Analysis of Signaling Mechanisms Essential to Mature B Cell Viability

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Abstract

The maintenance of mature peripheral B cells depends on at least two survival cues, tonic signaling from the B cell receptor (BCR) complex and the extracellular cytokine B cell activating factor of the TNF family (BAFF). In addition to enhancing viability, BAFF controls the functional efficiency of the peripheral B cell pool by regulating complex physiological processes including cell growth, metabolism, energy homeostasis and entry into the cell cycle. BAFF-mediated induction of two molecular mechanisms, namely activation of the Akt signal transduction pathway and upregulation of the oncogenic kinase Pim-2 results in the modification of effector proteins including transcription factors and regulators of protein synthesis which are capable of executing the observed cellular physiological changes. The classic protein kinase C β is instrumental in BAFF-induced Akt-activation and PKCβ-deficient B cells and mice show signs of partial refractiveness to BAFF. The protein tyrosine kinase Syk plays a role in early B cell development and is activated in mature B cells by immunogenic BCR-stimulation. Inducible ablation of Syk in mice results in the loss mature B cells from the peripheral lymphoid organs and reveals an indispensable function for Syk in tonic BCR survival signaling.

Kurze Zusammenfassung

Die Langlebigkeit reifer periphärer B Zellen ist abhängig von mindestens zwei Überlebenssignalen, einem tonischen Signal, welches vom B Zellrezeptor ausgeht und dem Zytokin B Zell aktivierender Faktor der TNF-Familie (BAFF). BAFF fördert nicht nur das Überleben von reifen B Zellen, sondern kontrolliert auch deren Funktionstüchtigkeit, indem es vielschichtige physiologische Prozesse wie Zellwachstum und –metabolismus, Energiehaushalt und Eintritt in den Zellzyklus reguliert. Zwei BAFF-induzierte molekulare Mechanismen, zum einen die Aktivierung des Akt Signaltransduktionsweges sowie die erhöhte Expression der onkogenen kinase Pim-2 zum anderen, führen zu Veränderungen in Effektorproteinen welche in der Lage sind diese physiologischen Zellveränderungen auszulösen. Die BAFF-induzierte Aktivierung von Akt hängt von der klassischen Proteinkinase C (PKC) β ab und sowohl PKCβ-defiziente B Zellen als auch Mäuse zeigen Anzeichen von Unsensitivität gegenüber BAFF-Stimulation. Die Proteintyrosinkinase Syk spielt eine Rolle während der frühen B Zellentwicklung und wird in reifen B Zellen durch Stimulation des B Zellrezeptors aktiviert. Induzierbare Inaktivierung von Syk in Mäusen führt zum Verschwinden reifer B Zellen aus den periphären lymphoiden Organen, was auf eine unverzichtbare Funktion von Syk in der Vermittlung des tonischen B Zellrezeptorsignals schliessen läßt.

FOR MY PARENTS

Abbreviations

Standard abbreviations used throughout this work include chemical symbols, SI units as well as one and three letter amino acid codes.

Non-standard abbreviations are explained in the text upon first citation and are listed in the following:

4E-BP1	4E binding protein 1
A1	BCL2-related protein A1
Act1	NF-κB activator 1
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
APRIL	a proliferation-inducing ligand
ATM	ataxia telangiectasia mutated
ATP	Adenosine triphosphate
Bad	Bcl-2-associated death promoter
BAFF	B cell activating factor of the TNF family
BAFF-R	BAFF receptor
Bak	BCL2-antagonist/killer
Bax	Bcl2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	Bcl2-like 1
Bcl10	B-cell lymphoma 10
BCMA	B-Cell Maturation Antigen
BCR	B cell receptor
Bid	BH3 interacting domain death agonist
Bim	Bcl-2 interacting mediator of cell death
BLAST	basic logical alignment search tool
BLNK	B cell linker protein
BrdU	bromodeoxyuridine
BSS	Hanks Balanced Salt Solution
Btk	Bruton's tyrosine kinase
Carma1 kinase	caspase-recruitment domain membrane-associated guanylate protein 1
Cbl	Casitas B-lineage lymphoma
CCCP	carbonyl cyanide m-chlorophenyl hydrazone

