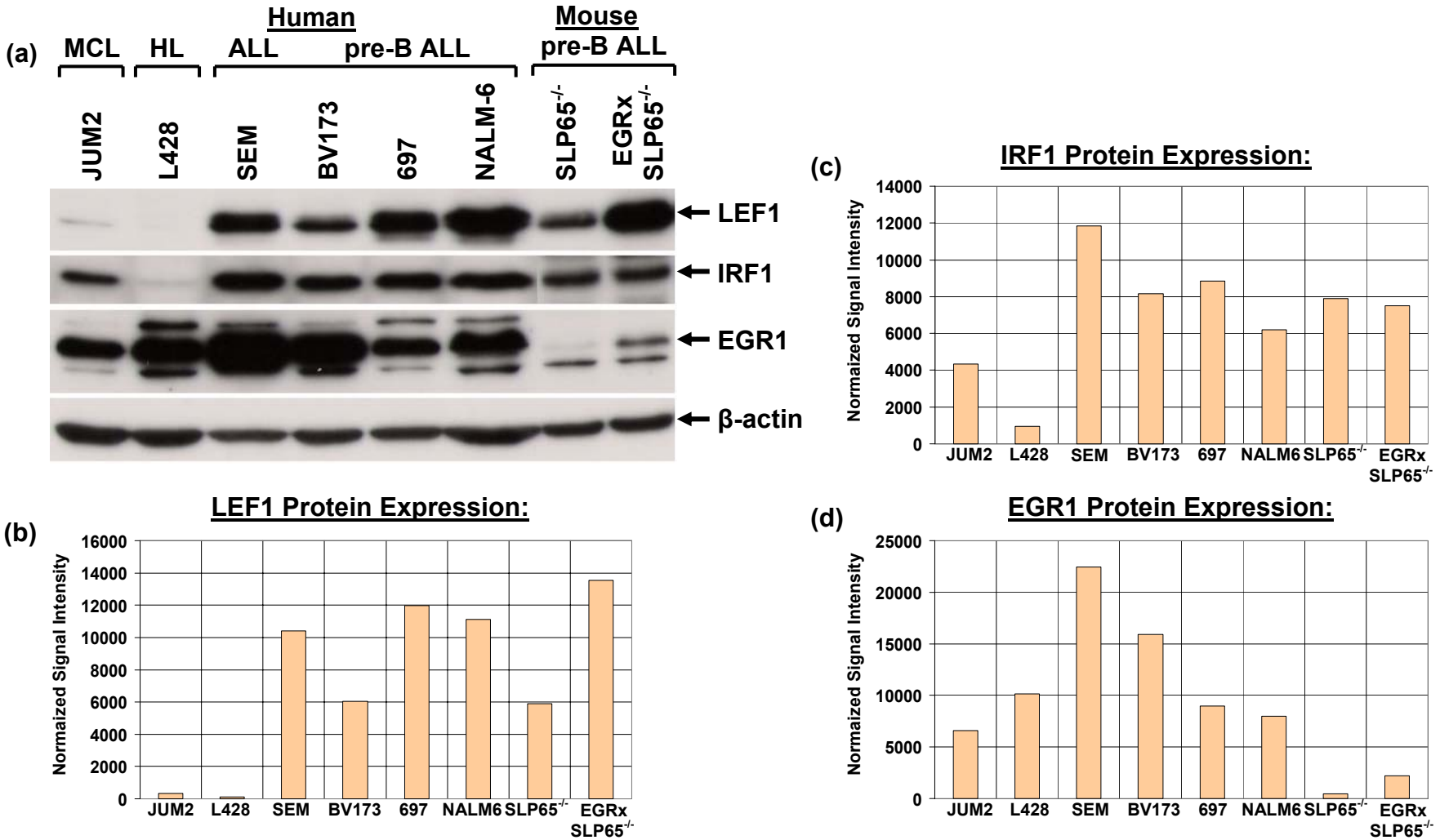


Figure 26: Human pre-B ALL cell lines express high levels of LEF1, IRF1 and EGR1 proteins. Immunoblot analysis of total cell lysates (a) from human lymphoma cell lines: JUM2 (mantle cell lymphoma), L428 (Hodgkin's lymphoma), SEM (acute lymphoblastic lymphoma), 3 pre-B cell acute lymphoblastic lymphoma cell lines: BV173, 697 and NALN-6 as well as SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mouse pre-B ALL cell lines. The diagrams show the relative expression levels of LEF1 (b), IRF1 (c) and EGR1 (d) proteins. β-actin was used as an internal control.

EGR1 functions as a transcriptional regulator that links common biochemical signaling pathways to the rapid modulation of downstream gene expression. In analogy to mature B cells, pre-B cells could transmit signals activating EGR1 via the pre-BCR signaling cascade. Although, we showed that EGR1 acts downstream of SLP65 in B cells, EGR1 protein expression and induction in the absence of SLP65 could be still possible and explained as follows. SLP-65$^{-/-}$ mice display a severe, but not complete block at the pre-B cell stage of development and it has been recently shown that the adaptor proteins LAT and SLP-76 are involved in pre-BCR signaling, thereby rescuing arrested murine SLP-65$^{-/-}$ pre-B cells (Su and Jumaa 2003). An initial step in transforming SLP65$^{-/-}$ pre-B cells into pre-B leukemic cells could be initiated by activating EGR1 transcription factor.

In order to check if pre-BCR stimulation in SLP65$^{-/-}$ B cells can induce EGR1, LEF1 and IRF1 protein expression we have stimulated *in vitro*-cultivated SLP65$^{-/-}$ pre-B cells with SL156 antibodies. As we expected, immunoblotting analysis showed that EGR1 protein can be induced upon pre-BCR stimulation in SLP65$^{-/-}$ pre-B cells. EGR1 protein was detectable both in the total cell lysates and nuclear extracts, whereas LEF1 protein was only detectable in the nuclear extracts and IRF1 protein was detected only in total cell lysates (Fig. 27, a and b). Interestingly, the results revealed a linear correlation of the induction kinetics of EGR1 and LEF1 proteins in SLP65$^{-/-}$ pre-B cells upon pre-BCR stimulation (Fig. 27c). Thus, the expression and induction (although to lower levels) of EGR1 in SLP65$^{-/-}$ pre-B cells could be due to the compensation of SLP65 by LAT and SLP76. Thus, the LEF1 protein could still be induced (although to a lower extent compared to the wild type pre-B cells) by EGR1 transcription factor in SLP65$^{-/-}$ pre-B cells. IRF1 protein expression kinetics did not correlate with the induction of EGR1 upon pre-BCR stimulation. These data suggested that although we observed high expression levels of IRF1 in EGRxSLP65$^{-/-}$ pre-B cells compared to the control and SLP65$^{-/-}$ pre-B cells, the higher levels of IRF1 might be not due to the direct effect of EGR1, but instead to a secondary effect caused by the forced expression of EGR1 in SLP65$^{-/-}$ pre-B cells.

3.3.5.Induction of LEF1 and IRF1 proteins upon differentiation

3.3.5.1 LPS stimulation in pre-B cells induces LEF1 protein induction

Lipopolysaccharides (LPS) are major components of the outer membrane of Gram-negative bacteria, which makes them prime targets for recognition by the immune system. Stimulation of B cells by LPS enhances their antigen-presenting capacity and is accompanied by B cell

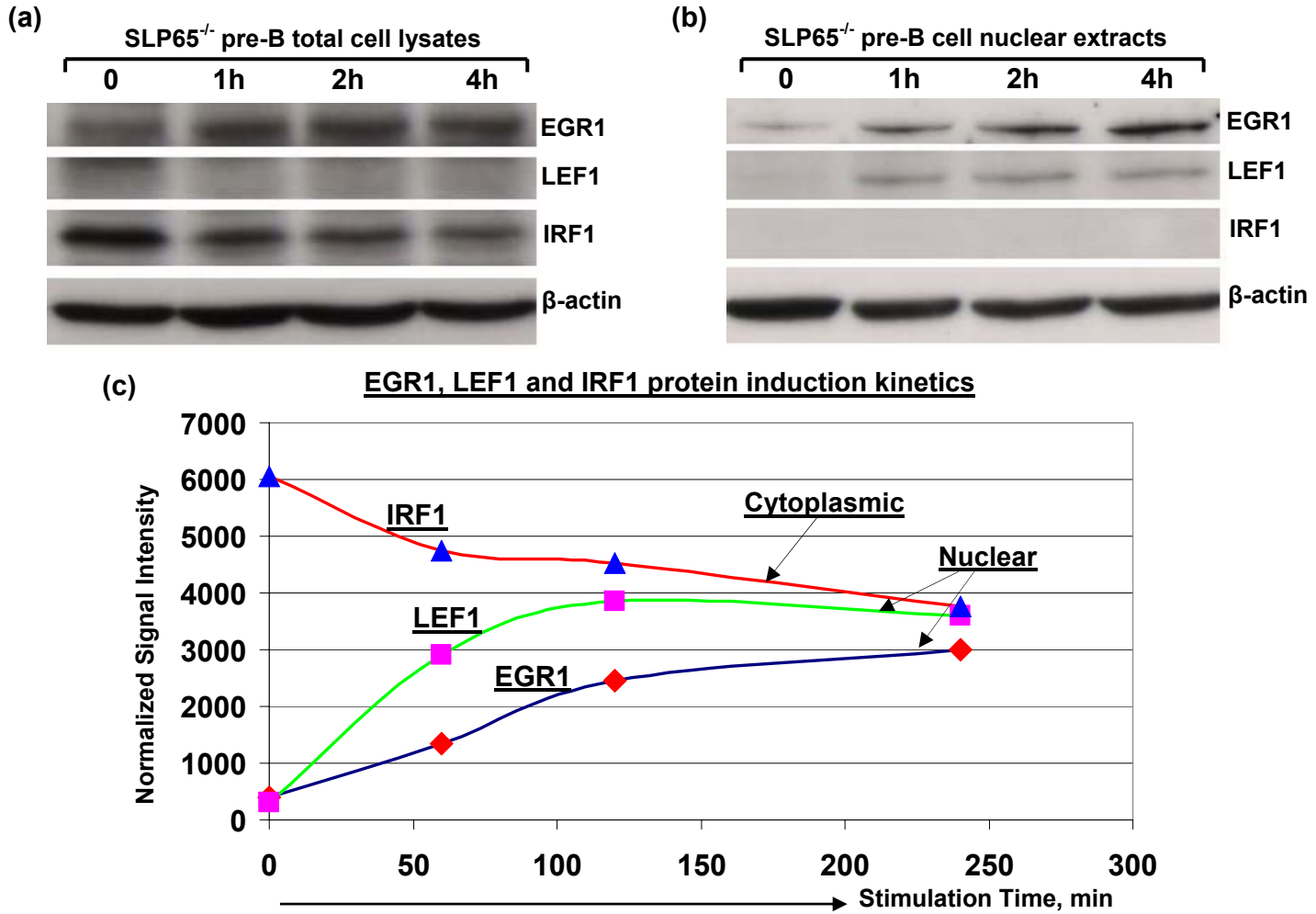


Figure 27: EGR1 and LEF1 proteins are induced upon pre-BCR stimulation in SLP65$^{-/-}$ pre-B cells. Immunoblot analysis of SLP65$^{-/-}$ pre-B cells for EGR1, LEF1 and IRF1 protein expression upon pre-BCR stimulation (a) in total cell lysates and (b) in nuclear extracts. The cells were stimulated for 0, 1, 2 and 4 hours with SL156 antibodies. The diagram (c) shows EGR1, LEF1 and IRF1 protein induction kinetics upon pre-BCR stimulation in SLP65$^{-/-}$ pre-B cells. β-actin was used as an internal control.

proliferation and secretion of large quantities of LPS-neutralizing antibodies (Kearney and Lawton 1975; Oliver et al., 1999). The responses of B cells to LPS are mediated by cell surface Toll-like receptor 4 (TLR4), which belongs to the multimember family of mammalian proteins homologous to the Toll protein of Drosophila melanogaster (Medzhitov et al., 1997; Rock et al., 1998). The murine pre-B cells respond to lipopolysaccharide (LPS) by kappa gene transcription and expression of surface IgM (sIg) leading to the differentiation of pre-B cells via NF-kappa B activation (Cohen et al., 1995). Recent investigations into the role of Bruton's tyrosine kinase (Btk) in LPS signaling has implicated Btk to be directly downstream of TLR4, both with respect to p38 MAPK activation and activation of the transcription factor NFkB. In fact Btk is activated by LPS and has been shown to directly bind TLR4 and the key proximal signaling proteins involved in LPS-induced NFkB activation, MyD88, Mal and IRAK-1 (Jefferies and O'Neill 2004). Although no experiments to provide evidence on the direct involvement of SLP65 in LPS-mediated proliferation and differentiation of B cells have been carried out yet, extrapolating from studies of BCR signaling, it is possible that the adaptor protein SLP65 is also involved in LPS-mediated responses of pre-B cells, since SLP65 couples Btk to PLC-γ activation and subsequent calcium mobilization as well as MAPK and NFkB activation in B cells.

To test this possibility, pre-B cells from control, $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ mice were stimulated with LPS for 1 hour, 2 hours and 4 hours. The immunoblot analysis of total cell lysates showed that LEF1 protein was induced during LPS-mediated differentiation of pre-B cells in wild type mice but was not induced in $SLP65^{-/-}$ pre-B cells (Fig. 28, a and b). $EGRxSLP65^{-/-}$ pre-B cells maintained LEF1 protein expression upon LPS stimulation. These data demonstrate that LEF1 protein might be involved in LPS-mediated differentiation of pre-B cells (since it is induced upon LPS stimulation) and strongly suggest regulation of LEF1 by EGR1 in pre-B cells, since EGR1 overexpression maintains LEF1 protein during LPS-mediated response of pre-B cells.

3.3.5.2 EGR1 maintains LEF1 protein expression during differentiation upon IL-7 withdrawal

IL-7 plays many fundamental roles during murine B lineage development. Murine pro-B cells require IL-7 for survival, proliferation and differentiation to the pre-B cell stage (Namen et al., 1988; Wei et al., 2000). *In vitro* cultures of B lineage cells supplied with exogenous IL-7 (with or without stromal cells) have shown that cells have rearranged Ig heavy chain gene but

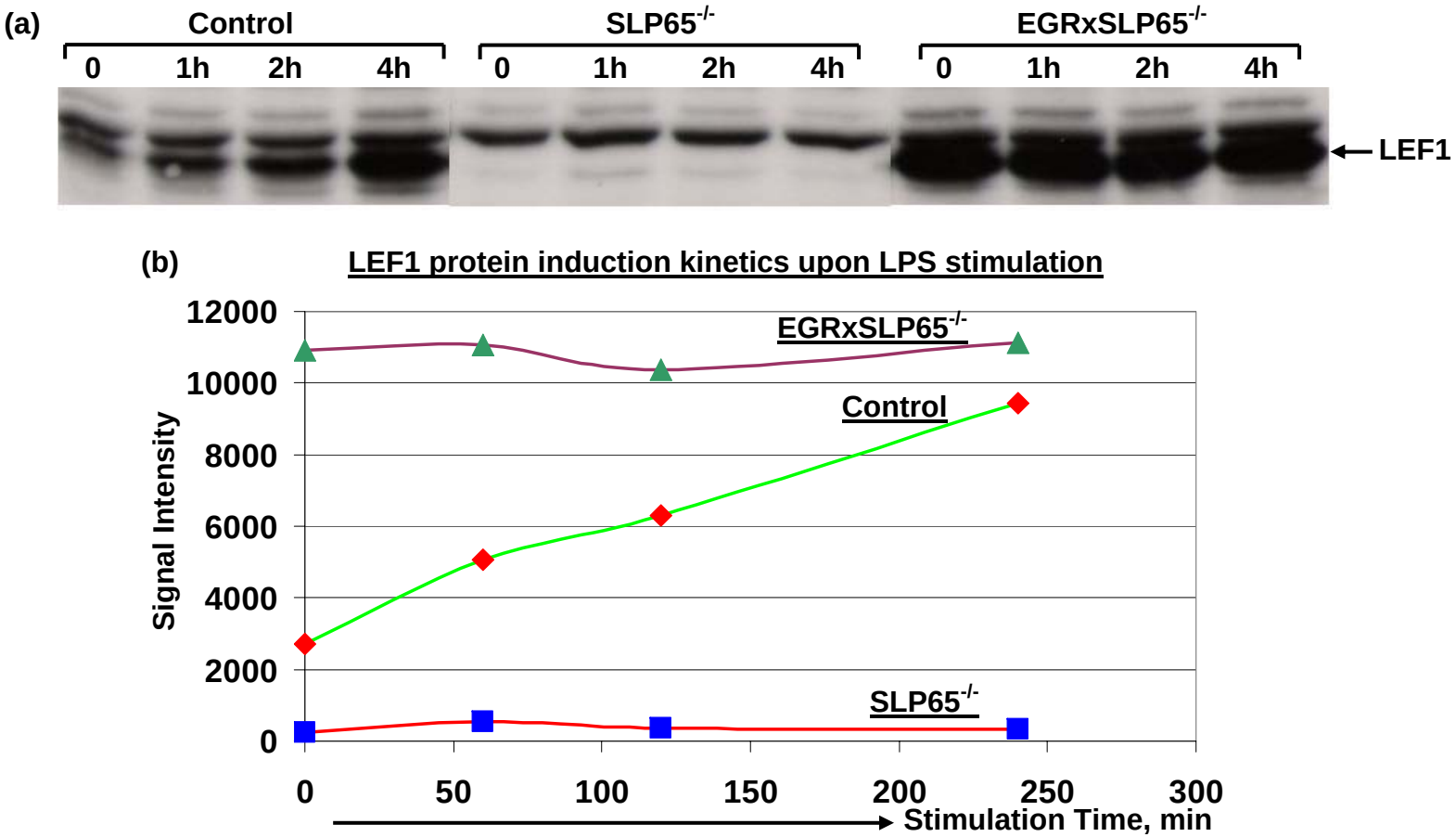


Figure 28: LPS stimulation in pre-B cells induces LEF1 protein induction. Immnublot analysis of total cell lysates of *in vitro*-cultivated pre-B cells from control (BALB/c), SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice. (a) pre-B cells were stimulated with LPS for 0, 1, 2 and 4 hours. (b) The diagram shows LEF1 protein induction kinetics upon LPS stimulation in pre-B cells.

have IgL genes in the germline configuration (Rolink et al., 1991; ten Boekel et al., 1995; Ray et al., 1998). Removing IL-7 from the culture medium results in an apparent increase in the differentiation of the population, including an increase in RAG expression, IgL rearrangements and expression of surface IgM. It was therefore postulated that IL-7 inhibits maturation to the surface IgM^+ stage and that IL-7 withdrawal from cultures of progenitors induces differentiation (Milne et al., 2004).

In order to examine LEF1 and IRF1 protein expression during differentiation mediated by IL-7 withdrawal, *in vitro*-cultivated pre-B cells from $SLP65^{-/-}$ and $EGRxSLP65^{-/-}$ mice as well as pre-B ALL tumor cells were cultivated in the IL-7 free medium for 4 hours and overnight (16 hours). Immunoblot analysis from total cell lysates showed that both LEF1 and IRF1 protein expression remained constant during 16 hours of IL-7 withdrawal in pre-B ALL cells (Fig. 29a). LEF1 protein expression was decreased in pre-B cells from $SLP65^{-/-}$ mice, whereas $EGRxSLP65^{-/-}$ pre-B cells maintained LEF1 protein expression during 16 hours of IL-7 withdrawal (Fig. 29, a and b). Upon IL-7 withdrawal, IRF1 protein was reduced in both $EGRxSLP65^{-/-}$ and $SLP65^{-/-}$ pre-B cells, but the expression levels of IRF1 was higher in $EGRxSLP65^{-/-}$ pre-B cells (Fig. 29, a and c). This also demonstrated that IRF1 might not be a direct target for EGR1 in pre-B cells and its higher levels of expression in $EGRxSLP65^{-/-}$ pre-B cells is associated with the secondary effects of EGR1 overexpression in $EGRxSLP65^{-/-}$ pre-B cells.

3.3.6 EGR1-mediated regulation of cyclin D2 and cyclin D3 genes

Since D-type (cyclin D1, D2, and D3) and E-type (cyclin E1 and E2) cyclins are regarded as essential links between the cell environment and the core cell cycle machinery and because cyclin gene promoters carry EGR binding sequences (Table 11), we have examined for changes in the expression of all three cyclin D and two cyclin E genes between $EGRxSLP65^{-/-}$ and $SLP65^{-/-}$ pre-B cells.

Differences in the expression of cyclin genes were tested by real-time RT-PCR using the expression of ribosomal protein L5 (RPL5) as an internal standard. After calculating the values and standard deviations of the Ct values for each gene, the relative quantities (Q) of the expression levels of cyclin genes between $EGRxSLP65^{-/-}$, $SLP65^{-/-}$ and wild type pre-B cells were assessed by the following equation: $Q = 1.84^{(\text{experiment-MEAN-Ct} - \text{baseline-MEAN-Ct})}$.

The analysis showed that a house-keeping gene RPL5 was amplified with the Ct value differences below 0.5 in all replicates and all samples compared, suggesting equivalent amount of input RNA in all replicates and between samples from $EGRxSLP65^{-/-}$, $SLP65^{-/-}$ and

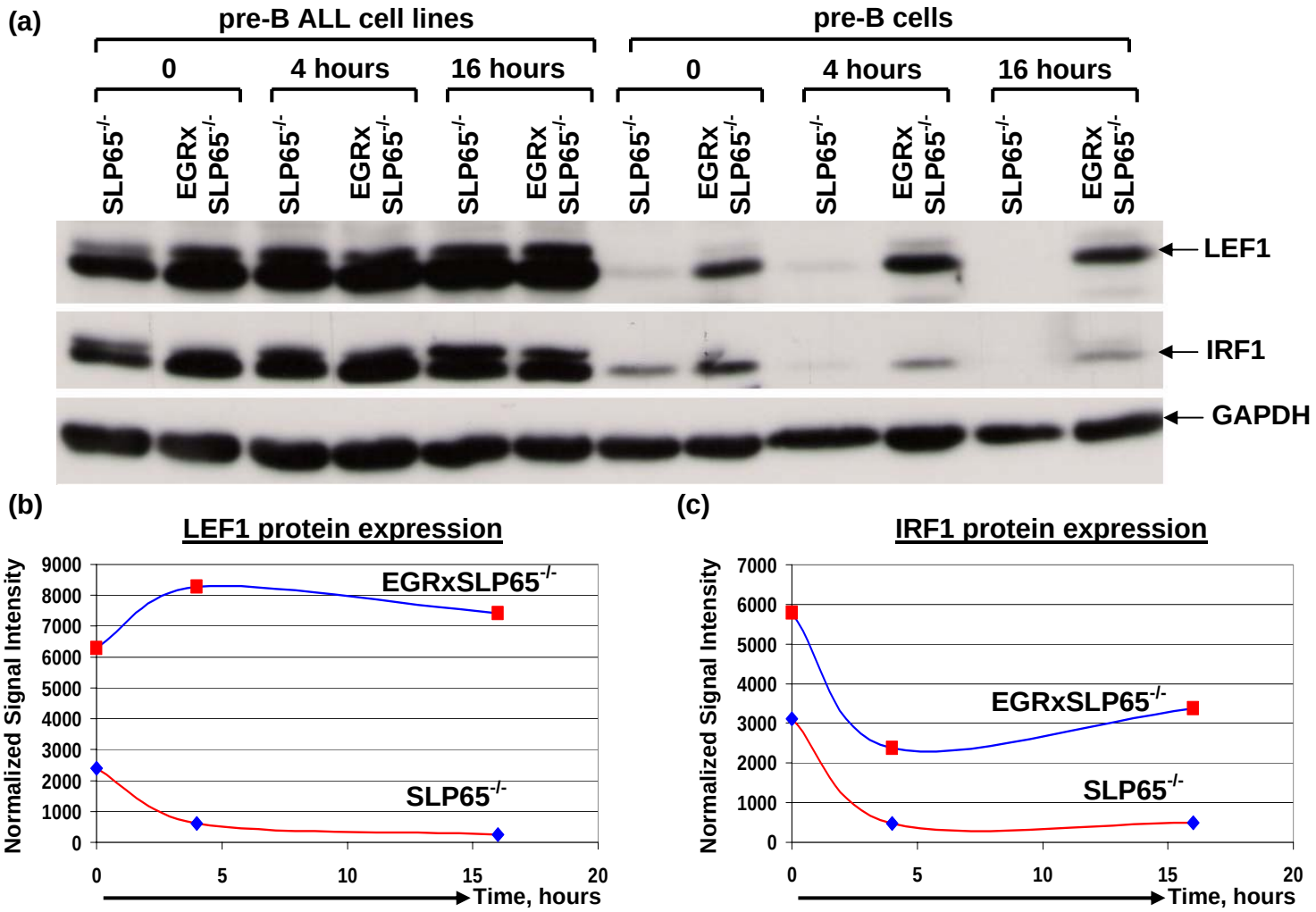


Figure 29: EGR1 maintains LEF1 and IRF1 protein expression during differentiation. (a) Immunoblot analysis of total cell lysates for the expression of LEF1 and IRF1 upon IL-7 withdrawal in SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ pre-B ALL cell lines as well as *in vitro*-cultivated pre-B cells from SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice. Total cell lysates were prepared either directly from cells in culture or after 4 hours and 16 hours of IL-7 withdrawal. The diagrams depict (b) LEF1 protein and (c) IRF1 protein induction kinetics during differentiation in pre-B cells from SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice.