CD	Cluster of Differentiation
Cdc	cell division cycle
Cdk	cyclin-dependent kinase
CFDA-SE	carboxyfluorescein diacetate succinimidyl ester
CFSE	carboxyfluorescein succinimidyl ester
CHX	cycloheximide
CLL	chronic lymphocytic leukemia
CRD	Cystein-Rich Domain
Cre	Cyclization recombination
Csk	C-terminal src-kinase
DAG	diacylglycerol
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
eIF4E	eukaryotic translation initiation factor 4E
Erk	extracellular signal-regulated kinase
ES	embryonic stem
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
Fc	fragment crystallizable
FITC	fluorescein isothiocyanate
FoxO	Forkhead box O
G1	Gap1
GAP	GTPase activating protein
GC	germinal center
GFP	green fluorescent protein
Gsk-3	gycogen synthase kinase 3
Gst	Glutathione S-transferase
HA	hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HK	hexokinase
Ig	immunoglobuline
IFN	interferon
ILK	integrin-linked kinase 1

IκB	inhibitor of nuclear factor-κB
IKK	IκB kinase
IP_3	inositol-1,4,5-trisphosphate
IPTG	isopropyl-β-D-thiogalactopyranoside
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
Jnk	Jun N-terminal kinase
LB	Luria-Bertani
loxP	locus of X-over of P1
MACS	magnetic cell sorting
MALT1	(mucosa-associated-lymphoid-tissue lymphoma-translocation gene 1
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia sequence 1
Mcm	minichromosome maintenance deficient
mTOR	mammalian target of rapamycin
MZ	marginal zone
neo	neomycin
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor-κB
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIP_3	phosphatidylinositol-3,4,5-triphosphate
PDK1	3-phosphoinositide-dependent kinase 1
PE	Phycoerythrin
PH	pleckstrin homology
PHLPP	PH domain leucine-rich repeat phosphatases
PI3K	phosphoinositide-3 kinase
PIKK	phosphoinositide-3-kinase-related kinase
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
PKC	protein kinase C
PLC	phospholipase C
Plk	Polo-related kinase
PRAS40	proline-rich Akt substrate 40kD
PTEN	phosphatase and tensin homolog deleted from chromosome 10

RA	rheumatoid arthritis
Rb	retinoblastoma
Rheb	Ras-homolog enriched in brain
RNA	ribonucleic acid
ROS	reactive oxygen species
S6	ribosomal S6 protein
S6K	ribosomal S6 kinase 1
SDS	sodium dodecyl sulfate
SLE	systemic lupus erythematosus
SH2	Src-homology 2
SHP-1	SH2-domain containing protein tyrosine phosphatase 1
Sos	Son of sevenless
Src	Rous sarcoma oncogene
Syk	spleen tyrosine kinase
T1	transitional 1
T2	transitional 2
TACI	Transmembrane Activator and CAML Interactor
TCA	trichloroacetic acid
TCR	T cell receptor
TEV	Tobacco Etch Virus
THD	TNF-homology domain
TNF	tumor necrosis factor
Tk	thymidine kinase
TMRE	tetramethylrhodamine ethyl ester
TRAF	TNF receptor associated factor
TSC	tuberous sclerosis
ZAP-70	zeta-chain associated protein kinase, 70kD

1 Introduction

The pool of peripheral B lymphocytes in mice consists of approximately a hundred million cells, the majority of which are mature, follicular B cells with a life-span ranging from several weeks to months (Forster and Rajewsky, 1990). Most of these cells are circulating the periphery in a quiescent state without actively contributing to an acute immunogenic response. This vast number of resting cells has to be sustained in order to assure the presence of a diverse B cell repertoire with the capacity to recognize foreign antigens. Lasting B cell persistence in the periphery has to be actively maintained and is dependent on survival signals which are transduced by cell surface receptors. An indispensable survival function has been established for two independent signals, the B cell receptor (BCR) complex and the cytokine B cell activating factor of the TNF-family (BAFF). The aim of this study was to illuminate the mechanism by which these signals accomplish the long-term survival and functional efficiency of mature B cells in the periphery.