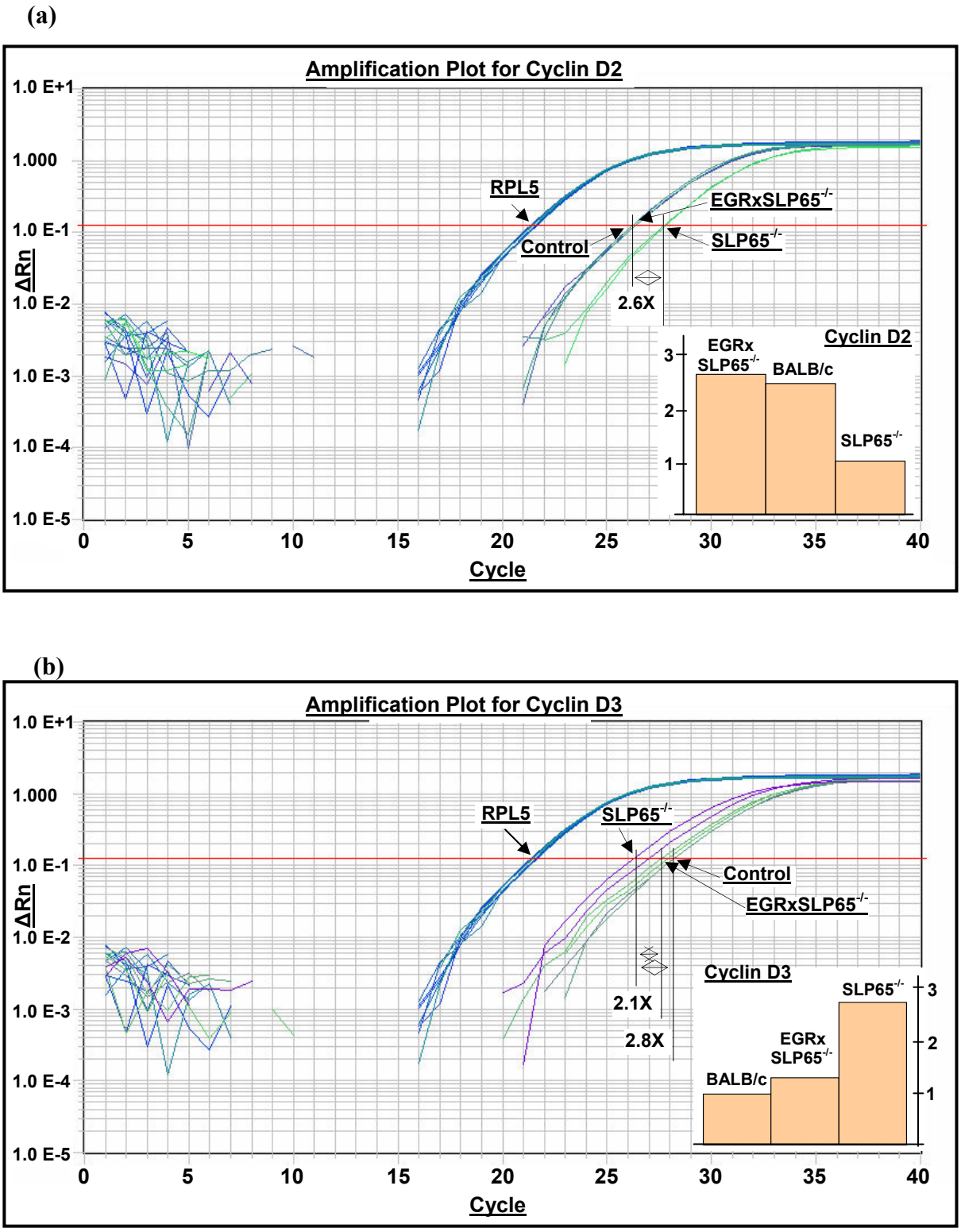


Figure 30: EGR1-mediated regulation of cyclin D2 and cyclin D3 genes. Real Time RT-PCR analysis for the relative expression of cyclin D2 (a) and cyclin D3 (b) genes in rapidly proliferating *in vitro*-cultivated BM derived B cells from SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice are shown. EGR1 activity leads to the upregulation of cyclin D2 and downregulation of cyclin D3 genes in proliferating SLP65$^{-/-}$ B cells.

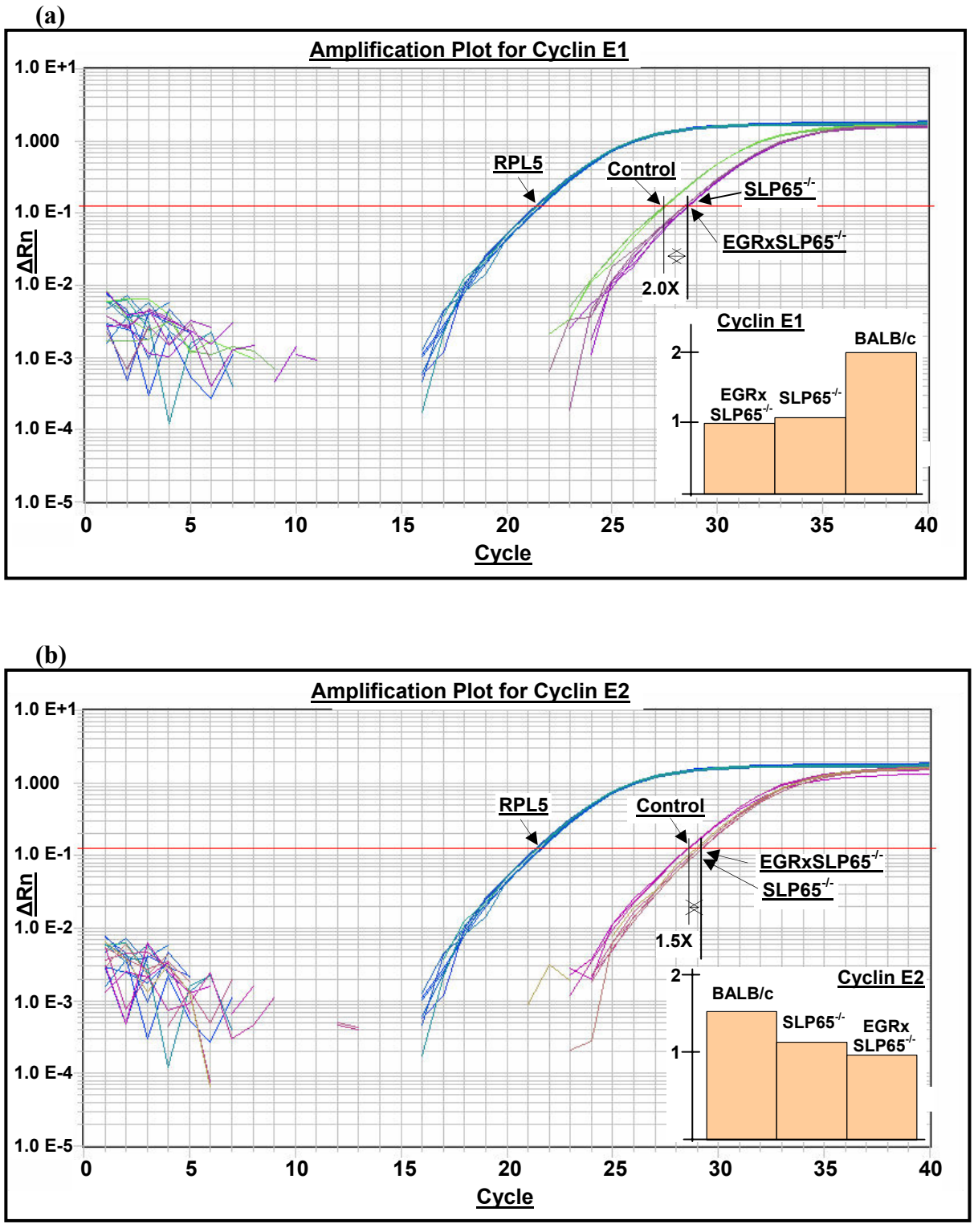


Figure 31: Real Time RT-PCR analysis for the relative expression of cyclin E1 and cyclin E2 genes in rapidly proliferating *in vitro*-cultivated BM derived B cells from SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice. Amplification plots for cyclin E1 (a) and cyclin E2 (b) showed no differences between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ proliferating pre-B cells.

wild type pre-B cells. Cyclin D1 could not be detected in any of the pre-B cell samples (data not shown). However, cyclin D2 gene expression is 2.4 fold lower in proliferating SLP65$^{-/-}$ pre-B cells compared to the wild type or EGRxSLP65$^{-/-}$ pre-B cells (Fig. 30a). The cyclin D3 gene was expressed at levels 2-3 fold higher in SLP65$^{-/-}$ pre-B cells compared to EGRxSLP65$^{-/-}$ or control pre-B cells (Fig. 30b). These data demonstrate that EGR1 activity in EGRxSLP65$^{-/-}$ upregulates cyclin D2 gene, whereas the lower levels of cyclin D2 gene expression might be compensated by higher levels of the expression of cyclin D3 gene in SLP65$^{-/-}$ pre-B cells.

Both SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ pre-B cells expressed equally low amounts of cyclin E1 gene expression, at levels about 2 fold lower than the control (Fig. 31a). In contrast, cyclin E2 gene expression was not significantly different between EGRxSLP65$^{-/-}$, SLP65$^{-/-}$ and control pre-B cells (Fig. 31b). Although further direct analysis of cell cycle and proliferation in EGRxSLP65-/- and SLP65-/- pre-B cells is necessary, our observation is that *in vitro*-cultivated pre-B cells from both mutant mice proliferate even more rapidly compared to the wild type pre-B cells. Thus, these preliminary data suggest that although both E-type cyclins (cyclin E1 and E2) are expressed at lower levels in EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ pre-B cells compared to the control, both mutant pre-B cells might employ additional mechanisms to compensate the reduced levels of the expression of cyclin E genes, required for the progression through the S-phase of the cell cycle.

4. Discussion

4.1 EGR1 overexpression cannot rescue the developmental block at the pre-B cell stage in the absence of SLP65

The signaling adaptor protein SLP65 is a key molecule in the formation of signaling complexes triggered by the pre-BCR and BCR. In the absence of SLP65, B cell development is partially blocked at the pre-BCR$^+$ large pre-B cell stage. Consequently, SLP65$^{-/-}$ mice show increased numbers of B220$^+$ CD43$^+$ pre-B cells and decreased numbers of Igκ$^+$ immature B cells in the bone marrow (Jumaa et al., 1999). At the early stages of B lymphopoiesis EGR1 promotes maturation and the transition from pro-B to pre-B cell stage in the bone marrow (Dinkel et al., 1998). Therefore, the first goal of our studies was to test the hypothesis if transcription factor EGR1 overexpression in SLP65$^{-/-}$ B cells can restore the developmental arrest at the large pre-B cell stage. The phenotypic comparison of bone marrow cells, from EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ as well as from BALB/c control mice, disproved this possibility that SLP65-deficient B cells overexpressing EGR1 can overcome the developmental block. In the bone marrow, B220^{low} c-kit$^+$ CD43$^-$ early pro-B and B220^{low} c-kit$^+$ CD43$^+$ late pro-B cell numbers did not significantly differ between wild type, SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ mice, suggesting that SLP65 adaptor protein is not important in pro-B cell stages. Both EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ mice showed significantly higher (about threefold) numbers of B220^{low} c-kit$^-$ CD43$^+$ pre-B cells compared to the control. Consistent with these data, the analysis of pre-BCR and Igκ expressing cells in the bone marrow revealed equally high numbers of pre-BCR$^+$ large pre-B cells and equally lower numbers of Igκ$^+$ immature B cell compartment in EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ mice compared to the wild type animals. Therefore, B220^{low} IgM$^+$ immature B cells were less frequent in the bone marrow of both mutant mice and as a consequence, there were lower numbers of B220^{hi} IgM^{low} recirculating mature B cells and higher numbers of B220^{hi} IgM^{hi} transtional B cells in EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ mice compared to the wild type control.

Pro-B cells express RAG-genes, which activate the V(D)J recombination and lead to the rearrangement of the IgH chains (Schatz et al., 1989; Oettinger et al., 1990). After successful V(D)J recombination in pro-B cells, Igμ chain expression results in the assembly of pre-BCR, composed of Igα, Igβ, μ-heavy chain and surrogate light chains (V-pre-B and λ5 polypeptides) (Pillai and Baltimore 1987; Tsubata and Reth 1990). In large pre-B cells, pre-BCR signaling downregulates the expression of RAG genes (Stanhope-Baker et al., 1996) and inhibits V(D)J recombination (Kitamura et al., 1991). Subsequently, the cells expand clonally

and allelic exclusion prevents additional heavy chain rearrangement. After clonal expansion pre-B I cells enter to the next stage, the small pre-B cell stage to allow light chain gene recombination through RAG activity. The expression of two transcription factors, IRF4 and SpiB is necessary and sufficient to induce germline Igκ transcription in small pre-B cells (Muljo and Schlissel 2003). In contrast to pre-B II cells, large pre-B cells express low levels of IRF4 and SpiB transcripts. Without SLP65 B cells are arrested at the pre-B I cell stage and this developmental block is not overcome by EGR1 as expression levels of RAG2, IRF4 and SpiB genes were equally low in both SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ BM-derived *in vitro*-cultivated pre-B cells. Higher levels of RAG2, IRF4 and SpiB expression in wild type pre-B cells suggest that wild type cells move to the pre-B II stage in culture, whereas SLP65-deficient B cells stay mainly at the pre-B I stage. Thus, these data together with the results of the phenotypic comparison of bone marrow cells suggest that EGR1 activity alone cannot rescue the developmental arrest at the large pre-B cell stage in the absence of SLP65 adaptor protein.

4.2 EGR1 mediates downregulation of IL-2R alpha chain (IL-2Rα) in B cells

Interleukin-2 (IL-2) is a pluripotent cytokine which plays a crucial role in the immune system response. IL-2 has broad activities in the immune system, enhancing cellular proliferation by acting as a cell cycle progression factor, as well as promoting functional differentiation of T and B lineage cells (Smith 1988; Willerford et al., 1995). The action of IL-2 requires the presence of a cell surface receptor. The high affinity IL-2 receptor (IL-2R) is composed of three component chains, IL-2Rα (p55, CD25, Tac antigen), IL-2Rβ (p75, CD122) and IL-2Rγ (p65) (Smith 1988; Waldmann 1991; Leonard et al., 1994). IL-2Rβ and IL-2Rγ are present constitutively in resting lymphocytes (Siegel et al., 1987; Kondo et al., 1994). Substantial proportions of resting B cells constitutively express low levels of IL-2Rα and anti-IgM stimulation can induce IL-2Rα expression (Nakanishi et al., 1992). IL-2Rα is mostly expressed only following activation (Smith 1988; Waldmann 1991; Taniguchi and Minami 1993). Together, the IL-2Rβ and IL-2Rγ chains form a low affinity IL-2R, which is sufficient to effect signal transduction upon ligation (Nakamura et al., 1994; Nelson et al., 1994). While the IL-2Rα chain is incapable of independently generating intracellular signals, its association with the IL-2Rβ and IL-2Rγ chains forms the high affinity IL-2R (Teshigawara et al., 1987; Wang and Smith 1987). Therefore, one function of IL-2Rα is to regulate the sensitivity of activated lymphocytes to IL-2 cytokine.

In pre-B cell development IL-2Rα expression defines a crucial stage and is a useful marker to distinguish pro-B cells from pre-B cells in bone marrow (Rolink et al., 1994). Its expression is initiated by functional rearrangement and expression of Igμ heavy chain gene and is downregulated when immature B cells mature and express IgD (Chen et al., 1994). Therefore, the transition from pro-B to pre-B cells is marked by the downregulation of c-kit (CD117) and by the upregulation of IL-2Rα chain. This transition can be visualized by monitoring CD117 (c-kit), CD43 and IL-2Rα expression: $CD117^+$ $CD43^+$ late pro-B cells do not express IL-2Rα, $CD117^-$ $CD43^+$ large pre-B cells express low levels of IL-2Rα and as the cells progress into the small pre-B cell stage, they become $CD43^-$ IL-$2R\alpha^{+/hi}$ (Rolink et al., 1994; Hayashi et al., 2000). In the absence of SLP65, pre-B cells fail to efficiently downregulate the membrane sialoglycoprotein CD43 and upregulate the expression of metallopeptidase BP-1, the adhesion molecule CD2 as well as IL-2Rα chain (Hayashi et al., 2000; Hayashi et al., 2003; Kersseboom et al., 2003). Surprisingly, in EGRxSLP65$^{-/-}$ mice we observed even lower levels of IL-2Rα expression than in SLP65$^{-/-}$ animals. Moreover, EGR1 transgenic mice on a wild type background also showed lower levels of the expression of IL-2Rα in bone marrow B cell subsets compared to the wild type mice. Inspection of the mouse IL-2Rα promoter revealed three putative EGR1 binding sites. Promoter activity experiments are needed to verify EGR1 mediated regulation of the IL-2Rα promoter but the above data suggest that IL-2Rα might be a target gene for EGR1 in B cells.

4.3 EGR1 protein expression and induction is impaired in SLP65$^{-/-}$ splenic B cells

As an immediate early gene, EGR1 is rapidly and transiently induced upon BCR stimulation in B cells (Seyfert et al., 1989; McMahon and Monroe 1995). EGR1 is induced through the different subgroups of mitogen-activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 MAPK pathways (McMahon and Monroe 1995; Silverman and Collins 1999). In B cells, upon BCR engagement SLP65 is phosphorylated by Syk and provide docking sites for Btk and PLCγ2 (Hashimoto et al., 1999; Su et al., 1999). In this complex, Btk can phosphorylate and activate PLCγ2 which leads to inositol 1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG) generation, and subsequently Ca^{2+} mobilization and protein kinase C (PKC) activation (Kurosaki et al., 2000; Niiro and Clark 2002), which in turn lead to the activation of ERK, JNK and p38 MAPK pathways and serum response factor (SRF) activation through which EGR1 transcription is induced. Therefore, we were interested to compare the expression levels of EGR1 protein in SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ B cells. Analysis of purified $B220^+$

splenic B cells revealed that SLP65$^{-/-}$ B cells express 2-3 fold lower basal levels of EGR1 protein compared to the wild type B cells, whereas EGR1 protein expression levels were restored in EGRxSLP65$^{-/-}$ B cells. These data demonstrated that indeed EGR1 protein expression is dependent on SLP65 in immature transitional and mature B cells. Further we analyzed the kinetics of EGR1 induction in SLP65$^{-/-}$ B cells upon BCR stimulation. As it was found for basal levels of EGR1 expression, also the inducible expression of EGR1 was reduced in SLP65-deficient B cells. However, since there was some residual induction of EGR1 in SLP65$^{-/-}$ B cells, SLP65 independent signaling cascades are likely to regulate EGR1 expression too. Btk, for example, can partially function as an adaptor molecule, independent of its kinase activity (Middendorp et al., 2003). Btk can associate with phosphatidylinositol (4)-phosphate 5-kinase, which is the enzyme that generates the substrate for PLCγ2 (Saito et al., 2003), and following activation of PLCγ2 induces the generation of $InsP_3$ and DAG, subsequent Ca^{2+} mobilization and PKC activation, which lead to the activation of downstream signaling pathways: ERK, JNK and p38MAPK (Niiro and Clark 2002). Thus, Btk and potentially additional adaptors in B cells might compensate for the lack of the activity of SLP65 and lead to the low levels of expression of EGR1 in SLP65$^{-/-}$ B cells. Nevertheless, our results show that EGR1 expression and induction in B cells is strongly dependent on SLP65 activity and that EGR1 acts downstream of SLP65 in splenic B cells.

4.4 Phenotypic comparison of follicular B cells

Two populations of splenic B cells were identified as precursors of mature B cells (Carsetti et al., 2004). Transitional B cells of type 1 (T1) are the recent immigrants from the bone marrow. Migrating T1 B cells expressing BCRs with high affinity to soluble self-antigens (Ag) in the blood are likely to die by negative selection by the way of Ag-induced death. On entry to the spleen, T1 B cells remain at the periarteriolar lymphoid sheath (PALS), where additionally blood-borne self-Ag trapped by the spleen may further drive negative selection. The remaining T1 B cells develop into transitional B cells of type 2 (T2), which are found exclusively in the primary follicles of the spleen (Loder et al., 1999). Situated in the microenvironment of splenic follicles, T2 B cells are shielded from the soluble antigens to which T1 B cells are exposed. Instead, T2 B cells likely encounter a unique set of Ag, possibly on follicular dendritic cells, which results in positive selection (Su and Rawlings 2002). The transition from immature transitional stages to mature B cells is characterized by a series of changes in surface marker expression (Carsetti et al., 1995; Carsetti et al., 2004).

Since basal levels of EGR1 protein expression is low and EGR1 protein induction is impaired in SLP65$^{-/-}$ B cells, the defects in development and maturation of SLP65$^{-/-}$ B cells might be partially due to the missing activity of EGR1 in these cells. In order to assess if restored levels of EGR1 could promote B cell maturation in SLP65$^{-/-}$ B cells, we analyzed spleen B cells from EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ mice. The phenotypic analysis revealed that EGR1 activity alone could not completely rescue the developmental arrest in SLP65$^{-/-}$ B cells at the transitional stages and did not result in completing maturation of B cells. Nevertheless, EGRxSLP65$^{-/-}$ B cells have more mature phenotype compared to SLP65$^{-/-}$ B cells. We found less numbers of immature T1 B cells (B220^{+} IgM^{hi} IgD^{low} CD21^{-} CD23^{-} pB493^{+} B cells) in the spleens of EGRxSLP65$^{-/-}$ mice in comparison to the SLP65$^{-/-}$ animals. Although, CD21 and CD23 expression on splenic B cells from both mutant mice did not show significant differences, there are less cells which are B220^{+} IgM^{hi} pB493^{+} B cells in EGRxSLP65$^{-/-}$ mice, suggesting that both T1 and T2 B cell compartments might be smaller in

EGRxSLP65$^{-/-}$ mice. Moreover, there are less B220^{+} IgM^{hi} IgD^{low} B cells and more B220^{+} IgM^{hi} IgD^{hi} as well as B220^{+} IgM^{low} IgD^{hi} B cells in the spleens of EGRxSLP65$^{-/-}$ mice. All together, our data suggest that EGR1 overexpression in SLP65$^{-/-}$ B cells partially rescues the SLP65-dependent developmental block in the spleen. These results prompted us to run gene expression profiling experiments in order to uncover the underlying molecular basis of EGR1-mediated phenotypic changes in EGRxSLP65$^{-/-}$ B cells as well as to identify additional EGR1 target genes B cells.

4.5 EGR1 target genes identified by microarrays and validation experiments

4.5.1 Lymphocyte 6 (Ly-6) antigens: Ly-6A/E, Ly-6D and Ly-6E

Expression profiling identified Ly-6A/E, Ly-6D and Ly-6E antigens to be upregulated in EGRxSLP65$^{-/-}$ B cells compared to the wild type B cells. Ly-6A/E was found to be expressed at high levels (19.7-fold upregulated) in SLP65$^{-/-}$ B cells and its expression levels are even higher (29-fold upregulated) in EGRxSLP65$^{-/-}$ B cells. Although the hybridizations failed to show differences in mRNA levels of Ly-6D and Ly-6E between SLP65$^{-/-}$ and wild type B cells, flow cytometric analysis revealed that SLP65$^{-/-}$ B cells express more Ly-6D and Ly-6E antigens than wild type B cells. This discrepancy between mRNA and protein levels may be due to differences between mRNA and protein stability and/or turnover.

The Ly-6 superfamily (Ly-6SF) derives its name from the identification of a lymphocyte antigen (Ag) that was observed on activated T cells (McKenzie et al., 1977). A large cluster of

cross-hybridizing genes is located on murine chromosome 15 (LeClair et al., 1987) and includes the known genes Ly-6A/E (Khan et al., 1990), Ly-6C (Bothwell et al., 1988), Ly-6F and Ly-6G (Fleming et al., 1993), Ly-6H (Apostolopoulos et al., 1999) and Ly-6I (Pflugh et al., 2000). Other related but non-hybridizing genes, such as ThB/Ly-6D (Gumley et al., 1992) and Ly-6E/TSA-1/Sca-2 (MacNeil et al., 1993), are also located near this cluster. Ly-6SF complex exists in two allelic forms, with Ly-6.1 alleles generally being expressed at lower levels than their Ly-6.2 counterparts (Gumley et al., 1995). Human homologues were first recognized with the identification of CD59 (Philbrick et al., 1990). Later, CD59 homologue has been characterized in the mouse (Powell et al., 1997). At the present time human homologues for several mouse Ly-6SF members are known, including ThB (Brakenhoff et al., 1995), Ly-6H (Horie et al., 1998) and Ly-6E/TSA-1/Sca-2/RIG-E (Capone et al., 1996; Mao et al., 1996). In addition, the human urokinase plasminogen activator receptor (uPAR/CD87) has been identified as a member of the Ly-6 superfamily (Palfree 1991).

Most hematopoietic cells express one or more members of the Ly-6 superfamily and the expression of Ly-6 proteins varies with development and activation (Gumley et al., 1995). Ly-6A/E is expressed on the hematopoietic stem cells (Kondo et al., 1997; Morrison et al., 1997), on distinct subpopulations of bone marrow and peripheral B lymphocytes (Ortega et al., 1986; Codias and Malek 1990) as well as thymic and peripheral T lymphocytes (Codias et al., 1989). Ly-6D has been detected on cortical thymocytes, small $sIg^{-}Thy\text{-}1^{-}$ and sIg^{+} lymphoid cells in the bone marrow, thymic medullary epithelial cells and peripheral B lymphocytes, but not on peripheral T lymphocytes (Eckhardt and Herzenberg 1980; Godfrey et al., 1990). Ly-6E is expressed on subsets of thymic medullary and cortical epithelial cells, thymic and splenic dendritic cells, activated peripheral T lymphocytes, bone marrow myeloid and B cells as well as peripheral B lymphocytes, but not on peripheral T cells (Godfrey et al., 1992; Tucek et al., 1992; Vremec et al., 1992; Kosugi et al., 1994). In addition, subsets of monocytes and granulocytes can express various levels of Ly-6C, Ly-6I and Ly-6G (Pflugh et al., 2002). Moreover, it has been shown that the murine Ly-6 superfamily members are involved in the progression of certain mouse tumors (Treister et al., 1998). The expression levels of several members of Ly-6 family was higher on highly malignant cells than on tumor cells expressing a lower malignancy phenotype (Witz 2000).