1.1 Brief overview of B cell development

The pool of mature peripheral B cells in adult mice is replenished by ongoing lymphopoesis in the bone marrow. The purpose of this process is the release of cells, each carrying a single type of functional antigen receptor whose specificity is unique among all others within the B cell repertoire. This aim is accomplished in an astounding combination of stochastic yet tightly coordinated events encompassing the random generation of diversity and allelic exclusion (for a comprehensive review, see (Janeway, et al., 2001). B lineage commitment begins with the differentiation of pro B cells from hematopoietic progenitors (Hardy and Hayakawa, 2001). Pro B cells are characterized by expression of the BCR accessory chains immunoglobuline α (Igα) and Igβ and by ongoing rearrangement at the Ig μ heavy chain locus (figure 1). Immunoglobuline gene recombination begins with diversity (D)- to junction (J_H)- gene segment fusion followed by rearrangement of DJ_H to variable (V_H) gene segments. Productive V_HDJ_H recombination inhibits potential processing of the second heavy chain allele and allows for the presentation of the transmembrane heavy chain molecule on the cell surface in conjunction with a surrogate light chain, V_{preB} or λ5, forming the pre-BCR. An adequate signal from the pre-BCR induces pre-B cell expansion and allows for V_L- to J_L- rearrangement of κ and, if necessary, λ light chain genes.

Once a functional BCR, composed of a μ heavy and κ or λ light chains and lacking self-reactive specificity, is expressed on the surface, the newly-formed B cells exit the bone marrow into the periphery where they have to compete with the existing B cells for entry into the stable pool of mature naive B lymphocytes in the secondary lymphoid organs. In the spleen, B cells newly immigrated from the bone marrow undergo further maturation through distinct stages which can be distinguished by surface marker expression (Loder, et al., 1999) and figure 1). With few exceptions, cell surface molecules on leukocytes are named after the CD nomenclature where CD denotes “Cluster of Differentiation” (Morse, 1992). Newly immigrated splenic B cells go from the IgM high CD21/35-negative (IgM^{hi} $CD21/35^{-}$) transitional 1 stage (T1) to the IgM high CD21/35 high (IgM^{hi} $CD21/35^{hi}$) T2 stage, whereas the mature follicular B cell compartment (M) is characterized by an intermediate expression level of both surface proteins (IgM^{int} $CD21/35^{int}$). A second

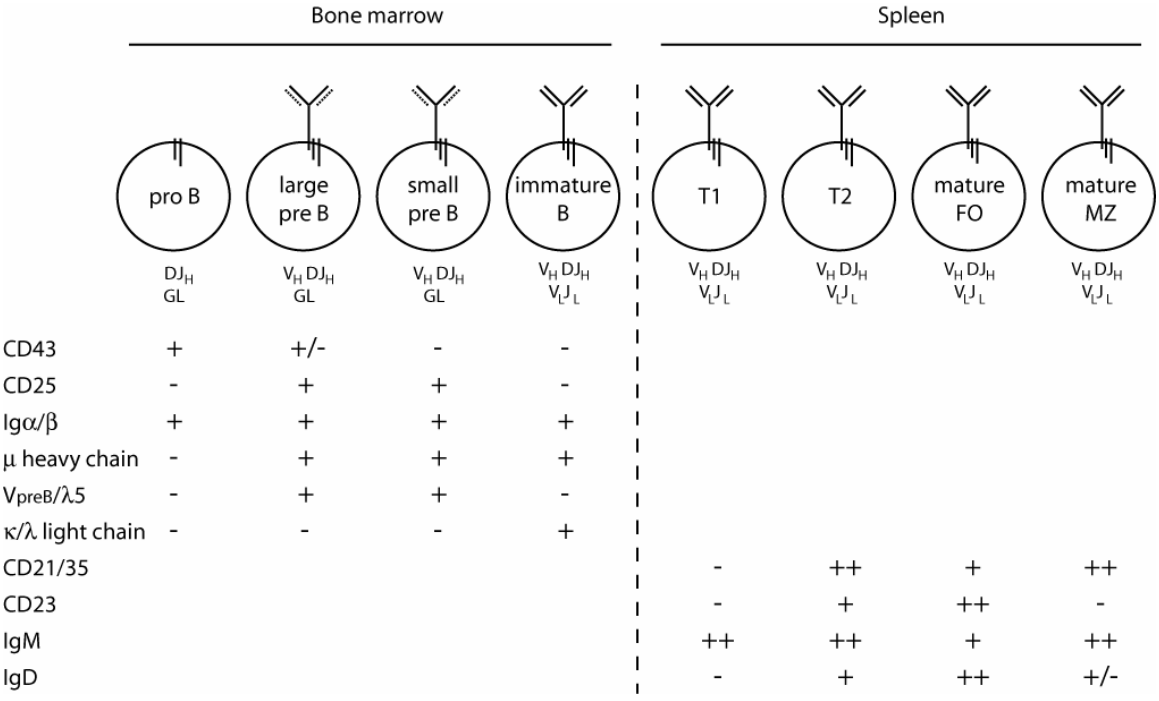


Figure 1: Overview of murine B cell development and maturation in the bone marrow and spleen. Diagrams represent different B cell developmental stages including the composition of the respective BCR. Abbreviations underneath indicate the state of the heavy (top) and light (bottom) chain immunoglobuline regions. The expression of selected surface markers commonly used to distinguish the different B cell developmental stages, is indicated at the bottom. See text for details (modified from Hardy and Hayakawa, 2001). GL = germline formation, T1 = transitional stage 1, T2 = transitional stage 2, FO = follicular B cells, MZ = marginal zone B cells