Given the number of known Ly-6 molecules and their apparent importance in the development and differentiation of most hematopoietic lineages, relatively little is known about the function of Ly-6 proteins. For example, CD59 binds complement components C8 and C9, subsequently blocks assembly of membrane attack complex (Davies et al., 1989;

Rollins and Sims 1990). Ly-6E has been shown to be physically associated with the TCR (Kosugi et al., 1998) and Ly-6A/E can influence T cell signaling and TCR expression (Lee et al., 1994). T cells from Ly-6A/E knockout mice are hyper-responsive (Stanford et al., 1997), suggesting Ly-6A/E may function to negatively regulate lymphocyte activation.

Transitional B cells express higher levels of Ly-6 antigens in comparison to the mature B cells. This is reflected by high expression levels of Ly-6A/E, Ly-6D and Ly-6E antigens in B cells from both EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ mice. Ly-6A/E might be a direct target gene for EGR1 in B cells, since its expression levels are even higher in EGRxSLP65$^{-/-}$ B cells compared to the SLP65$^{-/-}$ B cells and moreover, there is a putative EGR1 binding site in the Ly-6A/E promoter.

4.5.2 TATA-box-Binding Protein-Associated Factor, 30 kD ($TAF_{II}30$/TAF10)

Interestingly, gene expression profiling has identified TAF10 gene to be downregulated in SLP65$^{-/-}$ B cells, whereas its expression is restored in EGRxSLP65$^{-/-}$ B cells. We could verify these data on the protein levels showing TAF10 protein to be expressed in lower levels in SLP65$^{-/-}$ compared to the wild type B cells, whereas in EGRxSLP65$^{-/-}$ B cells its expression levels are partially restored. Furthermore, the analysis of TAF10 promoter sequence revealed 3 putative EGR1 binding sites in the mouse and interestingly, there is a single EGR1 binding site also in human TAF10 gene. These data suggest that TAF10 might be a potential EGR1 target gene in B cells.

Initiation of transcription at the eukaryotic class II promoters is a multistep process requiring the coordinated action of many proteins. In general, RNA polymerase II (Pol II) and a host of other factors, including the general transcription factors TFIIA, -B, -D, -E, -F, and –H, work together to form a preinitiation complex (PIC) and allow subsequent transcription (Mohan et al., 2003). The TATA-box-binding protein (TBP) and 14 evolutionarily conserved TBP-associated factors (TAFs) form TFIID (Hampsey 1998). Electron microscopy has shown that TFIID contains surfaces that could mediate both extensive core promoter and protein-protein interactions (Andel et al., 1999; Brand et al., 1999). Given its early requirement *in vitro*, as well as its capacity to direct PIC assembly on both TATA-containing and TATA-less promoters, TFIID was proposed to be a central component of the PIC (Mohan et al., 2003). As *in vitro* transcription experiments have suggested TAFs within TFIID function as coactivators by engaging in direct and selective interactions with transactivators and/or core promoter sequences (Bell and Tora 1999). Furthermore, TAF1 (formerly, $TAF_{II}250$; (Tora 2002)) has kinase, histone acetyltransferase (HAT) and ubiquitin conjugating/ligating

enzymatic activities (Wassarman and Sauer 2001), suggesting that TAFs can affect transcription at multiple levels.

Multiple TFIID complexes exists, indeed nuclear extracts contain at least two subpopulations of TFIID: those that contain TAF10 and those that do not contain TAF10 (Tora 2002). TAF10 is required for TFIID stability, cell cycle progression and transcription in the early mouse embryo (Mohan et al., 2003). To study the functions of TAF10, the two alleles of TAF10 gene have been targeted by homologous recombination in murine F9 embryonal carcinoma cells and subsequently disrupted using a Cre recombinase-loxP strategy (Metzger et al., 1999). Cells lacking TAF10 are blocked in G_0/G_1 phase of cell cycle and undergo apoptosis. In agreement with the G_1 arrest phenotype, the expression of cyclin E is decreased, phosphorylation of retinoblastoma (Rb) protein is decreased and p21 expression is increased in the TAF10-null cells (Metzger et al., 1999). Moreover, the authors have concluded that TAF10 is not indispensable for class II transcription in general, but seems to be required for the expression of a subset of genes.

It has been demonstrated that BCR-activated SLP65$^{-/-}$ B cells fail to enter the cell cycle, and this is due to their inability to induce the expression of the cell cycle regulatory proteins such as cyclin D2 and cyclin-dependent kinase 4 (Tan et al., 2001). Consistent with these data, low levels of TAF10 expression might account for additional defects at the G_0/G_1 phase of cell cycle in SLP65$^{-/-}$ B cells, whereas these defects might be at least partially restored in EGRxSLP65$^{-/-}$ B cells.

4.5.3 Schlafen 1 (SLFN1) and Schlafen 2 (SLFN2) genes

By gene expression profiling both SLFN1 and SLFN2 genes were found to be differentially expressed between SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ B cells, these results were confirmed by real-time RT-PCR. SLP65$^{-/-}$ B cells express relatively high levels of SLFN1 and lower levels of SLFN2 genes compared to the wild type B cells.

In the literature, there is the only report describing a new family of growth regulatory genes - the Schlafen (SLFN) gene family. In mice, SLFN gene family consists of at least seven highly related members (Schwarz et al., 1998). These genes encode proteins that fall into two distinct groups based on size; a short form encoded by SLFN1 and SLFN2, and a long form encoded by SLFN3 through SLFN7.

SLFN1, 2, 4 and 7 were found to be mainly expressed in the thymus, lymph nodes and spleen, whereas the highest expression levels of other members of the family were detected in the testis, although there was a small amount detected in the thymus and heart. The analysis of the

four major thymic subsets; immature $CD3^-$ $CD4^-$ $CD8^-$ triple-negative (TN) cells, $CD4^+$ $CD8^+$ double positive (DP) cells, mature single positive (SP) $CD4^+$ and $CD8^+$ T cells, revealed that SLFN1 is upregulated approximately 100-fold during the DP to SP transition. SLFN2 expression is also increased during this transition, but the increase was found to be only 5- to 10-fold. Following T cell activation with anti-CD3 antibodies, there was an increase in the expression of SLFN3, 4, 6 and 7, and concomitant decrease in the expression of SLFN1 and 2. These changes were more pronounced following the costimulation with CD3 and CD28. These data suggested that the expression of SLFN gene family is linked to cell growth. Indeed, using a repressible promoter system to drive the expression of SLFN1 in NIH-3T3 fibroblasts, the authors demonstrated that the induction of SLFN1 causes a cell cycle arrest such that cells fail to initiate DNA synthesis and are consequently arrested at the G_0/G_1 stage of the cell cycle. Although, the mechanism, how SLFN genes perturb the cell cycle, is not known yet, they must act on the fundamental machinery involved with cell cycle control, since the lymphocyte specific members can arrest growth of fibroblast.

Consistent with the notion that $SLP65^{-/-}$ B cells fail to enter the cell cycle (Tan et al., 2001) and our findings that $SLP65^{-/-}$ B cells express low levels of TAF10, the cell cycle arrest at the G_0/G_1 stage might be even more profound also due to the differential expression of SLFN1 and SLFN2 genes in $SLP65^{-/-}$ B cells. These defects might be restored in $EGRxSLP65^{-/-}$ B cells, since they express both SLFN1 and SLFN2 genes close to the levels observed in wild type B cells. Interestingly, in the regulatory regions of both SLFN genes, there are EGR1 binding sites. Although direct promoter assay experiments are still needed to confirm the regulation of SLFN genes by EGR1, the above data suggest that EGR1 might directly regulate both SLFN1 and SLFN2 genes in B cells. Moreover, according our microarray data, the expression of SLFN4 gene is 11-fold and 22-fold increased in wild type B cells upon BCR stimulation for 45 min and 6 hours, respectively. Although in resting B cells the expression levels of SLFN4 gene is not significantly different between $SLP65^{-/-}$ and wild type B cells, upon BCR cross-linking SLFN4 gene is expressed at even higher levels in $SLP65^{-/-}$ B cells, being 28-fold and 39-fold increased after 45 min and 6 hours, respectively. Thus, our results here demonstrate for the first time that SLFN1, SLFN2 and SLFN4 genes are also expressed in B cells, which suggest that SLFN genes might be also involved in growth control of B lymphocytes.

4.5.4 S100 calcium binding protein A8 (S100A8) - Calgranulin A (CalA)

We found by the means of two independent methods, microarrays and real-time RT-PCR that the expression levels of CalA gene is significantly high in SLP65$^{-/-}$ B cells compared to the wild type B cells. EGRxSLP65$^{-/-}$ B cells express intermediate levels, CalA gene expression is decreased in EGRxSLP65$^{-/-}$ cells compared to the SLP65$^{-/-}$ B cells.

Myeloid-related proteins (MRPs) are involved in neutrophil migration (Guignard et al., 1995). Two members of MRPs, CalA and CalB (10.6 and 13.5 kDa, respectively (Odink et al., 1987)) are calcium-binding proteins that belong to the S100 protein family (Schafer and Heizmann 1996). They are expressed almost exclusively by cells of myeloid lineage (Ryckman et al., 2003). CalA and CalB form noncovalent homodimers and a heterodimer (CalA/B) in a calcium dependent manner (Hunter and Chazin 1998). The MRPs are constitutively expressed in the cytosol of neutrophils where they comprise up to 30% of the cytosolic proteins (Hessian et al., 1993). CalA/B is the main form found in the extracellular milieu and its *in vitro* release by neutrophils, activated monocytes and macrophages has also been demonstrated (Pechkovsky et al., 2000). CalA, CalB and CalA/B are potent stimulators of neutrophils and involved in neutrophil migration to inflammatory sites (Ryckman et al., 2003).

Little is known about the expression and function of calcium binding proteins in B cells. In one report the authors have characterized a B cell surface antigen with homology to the CalA (Shapiro et al., 1999). Similarly to CalA in myeloid cells, this antigen is expressed in the cytoplasm of B cells and is secreted by LPS-induced activated B cells. The authors concluded that this surface antigen is not B cell specific and is a new member of S100 calcium binding protein family, since CalA is not expressed by lymphoid cells (Shapiro et al., 1999). Recent data indicate that, in gastric B cell mucosa-associated lymphoid tissue lymphomas, the transition to destructive lymphoepithelial lesions and malignant lymphoma is marked by an increase in transcription of a single gene encoding CalA (Mueller et al., 2003).

Since, regulatory region of the CalA gene contains putative EGR1 binding sites, these data suggest that EGR1 might directly downregulate or suppress the expression of CalA gene in B cells.

4.5.5 Chemokine C-C motif Receptor 2 (CCR2)

Interestingly, we found that SLP65$^{-/-}$ B cells express relatively high levels of CCR2 gene compared to the wild type B cells, whereas CCR2 is expressed in EGRxSLP65$^{-/-}$ B cells close

to the levels observed in the wild type B cells. These results were also verified by real-time RT-PCR analysis.

Chemokines are the proinflammatory cytokines that function in leukocyte chemoattraction and activation. Monocyte chemoattracktant protein 1 (MCP1) is produced by endothelial cells, smooth muscle cells and macrophages in response to variety of meditators. MCP1 and its receptor CCR2 may be involved in inflammatory processes in disorders including rheumatoid arthritis, collagen-induced arthritis, alveolitis and tumor infiltration (Charo et al., 1994; Bruhl et al., 2004). CCR2 is expressed in monocytes, activated T cells as well as in B lymphocytes (Frade et al., 1997). CCR2-deficient mice have been generated (Kuziel et al., 1997). CCR2 is expressed in immature T1 B cells, while being dramatically downregulated at the T2 B cell and mature B cell stages (Flaishon et al., 2004). Immature transitional B cells are targeted to specific areas in the spleen, where a fraction of these cells receive signals that induce them to mature and participate in the immune response. CCR2-deficient cells exhibit upregulation of chemokine-induced actin polymerization, migration and homing to the lymph nodes of immature B cells (Flaishon et al., 2004). Thus, CCR2 is involved in the homing regulation of immature B cells and their targeting to the spleen for their final maturation.

Taking into account, that CCR2 is expressed only in T1 B cells and is downregulated in T2 and mature B cells, a possible interpretation of these data might be that there are more T1 B cells in SLP65$^{-/-}$ mice in comparison to the wild type as well as EGRxSLP65$^{-/-}$ littermates, which is also consistent with the results of phenotypic comparison by FACS. In addition, CCR2 might be directly downregulated by EGR1 in B cells, since both the mouse and human CCR2 genes reveal EGR1 binding sites in their promoter regions.