subtype of splenic B cells which resides in the marginal zone (MZ) surrounding the lymphatic follicles and is hence named MZ B cells is also CD21/35^{hi} IgM^{hi}, but differs from T2 cells in the low expression of the surface marker CD23. The lineage commitment of the latter splenic B cell subset has not been entirely resolved, but it appears increasingly likely that follicular and MZ B cells originate from a common transitional B cell precursor (Pillai, et al., 2005).

While signals originating from the surface expressed BCR or pre-BCR regulate the survival of B cells throughout their development, it is at the T2 stage that they become simultaneously sensitive to as well as dependent on the cytokine BAFF for viability (see below).

The developmental pathway described above applies for conventional B-2 lineage cells which constitute the vast majority of the mature peripheral B cell pool and represent the B cell component of the adaptive immune system. In contrast, B-1 cells originate from a different source whose nature is still largely elusive. The fraction of B-1 cells is low in lymphoid compartments including spleen, lymph nodes and peripheral blood, but they constitute a significant portion of lymphocytes in the peritoneum and an important component of the innate immune response (Hardy, 2006).

1.2 Mature B cell survival through tonic BCR signaling

1.2.1 Composition of the BCR-complex and its signaling mechanisms

Signaling from the BCR or pre-BCR has the astounding capacity to induce dramatically different cell fates. In mature naive B cells, BCR engagement with a foreign antigen represents the first step towards the induction of an immunogenic program which comprises clonal expansion, BCR maturation through class-switch recombination and somatic hypermutation as well as B cell differentiation into memory B or antibody-secreting plasma cells (Janeway, et al., 2001). Conversely, triggering of the same type of receptor by an auto-antigen has the opposite effect. Depending on the developmental stage and co-stimulatory signals, B cell encounter with a self-antigen leads to deletion, tolerance induction or alteration of receptor specificity through receptor editing (Healy and Goodnow, 1998). Importantly, some crucial signals originate from the BCR even in the absence of a discernable receptor ligand. This so-called tonic BCR-signal is critical to the survival of mature

naive B cells in the periphery (see below).

In mature naive B cells, the BCR complex is composed of a transmembrane Ig heavy chain of the μ or δ type (IgM or IgD) which is covalently linked to Ig light chains. Together, heavy and light chains form the antigen-binding unit of the BCR whose specificity is identical between all the receptors of a single B cell, but unique among all other receptors in the peripheral B cell pool. The BCR's signal transduction capacity is conveyed through the non-covalently associated BCR-accessory chains Igα and Igβ. Each chain contains a single immunoreceptor tyrosine-based activation motif (ITAM) in which two tyrosine residues are surrounded by a characteristic consensus sequence. Receptor cross-linking causes phosphorylation of the Igα-Igβ heterodimer on ITAM-tyrosines by cytoplasmic protein tyrosine kinases, creating docking sites for a plethora of Src homology 2 (SH2)-domain containing proteins (figure 2 and (Kurosaki, 2002: ; Monroe, 2006). Ligand-induced ITAM-phosphorylation is carried out by cytoplasmic protein tyrosine kinases of the Src- and Syk-family. In B cells, the most prominent members of theses families are Lyn and Syk, respectively. Although Lyn is placed upstream of Syk in most BCR-signaling models, both are capable of ITAM-phosphorylation *in vitro* and *in vivo* (see below). In any case, both appear to play a crucial role since the sole absence of either one compromises BCR-mediated signal transduction (Takata, et al., 1994). The kinase activities of Syk and Lyn are themselves stimulated by binding of phospho-ITAMs to their respective SH2-domains. ITAM-phosphorylation and proximal tyrosine kinase activation lead to the formation of multi-protein signaling complexes, sometimes called signalosomes, which ultimately relay the signal to changes in cellular architecture and gene expression. The adaptor protein B cell linker protein (BLNK, also known as SLP-65) has a major role in orchestrating the activation of Bruton's tyrosine kinase (Btk) and phospholipase C γ 2 (PLCγ2) and thus in the generation of second messengers inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). BLNK also functions in the activation of guanine nucleotide exchange factors Vav and Son of sevenless (Sos) which are instrumental for cytoskeletal rearrangements and for triggering of the mitogen-activated protein kinase (MAPK) pathway, respectively. IP_3 signals the release of Ca^{2+}-ions from intracellular stores and mediates activation of the Ca^{2+}-sensitive transcription factor nuclear factor of activated T cells (NFAT) and the Ca^{2+}-dependent protein kinase C (PKC) isoforms. DAG also functions in PKC-activation and this has been linked to the activation of the nuclear factor-κB (NF-κB) family of transcription factors. Another cluster of adaptor proteins