4.5.6 Octamer-binding Transcription Factor 1 (Oct1/POU2F1)

We found that Oct1 gene is downregulated in EGRxSLP65$^{-/-}$ B cells compared to SLP65$^{-/-}$ B cells. In EGRxSLP65$^{-/-}$ B cells, Oct1 gene expression pattern is similar to the wild type B cells. Although, microarray data indicated that Oct1 gene is downregulated in SLP65$^{-/-}$ B cells compared to the wild type control, real-time RT-PCR assays detected significantly high levels of Oct1 in SLP65$^{-/-}$ B cells. These data suggest that Oct1 transcription might be downstream of SLP65 signaling and Oct1 might be regulated directly by EGR1 in B cells, since Oct1 gene promoter contains EGR1 binding sites.

B cell development consists of an ordered program of transcriptional events, leading ultimately to the rearrangement and expression of immunoglobulin (Ig) molecules. Although the Ig heavy and light chain enhancers contain binding sites for numerous different classes of

transcription factors, variable region promoters seem to be less complex and prominently contain a motif termed the octamer element (5'-ATGCAAAT-3') (Falkner and Zachau 1984; Wirth et al., 1987). The canonical octamer or its reverse complement is conserved in the majority of Ig heavy and light chain variable region promoters (Matsuda et al., 1998). The importance of the octamer element in mediating Ig transcription has been demonstrated by using transgenic mice: a point mutation in the octamer reduces the expression of an Ig-transgene by over 20-fold (Jenuwein and Grosschedl 1991). Several studies have indicated that, when attached to heterologous promoter, the octamer element can confer B cell specificity (Wirth et al., 1987). Octamer or octamer like sequences have been implicated in the regulation of a number of lymphoid specific genes such as CD20, CD21, CD36, IL-2, IL-4 and Pax5 (Wang et al., 2004). However, octamer elements are also important in the regulation of ubiquitously expressed genes such as U1, U2 and U6 small nuclear RNA and histone H2B (Wang et al., 2004).

The POU domain containing proteins Oct1 and Oct2 were identified as protein activities that selectively interact with the octamer sequence (Singh et al., 1986; Clerc et al., 1988; Staudt et al., 1988). The DNA-binding POU domain consists of two subdomains (the POU-specific and POU-homeodomain) tethered by a short linker sequence (Sturm and Herr 1988; Verrijzer et al., 1992). The DNA-binding domains of Oct1 and Oct2 are highly homologous and both proteins bind octamer DNA with equal affinity *in vitro* (LeBowitz et al., 1988). Oct1 is widely expressed whereas Oct2 expression is restricted to the lymphoid and neuronal compartments (Muller et al., 1988; Stoykova et al., 1992). Because of its expression pattern, Oct2 was thought to be an important regulator of Ig expression. However, B cell development in $Oct2^{-/-}$ animals is normal. Analysis of $Oct2^{-/-}$ fetal liver revealed the presence of pre-B cells with rearranged Ig genes in numbers similar to that of wild type littermates. The transcription rates of several genes, including Igμ and Igκ were also normal in the fetal liver pre-B cells (Corcoran et al., 1993). The discovery of Oct-binding factor 1 (OBF1/Bob1/OCA-B), a B cell-specific cofactor that interacts with both Oct1 and Oct2 (Gstaiger et al., 1995; Strubin et al., 1995), provided a possible explanation for the B cell-restricted activity of the octamer element. Bob1 is largely B cell restricted but can be induced in T cells with phorbol-esters and ionomycin (Zwilling et al., 1997). However, Bob1-deficient mice are viable and fertile and have normal B cell numbers in the bone marrow and slightly reduced B cell numbers in the spleen. Most strikingly, these animals show a complete lack of germinal centers (Kim et al., 1996; Nielsen et al., 1996). Even in the absence of both Oct2 and Bob1, B cells with normal levels of surface Ig can be generated (Schubart et al., 2001). These data suggested a model

whereby Oct1 plays a key role in mediating B cell specificity at the Ig locus either by compensating for Oct2 in its absence or by conferring a unique role independently of Oct2. However, the studies on Oct1 knockout mice revealed that Oct1 protein is dispensable for B cell development and Ig transcription (Wang et al., 2004), most probably due to the compensation of missing Oct1 activity by Oct2 and Bob1. Thus, generation of compound mutants of Oct1 with Oct2, Bob1 and other factors thought to be important in the control of Ig gene expression may provide additional insights into the role of these proteins in B cell development.

Interestingly, gene expression profiling analysis revealed that a few Igκ light chain variable region genes are upregulated after 45 min of BCR stimulation in SLP65$^{-/-}$ B cells. A possible interpretation of these data might be that high levels of Oct1 expression in SLP65$^{-/-}$ B cells might lead to the deregulation (upregulation) of Igκ genes upon BCR cross-linking.

4.6 IRF1 induction upon BCR cross-linking is impaired in SLP65$^{-/-}$ B cells

Microarray analysis revealed that IRF1 is induced after 6 hours of BCR cross-linking in wild type B cells, suggesting that IRF1 might be involved during the secondary responses upon BCR engagement in B cells. Interestingly, SLP65$^{-/-}$ B cells showed improper regulation of IRF1 during the first events upon B cell activation and downregulation of IRF1 after 6 hours, which was confirmed on the protein levels by immunoblotting assay. These data suggest that IRF1 may act downstream of SLP65 in B cells upon Ag encounter and BCR cross-linking.

Interferon (IFN) regulatory factors (IRFs) play a role in antiviral responses, immune responses and hematopoietic development (Nguyen et al., 1997). The IRF family consists of nine members that share homology in their N-terminal domain, which confers binding to DNA, but diverges in their C-terminal regions. Many names have been given to the specific DNA element that IRFs bind, including: 1) the IFN-stimulated response element, A/G NGAAANNGAAACT, found in most IFN-inducible promoters; 2) IFN consensus sequence, GAAA G/C T/C, found in the MHC class I promoter; 3) the IRF element, G(A)AAA G/C T/C GAAA G/C T/C, in the IFN-β promoter (Mamane et al., 1999).

Although IRFs are important in antiviral responses, they are also important in hematopoietic cells. IRF1, IRF2 and IRF3 are constitutively expressed at low levels in most cell types (Taniguchi 1997; Sato et al., 2000), while IRF4 (Eisenbeis et al., 1995), IRF7 (Zhang and Pagano 1997), and IRF8 (Tamura and Ozato 2002) are expressed primarily in hematopoietic cells. Gene targeting of IRF4 and IRF8 has established their importance in lymphocyte and macrophage maturation, respectively (Mittrucker et al., 1997; Tamura et al., 2000). Mice

deficient in IRF2, but not mice deficient in IRF1, exhibit defects in B cell development (Matsuyama et al., 1993). However, mice expressing an IRF1 transgene driven by Eμ have decreased B cells (Yamada et al., 1991). IRF1 and IRF2 also affect cell proliferation: IRF1 is a negative regulator of cell proliferation and IRF2 antagonizes IRF1 (Taniguchi 1997). IRF1 and IRF2 target genes in B cells are not completely defined, but may include c-myc, c-myb, IL-7R and p21 (Pleiman et al., 1991; Tanaka et al., 1996; Coccia et al., 1999; Manzella et al., 2000). Moreover, the consensus sequence for Blimp1 (B lymphocyte-induced maturation protein 1), a key transcription factor during terminal differentiation of B cells to plasma cells, has been found to be similar to a subset of previously determined consensus sequences for IRF proteins (Kuo and Calame 2004). Indeed, *in vitro* binding studies, cotransfections and *in vivo* chromatin immunoprecipitation (ChIP) assays showed overlapping binding of Blimp1, IRF1 and IRF2 on sites containing a GAAAG sequence and functional competition between Blimp1 and IRF1 (Kuo and Calame 2004).

Thus, one possible interpretation of our results might be that the defects in the secondary response to Ag in SLP65$^{-/-}$ B cells might be in part due to the improper regulation and decrease of IRF1 upon stimulation and as a consequence, the defective regulation of IRF1 target genes in these cells.

4.7 General conclusions from microarray experiments

High-throughput analysis of gene expression is now feasible with the use of microarrays. However, microarray results can be influenced by each step of the complex assay, from array manufacturing to sample preparation (extraction, labeling, hybridization) and image analysis. Validation of expression differences is accomplished with alternative methods such as Northern blot hybridization, RNase protection assay, conventional reverse transcription (RT)-PCR and real-time (kinetic) RT-PCR methods. Real-time (kinetic) RT-PCR evaluates product accumulation during the log-linear phase of the reaction and is currently the most accurate and reproducible approach to gene quantification. Two step real-time RT-PCR using SYBR Green I dye detection with product verification by melting curve analysis is a rapid and quantitative method (Rajeevan et al., 2001).

In this study we have selected 21 genes to further verify the microarray results. 6 genes were analyzed by FACS, 8 genes by immunoblotting and 7 genes analyzed by real-time RT-PCR. FACS analysis could confirm only 50% (3 out of 6 selected genes) of microarray results, whereas this number was even lower by immunoblotting assays, only 25% (2 out of 8 selected genes) of microarray data could be validated. However, by real-time RT-PCR, microarray

results were verified in about 80% of comparisons (in 11 out of 14 comparisons = 7 genes x 2 comparisons), demonstrating that real-time RT-PCR is the most robust and the most sensitive method for the validation of microarray hybridizations. These big variations in the numbers of confirmed genes by each of the above techniques might be explained by the specific procedures used in each method. For example, it is relatively difficult to exactly quantitate protein amounts by immunoblotting compared to FACS analysis, because of the difficulties in exact protein quantification from signal intensities or due to the variations in blotting efficiencies in immunoblotting assays. Moreover, in validation experiments by immunoblotting and FACS analysis, protein levels are compared to the transcript levels detected by microarrays, whereas by real-time RT-PCR, mRNA levels are compared to the mRNA levels identified by gene expression profiling.

There are certain advantages and disadvantages of Affymetrix GeneChip® Murine Genome U74Av2 oligonucleotide microarrays, which we used in our experiments. Firstly, the use of multiple (16 probe pair) and short sequence (25-mer) detectors enables splice variants and closely related members of a gene family to be distinguished. Secondly, for each probe designed to be perfectly complementary to a target sequence, a partner probe is generated that is identical except for a single base mismatch in its center. This probe mismatch strategy, along with the use of multiple probes for each transcript, helps identify and minimize the effects of non-specific hybridization as well as background signal and allows the direct subtraction of cross-hybridization signals and discrimination between real and nonspecific signal. A main disadvantage might be that although short-sequence probes confer high specificity, they may have decreased sensitivity or binding compared with cDNA microarrays. Low sensitivity can be compensated for by employing multiple spotting of target oligonucleotides.

One or two rounds of mRNA amplification techniques are used to analyze gene expression from small amount of total RNA, for example, for total RNA from fine needle aspirate samples, samples isolated using laser capture microdissections or samples from sorted cells (Feldman et al., 2002). Due to the limited yield of RNA from purified B cells we also employed two rounds of antisense RNA amplification procedure in gene expression profiling experiments. For some genes selected for validation experiments using real-time RT-PCR, the relative expression levels identified by microarrays were higher or respectively lower than that of observed by real-time RT-PCR. One possible explanation of these observations might be that two rounds of amplification might introduce additional amplification of signals for some genes. In total, only about 50% (11 out of 21) of genes identified by microarrays could

be validated by the means of alternative methods. Therefore, one general conclusion from microarray hybridization experiments might be that microarray results should be considered as true changes only after validation of data by an alternative approach and it is easier to verify larger (for example, ≥ 3-fold) differences than smaller changes on the protein levels, whereas in validation experiments at the transcript levels by real-time RT-PCR even lower (for example, ≥1.5-fold) differences might be also considered.

4.8 EGRxSLP65$^{-/-}$ B cells express low levels of TACI

BAFF (B cell Activating Factor belonging to the TNF Family) is necessary for normal B cell development and survival (Schneider et al., 1999; Nardelli et al., 2001). BAFF exerts its function on B cells by binding to BAFF-R and regulates B cell expansion and survival (Mackay et al., 2005). FACS analysis of spleen cells with recombinant BAFF protein revealed no significant differences in the overall expression of the receptors for BAFF (BAFF-binding proteins) between SLP65$^{-/-}$, EGRxSLP65$^{-/-}$ and wild type mice. BAFF binds to two receptors with different affinities: BAFF-R and TACI (Gras et al., 1995; Gross et al., 2000; Wu et al., 2000). However, further analysis of the cells for the expression of TACI showed that

SLP65$^{-/-}$ B cells express low levels of TACI compared to the wild type B cells and TACI is expressed to even drastically lower extent in B cells from EGRxSLP65$^{-/-}$ mice. The inspection of the regulatory regions did not reveal EGR1 binding sites in the mouse TACI gene promoter. Thus, these changes in the expression of TACI might be indirect (secondary) effects of EGR1 overexpression in SLP65$^{-/-}$ B cells.

TACI acts as a receptor for both APRIL and BAFF ligands and plays an important role in B cell development and function. TACI-deficient mice have normal numbers and phenotypes of B lineage cells in the bone marrow, but have twice the number of circulating and splenic B cells, with expanded follicular and marginal zone areas in the peripheral lymphoid organs (Yan et al., 2001). The loss of TACI causes fatal lymphoproliferation and autoimmunity in mice (Seshasayee et al., 2003). Therefore, the downregulation of TACI expression in EGRxSLP65$^{-/-}$ B cells would be an interesting subject for further investigations.

Furthermore, SLP65$^{-/-}$ B cells might have defects in isotype switching due to the low levels of TACI expression, since recent data indicate that both BAFF-R and TACI are able to transduce signals that result in isotype switching in B cells (Castigli et al., 2005), and these defects might be even more profound in EGRxSLP65$^{-/-}$ mice because of the almost complete absence of TACI on EGRxSLP65$^{-/-}$ B cells.

4.9 Higher incidence of pre-B ALL cases is associated with high levels of LEF1 and IRF1 expression in EGRxSLP65$^{-/-}$ mice

A B cell-specific adaptor protein SLP65 acts as a tumor suppressor in pre-B cells by limiting pre-B cell expansion. SLP65$^{-/-}$ mice develop spontaneous pre-B cell tumors and pre-B cell lymphomas (Flemming et al., 2003). Murine SLP65$^{-/-}$ pre-B cell leukemia resembles human childhood pre-B cell acute lymphoblastic leukemia (pre-B ALL) (Jumaa et al., 2003). Pre-B ALL leukemia was observed in 5-10% of SLP65$^{-/-}$ mice (Flemming et al., 2003). Consistent with these data we found in 6.78% (4 out of 59 mice analyzed) of SLP65$^{-/-}$ mice pre-B ALL leukemia. To our surprise, the incidence of pre-B ALL leukemia in EGRxSLP65$^{-/-}$ mice was twice as high (13.33%, i.e., in 12 out of 90 mice analyzed) than in SLP65$^{-/-}$ mice. These unexpected observations as well as the ability of pre-B ALL cells from an EGRxSLP65$^{-/-}$ mouse to form tumors *in vivo* after injection them into immune-deficient RAG2/γc$^{-/-}$ mice prompted us to further investigate the possible causes of this phenomenon. Candidate genes, which might be regulated by the EGR1 transgene activity in EGRxSLP65$^{-/-}$ pre-B cells, would be the genes involved in pre-B cell proliferation as well as cell cycle control. The differential expression of such genes between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ pre-B cells might lead to the higher frequency of pre-B ALL in EGRxSLP65$^{-/-}$ mice.

There are two parallel and/or alternative pathways described for pro-B and pre-B cells, through which they proliferate, survive and further differentiate. The survival and proliferation of pro-B and pre-B cells depends on IL-7 signaling and IL-7 exerts its function through its receptor IL-7R on progenitor B cells (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). IL-7R composed of IL-7Rα chain and common γ chain (CD132/IL-7Rγc). IL-7Rα chain expression is highly regulated in a developmental-stage-specific manner in progenitor B cells, whereas IL-7γc expression is relatively ubiquitous (Kang and Der 2004). A similar role in pro-B and pre-B cell survival and proliferation has been described for Wnt signaling based on targeted inactivation of LEF1, which regulates the transcriptional response of the canonical Wnt/β-catenin pathway (Reya et al., 2000). Thus, candidate genes would be IL-7Rα and LEF1 to further investigate for the differences in the expression between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ pre-B cells. Other possible candidate genes leading to the high frequency of leukemia would be the genes which are involved in the control of cell cycle. For example, IRF1 has been shown to be implicated in the regulation of IL-7R (Pleiman et al., 1991) and p21 (Coccia et al., 1999) or another possibility is that cyclin genes (all tree D-type and two E-type cyclins) might be differentially regulated between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ pre-B cells, since it has been shown that upon BCR stimulation SLP65$^{-/-}$ B cells fail to enter the cell

cycle due to their inability to induce cyclin D2 (Tan et al., 2001). Moreover, it has been reported that EGR1 directly regulates the expression of p21 gene in human acute myeloid leukemia cell lines (Ragione et al., 2003) and cyclin D2 gene in mouse aggressive tumorigenic prostate cancer cells (Virolle et al., 2003). Interestingly, the analysis of gene regulatory region sequences of all the above mentioned nine genes revealed putative EGR1 binding sites both in the mouse and human genes, except for cyclin D3.

Although SLP65 has been shown to be involved in the transcriptional repression of IL-7Rα chain (Hayashi et al., 2003), we could not find any significant differences in the expression of IL-7Rα chain between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ pre-B cells or between pre-B cells of these two mutant strains and wild type littermates. We were also unable to detect the expression of p21 protein either in pre-B cells or spleen B cells by immunoblotting analysis, whereas p21 was detectable in NIH-3T3 cells after overnight starvation and after 6 hours of serum response. A possible explanation here might be that B cells express very low or undetectable levels of p21 compared to NIH-3T3 cells. Thus, these two genes were excluded from further analysis.

Interestingly, we found that EGRxSLP65$^{-/-}$ pre-B cells express significantly high levels of both LEF1 and IRF1 proteins compared to the SLP65$^{-/-}$ and wild type pre-B cells. Further analysis showed that both proteins (LEF1 and IRF1) are expressed at even higher levels in both EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ pre-B ALL cells, suggesting that high levels of expression of LEF1 and IRF1 might be associated with tumor formation. Indeed, *ex vivo* pre-B ALL cells from both EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ mice supported this hypothesis showing comparable high levels of LEF1 and IRF1 protein expression. Next, we questioned whether human pre-B ALL cells also express LEF1 and IRF1 proteins, since SLP65$^{-/-}$ pre-B cell leukemia resembles human pre-B ALL (Jumaa et al., 2003). We have analyzed 6 human lymphoma cell lines together with SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ pre-B ALL cells. Indeed, LEF1 was specifically expressed only in acute lymphoblastic leukemia cell lines from both mouse (SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ pre-B ALL cell lines) and human (NALM-6, 697 and BV173 - pre-B ALL as well as SEM - ALL cell lines), suggesting its specific association with pre-B ALL and ALL leukemia formation. IRF1 was also detected in all pre-B ALL and ALL cell lines tested from both human and mouse and in addition, JUM2 (a mantle cell lymphoma) cells also expressed comparably high levels of IRF1 protein. Most interestingly, all human lymphoma cell lines expressed significantly high levels of EGR1 protein compared to the EGRxSLP65$^{-/-}$ pre-B ALL cells. These data support our hypothesis and suggest that high levels of expression of

EGR1, LEF1 and IRF1 proteins might be indeed associated with pre-B ALL lymphoma formation both in the mouse and in humans.

4.10 Possible cause of high pre-B ALL incidence in EGRxSLP65$^{-/-}$ mice might be due to the direct regulation of LEF1 by EGR1 in pre-B cells

WNT proteins and WNT signaling have key roles in diverse biological phenomena, such as specification of cell fate, proliferation of progenitor cells, establishment of the dorsal axis and control of asymmetric cell division in species ranging from the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* to mice and men (Staal and Clevers 2005). The term WNT is derived from a contraction of the names for the *D. melanogaster* segment-polarity gene *wingless* and its mouse orthologue *Int1*, a mouse proto-oncogene discovered as an integration site for mouse mammary-tumor viruses (Nusse and Varmus 1982). There are 19 known WNT genes in the human and the mouse genome, all of which encode lipid-modified secreted glycoproteins (van Noort and Clevers 2002).

WNT signaling is initiated by the binding of a WNT protein to the cystein-rich domain of a receptor of the frizzled (FZ) family and a co-receptor of the low-density-lipoprotein-receptor-related protein family, namely LRP5 or LRP6 (Mao B et al., 2001; Mao J et al., 2001). At least three intracellular signaling pathways have been reported to emanate from the WNT-FZ complex: the first, well defined canonical WNT pathway, with β-catenin and proteins of the T cell factor (TCF)/lymphoid enhancer-binding factor (LEF) family as important components; the second, a partially understood pathway that involves calcium ions; and the third, planar-cell polarity pathway (Staal and Clevers 2005). The ultimate events that are triggered by the canonical WNT pathway are the nuclear translocation of β-catenin and its physical binding to and activation of TCF and/or LEF transcription factors (Staal and Clevers 2005).

LEF1 is expressed by the all cells of the T lineage (Bruhn et al., 1997). The first insights into the role of LEF1 in B cells have been provided through studies using LEF1-deficient mice (Reya et al., 2000). It has been shown that LEF1 is expressed in developing progenitor B cells in a temporally regulated manner and the absence of LEF1 leads to reduced proliferation and increased apoptosis of pro-B cells (Reya et al., 2000). Furthermore, soluble WNT3a protein has been shown to act directly on pro-B cells to induce entry into the cell cycle and cell proliferation and that the absence of LEF1 impairs the ability of pro-B cells to respond to WNT signaling (Reya et al., 2000).

Dysregulated WNT signaling has become a hallmark of some type of solid tumors, most notably colon carcinomas (van Es et al., 2003). Constitutive activation of the WNT signaling

pathway has been shown to be a root cause of many colon cancers. Activation of this pathway is caused by genetic mutations that stabilize the β-catenin protein, allowing it to accumulate in the nucleus and form complexes with LEF1 and TCF family (TCF1, TCF3, TCF4) of transcription factors (referred to collectively as LEF/TCFs) to activate transcription of target genes (Polakis et al., 1999; Roose and Clevers 1999). Target genes such as myc, cyclin D1, MMP7 and TCF7 are normally expressed in colon tissue, so it has been proposed that abnormal expression levels or patterns imposed by β-catenin/TCF complexes have a role in tumor progression (Hovanes et al., 2001). LEF1 transcription is activated by β-catenin/TCF complexes and the activities of the LEF1 and TCF1 protein isoforms that are expressed in colon cancer cells have been shown to be in direct competition, with TCF1 dampening the effects of the oncogenic WNT-pathway mutations and LEF1 enhancing these effects (Hovanes et al., 2001). Moreover, LEF1 has been recently identified as one of the tumor-inducing genes among the other 18 candidate cancer genes in T cell lymphomas induced by the retrovirus SL3-3 (Kim et al., 2003).

The specific co-expression of LEF1 protein together with high levels of EGR1 protein in EGRxSLP65$^{-/-}$ pre-B cells and pre-B ALL lymphoma cell lines raised another interesting question, whether LEF1 is regulated directly by EGR1 in pre-B cells, since there are a few putative EGR1 binding sites in both the mouse and human LEF1 gene promoter. Indeed, anti-pre-BCR and LPS stimulations in pre-B cells revealed a linear correlation of the induction kinetics of EGR1 and LEF1, strongly suggesting that LEF1 might be regulated directly by EGR1 in pre-B cells. Moreover, consistent with these data, EGR1 activity maintained the expression of LEF1 protein during differentiation induced by IL-7 withdrawal. Furthermore, recently regulation of LEF1 promoter by EGR1 has been confirmed by two independent methods: by a reporter gene assay using the LEF1 promoter and transient transfection of 697 human pre-B ALL cells with dominant negative construct of EGR3 (dnEGR3), in which dnEGR3 binds to and thus blocks EGR binding sites in the promoters leading to the suppression of the expression of target genes (Lisa Rozenkranz, personal communications). Thus, in pre-B cells EGR1 seems to regulate LEF1 expression. Due to the high levels of LEF1 expression, EGRxSLP65$^{-/-}$ pre-B cells might have higher proliferative and survival capacity which might lead to the pre-B ALL formation and this might cause the higher incidence of pre-B ALL leukemia in EGRxSLP65$^{-/-}$ mice compared to SLP65$^{-/-}$ littermates. These observations also suggest that despite its known role as a tumor-suppressor in several other types of cancers (Huang et al., 1997), EGR1 might be involved in pre-B ALL leukemia progression.

4.11 IRF1 is not directly regulated by EGR1 but high levels of IRF1 might be still associated with tumor formation

Although the initial results of immunoblotting assay and the promoter sequence analysis suggested that IRF1 might be a direct EGR1 target gene in pre-B cells, the further analysis disproved this hypothesis. In contrast to the LEF1, anti-pre-BCR and LPS stimulations as well as IL-7 withdrawal experiments revealed a non-linear correlation of the induction kinetics of IRF1 and EGR1 proteins in pre-B cells. Consistent with these data, microarray analysis did not show significant differences in the expression of IRF1 gene between EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ or between EGRxSLP65$^{-/-}$ and wild type resting B cells in the spleen. A possible interpretation of these data might be that high levels of IRF1 protein expression is caused by indirect effects triggered by the EGR1 overexpression or another possibility might be that IRF1 is associated with tumor formation independent of EGR1 activity in pre-B cells.