composed of BCL-10 (B-cell lymphoma 10), CARMA1 (caspase-recruitment domain membrane-associated guanylate kinase protein 1) and MALT1 (mucosa-associated-lymphoid-tissue lymphoma-translocation gene 1) functions in the activation of the IKK (inhibitor of nuclear factor-κB (IκB) kinase) complex, a prerequisite for NF-κB-activation. BCR-ligation also enhances the formation of the lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) through activation of phosphoinositide-3 kinase (PI3K). This is a key event for the activation of many pleckstrin homology (PH)-domain-containing signaling proteins including the oncogenic kinase Akt (see below).

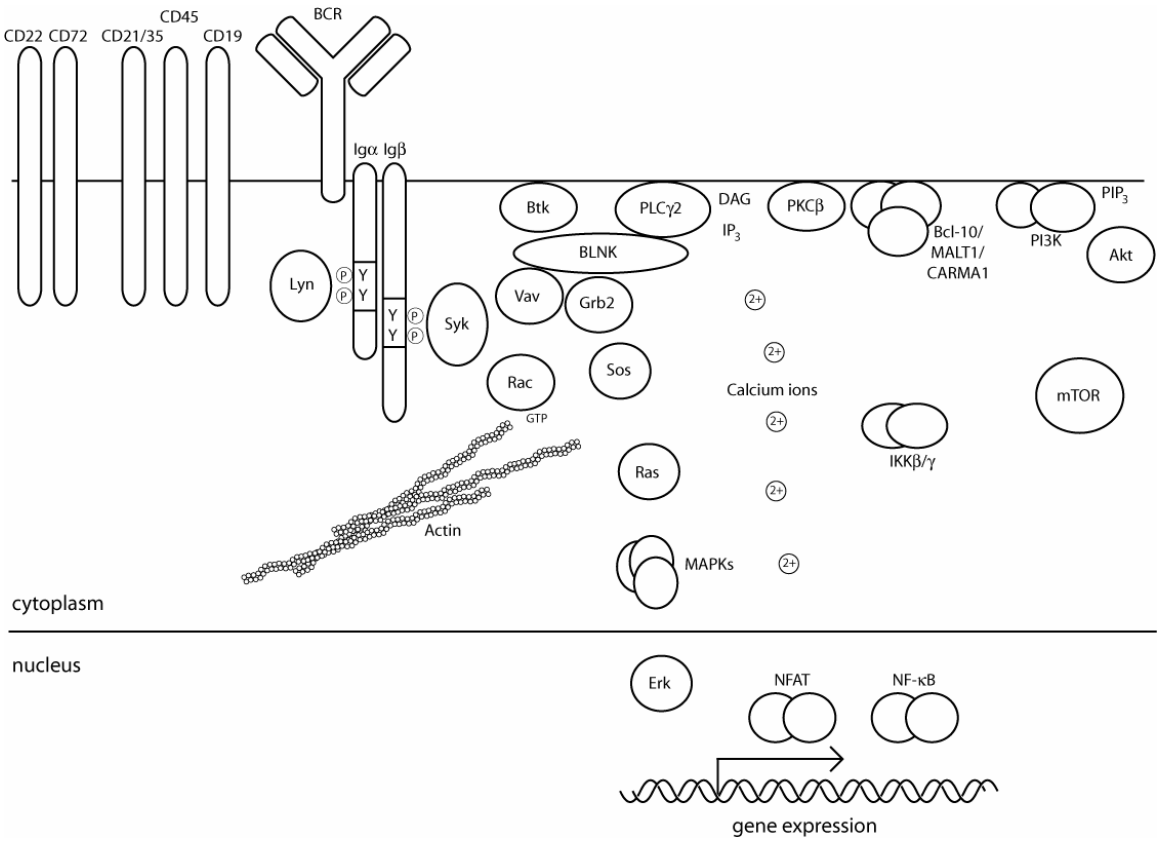


Figure 2: Overview of BCR-signaling. See text for details (modified from Monroe, 2006).