Deletions and/or translocations involving one or both IRF1 alleles are frequently observed in human cancers and are thought to play a role in tumorigenesis (Miura et al., 1992; Willman et al., 1993; Ogasawara et al., 1996; Ransom et al., 1996; Sugimura et al., 1997; Zhao et al., 1997). Recent studies showed that all 7 malignant natural killer (NK) cell lines tested as well as 5 malignant NK-like T (NKT) cell lines, 5 T-cell acute lymphoblastic leukemia (ALL) cell lines and 3 B cell precursor ALL cell lines were positive for IRF1 transcription factor expression (Matsuo et al., 2004). In mice, loss of IRF1 alone (IRF1$^{-/-}$) has not been shown to be associated with spontaneous tumor development, but is assosiated with greatly increased tumor susceptibility in combination with tumor predispositions caused by cHa-Ras expression or p53 null zygosity (p53$^{-/-}$) (Nozawa et al., 1999). In this study, no lymphomas were reported in about 200 days old IRF1$^{-/-}$ mice. Recently in another report, development of CD30+ lymphoproliferative disease was observed in 330-days-old IRF1$^{-/-}$ mice (Eason et al., 2003). The age of mice analyzed has been interpreted to be the only reason for these two contradicting observations. Additionally, there are other contradicting data, that IRF1 expression became constitutive in ras-transformed NIH 3T3 cells and in cells transformed by oncogenes ets, fes, fos, her2/neu, met, mos, raf, or trk suggesting that deregulated IRF1 expression may be associated with loss of growth control (Contente et al., 2003). The deregulated overexpression of other transcription factors involved in growth and cell cycle control has been also observed clinically in tumors and leukemias. For example, overexpression of E2F-1, at both the transcriptional and translational levels, was demonstrated in non-small cell lung carcinomas and was associated with increased growth induction (Gorgoulis et al., 2002). Deregulation of promyelocytic leukemic zinc finger protein has been

linked to poor prognosis in patients with B cell chronic lymphocytic leukemias (B-CLL) (Parrado et al., 2000). Therefore, although loss of IRF1 expression may certainly be deleterious, overexpression or inappropriate expression of IRF1 may also be dangerous and this might be the case with EGRxSLP65$^{-/-}$ mice. Thus, although not directly regulated by EGR1, high levels of IRF1 expression in EGRxSLP65$^{-/-}$ pre-B cells might contribute to pre-B ALL in EGRxSLP65$^{-/-}$ animals.

4.12 Differential regulation of cyclin genes in EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ pre-B cells

It has been shown that B lymphocytes lacking the adaptor protein SLP65 do not proliferate in response to BCR engagement. Upon BCR cross-linking SLP65$^{-/-}$ B cells fail to enter the cell cycle and this is due to their inability to induce the expression of the cyclin D2 and cdk-4 (Tan et al., 2001). Consistent with these observations, cyclin D2 expression was lower in proliferating SLP65$^{-/-}$ pre-B cells compared to the wild type control, suggesting that the failure to induce cyclin D2 in SLP65$^{-/-}$ B cells might be also true at the pre-B cell stage. Cyclin D2 has been shown to be overexpressed by EGR1 in both human and mouse prostate cancer cells (Virolle et al., 2003). In agreement with these data, we found that cyclin D2 expression was upregulated in EGRxSLP65$^{-/-}$ pre-B cells. Interestingly, cyclin D3 gene was upregulated in SLP65$^{-/-}$ pre-B cells compared to EGRxSLP65$^{-/-}$ or wild type pre-B cells. Previous studies on cyclin D2$^{-/-}$ mice revealed that in the bone marrow progenitors of B2 cells the lack of cyclin D2 can be compensated by cyclin D3 (Mohamedali et al., 2003). Although here we do not provide a direct functional evidence, by extrapolating the data from the above observations, one possibility would be that although proliferating SLP65$^{-/-}$ pre-B cells express lower levels of cyclin D2 gene, the cell cycle entry and progression might be compensated by high levels of cyclin D3 expression in these mutant B cells. Cyclin D3 was downregulated in EGRxSLP65$^{-/-}$ pre-B cells. This downregulation might not be a direct effect of EGR1 activity, because in contrast to the murine cyclin D3 promoter there are no EGR1 binding sites in human cyclin D3 promoter. Thus, EGR1 activity in EGRxSLP65$^{-/-}$ pre-B cells may upregulate cyclin D2, whereas low levels of cyclin D2 gene might be compensated by higher levels of cyclin D3 gene in SLP65$^{-/-}$ pre-B cells.

We could not detect cyclin D1 gene in any of the tested pre-B cell samples. The cyclin D1 gene can be transcriptionally activated in lymphoid tumours as a result of chromosomal rearrangements but is normally silent in B and T lymphocytes (Milne et al., 2004). Consistent with this notion, probably cyclin D1 is not expressed in proliferating wild type and normal

SLP65$^{-/-}$ and EGRxSLP65$^{-/-}$ pre-B cells and might be present only in malignant lymphoid cells.

The expression of both E-type cyclins (cyclin E1 and E2) was equally downregulated in EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ pre-B cells compared to the wild type pre-B cells. B-cell proliferation involves entry into the cell cycle from a resting or quiescent state (G_0 phase) and successful progression through G_1 (gap phase 1), S (DNA synthesis), G_2 (gap phase 2) and M (mitosis) of the cell cycle. Progression through each phase of cell cycle is controlled by cooperative activity of specific cyclin-dependent kinases (CDKs) and their regulatory subunits, cyclins (Lauper et al., 1998; Sherr and Roberts 1999; Sherr 2000). Essentially, the D-type cyclins (cyclin D1, D2 and D3) in association with CDK4 and CDK6 are responsible for G_1 phase progression, while CDK2-cyclin E (E1 and E2) complex is important for initiation of S phase (Glassford et al., 2003). Our results suggest that although both E-type cyclins (cyclin E1 and E2) are expressed at lower levels in EGRxSLP65$^{-/-}$ and SLP65$^{-/-}$ pre-B cells compared to the control, in the absence of SLP65 there might exist additional yet unknown mechanisms that allow the cell cycle progression through the S phase, since a previous report on SLP65$^{-/-}$ pre-B cells (Flemming et al., 2003) showed that in the absence of SLP65 pre-B cells expand and proliferate even more rapidly in comparison to the wild type pre-B cells.

Summary

B cell development is severely impaired in the absence of SLP65 adaptor protein. SLP65-deficient B cells are arrested at the large cycling pre-B cell stage in the bone marrow and transitional B cell stages in the spleen of mice. The zinc-finger transcription factor EGR1 is an immediate early gene induced upon Ag-binding and BCR engagement in B cells. EGR1 promotes B cell differentiation and maturation at the progression from pro-B to pre-B and into the mature B cell stages. The main hypothesis of this study was that EGR1 expression is regulated through a SLP65-dependent signaling chain and therefore, EGR1 overexpression in $SLP65^{-/-}$ mice could restore or at least partially rescue developmental blocks caused by the absence of SLP65 in B cells. The results revealed that both $EGRxSLP65^{-/-}$ and $SLP65^{-/-}$ B cells appear to be arrested at the same developmental (large pre-B cell) stage, suggesting that in the absence of SLP65, EGR1 activity alone is not sufficient to rescue the developmental block in the bone marrow. However, independent of this developmental arrest EGR1 overexpression mediates downregulation of IL-2Rα chain in B cells. Further analysis showed that although EGR1 could not completely rescue the developmental arrest at the transitional B cells stages in $EGRxSLP65^{-/-}$ mice, splenic B cells from $EGRxSLP65^{-/-}$ mice have a more mature phenotype compared to splenic B cells from $SLP65^{-/-}$ animals. In order to determine the underlying molecular basis of these EGR1-mediated phenotypic changes in $EGRxSLP65^{-/-}$ B cells as well as to identify additional EGR1 target genes in B cells, gene expression profiling experiments were performed. By microarray hybridizations and several validation methods at least nine genes were found to be differentially regulated in $EGRxSLP65^{-/-}$ B cells. Inspection of the promoter sequences suggests that seven genes: Ly-6A/E, TAF10, SLFN1, SLFN2, CalA, CCR2 and Oct1 might be potential EGR1 target genes in B cells. Ly-6D and Ly-6E are upregulated in both $EGRxSLP65^{-/-}$ and $SLP65^{-/-}$ B cells, since the cells are T1 and T2 B cells. We also could show that IRF1 is downregulated upon BCR cross-linking in $SLP65^{-/-}$ B cells. In addition, we found that the $SLP65^{-/-}$ B cells express low levels of TACI and the expression levels of TACI is drastically reduced in $EGRxSLP65^{-/-}$ B cells.

Due to the block at the large pre-B cell stage $SLP65^{-/-}$ mice develop pre-B ALL leukemia in 5-10% of cases. Consistent with these data we found pre-B ALL formation in about 6.78% of $SLP65^{-/-}$ mice. Surprisingly, the pre-B ALL incidence was twice as high (13.33%) in $EGRxSLP65^{-/-}$ mice. Searching for possible candidate genes which might be involved in pre-B ALL formation, we found that LEF1 and IRF1 might be associated with tumor formation in pre-B cells. Our results strongly suggest that EGR1 might play a role in pre-B ALL progression by directly regulating LEF1 in pre-B cells.

Zusammenfassung

Die B-Zellentwicklung wird durch Abwesenheit des Adaptorproteins SLP65 stark beeinträchtigt. SLP65-defizienten Mäusen differenzieren B-Zellen nur bis zum Stadium der großen, sich teilenden Prä-B-Zellen im Knochenmark sowie zu Transitional-B-Zellen in der Milz. Der Transkriptionsfaktor EGR1 gehört zur Gruppe der unmittelbar frühen Gene, welche nach Antigenaufnahme und B-Zellrezeptorstimulation in B-Zellen induziert werden. EGR1 fördert die Differenzierung und Reifung von B-Zellen beim Übergang vom Pro-B zum Prä-B-Stadium und zu reifen B-Zellstadien. In der dieser Arbeit zugrundeliegenden Hypothese wurde von einer Regulation der EGR1-Expression durch eine SLP65-abhängige Signalkaskade ausgegangen. Eine Überexpression von EGR1 in SLP65$^{-/-}$-Mäusen sollte daher die Blockaden in der B-Zellentwicklung, welche durch die Abwesenheit von SLP65 hervorgerufen werden, ganz oder zumindest teilweise überwinden. Die Ergebnisse zeigten, dass sowohl EGRxSLP65$^{-/-}$, als auch SLP65$^{-/-}$ B-Zellen im Stadium der großen Prä-B-Zelle verbleiben. Dies legt nahe, dass die Aktivität von EGR1 in Abwesenheit von SLP65 nicht ausreicht, um den Entwicklungsblock im Knochenmark aufzuheben. Unabhängig von dieser Entwicklungsblockade wird jedoch durch die Überexpression von EGR1 in den B-Zellen die Expression der IL-2Rα-Kette herunterreguliert. Aus weiteren Analysen ergab sich, dass der Entwicklungsblock in den Transitionalstadien in EGRxSLP65$^{-/-}$-Mäusen zwar von EGR1 nicht aufgehoben werden kann, dass aber B-Zellen aus den Milzen dieser Mäuse einen reiferen Phänotyp aufweisen, als es bei B-Zellen aus den Milzen von SLP65$^{-/-}$-Mäusen der Fall ist. Um die dafür verantwortlichen, molekularen Grundlagen der EGR1-vermittelten phänotypischen Veränderungen in EGRxSLP65$^{-/-}$-B-Zellen zu erforschen, und um zusätzliche EGR1-Zielgene in B-Zellen aufzudecken, wurden Genexpressionsanalysen durchgeführt. Durch Microarray-Hybridisierungen und mehrere, die Ergebnisse bestätigende Methoden wurden mindestens neun Gene gefunden, die in EGRxSLP65$^{-/-}$ B-Zellen differenziell reguliert werden. Die Untersuchung der Promotersequenzen deutet darauf hin, dass es sich bei den sieben Genen Ly-6A/E, TAF10, SLFN1, SLFN2, CalA, CCR2 und Oct1 um potentielle EGR1-Zielgene in B-Zellen handelt. Ly-6D und Ly-6E werden sowohl in EGRxSLP65$^{-/-}$ als auch in SLP65$^{-/-}$ B-Zellen verstärkt exprimiert, da diese Zellen T1 und T2 B-Zellen darstellen. Wir konnten weiterhin zeigen, dass in SLP65$^{-/-}$-B-Zellen nach BCR-Kreuzvernetzung IRF1 herunterreguliert wird. Darüberhinaus fanden wir eine geringere Expression von TACI in SLP65$^{-/-}$ B-Zellen, die sich in EGRxSLP65$^{-/-}$ B-Zellen noch als deutlich stärker vermindert erwies.

Aufgrund des Blockes im großen Prä-B-Zellstadium entwickeln SLP65$^{-/-}$-Mäuse in 5-10% der Fälle Prä-B ALL Leukämien. In Konkordanz mit diesen Daten konnten wir die Entwicklung von Prä-B ALL in etwa 6,78% der SLP65$^{-/-}$- Mäuse beobachten. Überraschenderweise war das Auftreten von Prä-B ALL in EGRxSLP65$^{-/-}$-Mäusen etwa doppelt so hoch (13,33%). Die Suche von möglichen Kandidatengenen, welche an der Entwicklung von Prä-B ALL beteiligt sein könnten, ergab eine mögliche Assoziation von LEF1 und IRF1 mit der Tumorentstehung in Prä-B-Zellen. Unsere Ergebnisse legen eine Rolle von EGR1 bei der Progression von Prä-B ALL durch die direkte Regulation von LEF1 in Prä-B-Zellen nahe.

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