In addition to the BCR core elements surface Ig and Igα-Igβ heterodimer, the complex can contain several co-receptors which have the capacity to modulate BCR-induced signaling (Nitschke, 2005: ; Poe, et al., 2001). Examples for important co-stimulants are CD19, CD21/35 and CD45, whereas CD22 and CD72 transduce inhibitory signals through motives termed immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in analogy to the ITAMs. ITIMs function in the recruitment of SH2-domain containing protein tyrosine phosphatases which are not only important in the down-regulation

and termination of the BCR-induced response, but which also possess the important capacity to modulate the strength of the BCR-mediated signal by directly opposing the action of proximal protein tyrosine kinases.

Signaling form the BCR or pre-BCR can direct dramatically different cell fates ranging from survival and proliferation to anergy and cell death. Despite the wealth of information on BCR-induced signaling pathways, establishing a certain cellular outcome as the consequence of specific molecular events has proven challenging. It is likely that key signaling factors are important for several potential cell fates, but there could be specific differences in their respective regulation through feedback interactions or signaling input from additional receptors. Conversely, some components of the BCR-related signaling web could function as unique features in promoting a specific cellular destiny. Most likely, a combination of both quantitative and qualitative differences enables the diversification from a single inducer to a range of cellular outcomes. For example, the MAPK Erk (extracellular signal-regulated kinase) and the transcription factor NFAT are responsive to antigenic stimulation in both naive and auto-reactive B cells, albeit to a different extend, whereas activation of the MAPK Jnk (Jun N-terminal kinase) and the transcription factor NF-κB occurs only in naive cells (Healy and Goodnow, 1998). These unique BCR-induced signaling profiles coincide with opposing cell fates, namely the clonal expansion of activated versus the inactivation and deletion of auto-reactive cells.

Several lines of evidence strongly suggest that the BCR complex has a critical signaling function even in the absence of ligand binding. Loss of the Ig portion of the BCR complex on mature B cells causes their demise and disappearance from the peripheral B cell pool (Lam, et al., 1997). The same is true for ablation of the ITAM-containing signaling portion of the Igα and Igβ accessory chains (Kraus, et al., 2004: ; Meffre and Nussenzweig, 2002). Conversely, expression of a plasma membrane targeted chimeric Igα-Igβ protein in bone marrow progenitor cells which lack a functional Ig heavy chain coding region, is sufficient to generate peripheral follicular B cells which persist in the absence of surface BCR-expression (Bannish, et al., 2001). These studies demonstrate that the maintenance of mature resting B cells in the periphery requires a constitutive survival signal which originates from the signaling portion of the BCR-complex and the expression of this segment is the minimal unit required to sustain peripheral B cell development and viability.

Several hypotheses have been put forward to explain the nature of the so-called tonic BCR-mediated survival signal(Monroe, 2006). It has been proposed that a potential oligomeric assembly of BCR-complexes on the cell surface even in the absence of a cross-linking agent may be sufficient to generate a transducible event through the physical vicinity of signaling modules (Schamel and Reth, 2000). However, most of the current data suggest that BCR-complexes are rather dispersed throughout the plasma membrane in a monomeric state in resting naive cells, whereas stimulation causes their clustering, cap formation and eventual internalization (Tolar, et al., 2005). An alternative model focuses on BCR-localization in specialized ganglioside-rich membrane domains called lipid rafts. These membrane micro-domains are believed to function as signaling platforms and BCR-aggregation into lipid-raft localized clusters occurs upon receptor cross-linking. As a fraction of BCR-complexes is lipid-raft associated in resting unstimulated B cells, they might represent the source of the tonic BCR-signal (Guo, et al., 2000). This model is complicated by the observation that many of the signaling proteins believed to be essential for BCR-mediated signaling, including Syk and PLCγ2, only localize to lipid rafts upon receptor cross-linking. Furthermore, BCR-localization in lipid rafts appears to be dispensable for its tonic signaling to occur (Fuentes-Panana, et al., 2005).

Finally, Monroe and others have proposed the homeostatic equilibrium model. Tonic BCR-signaling in this model represents a steady-state balance between stochastic phosphorylation of BCR-ITAMs by receptor-associated protein tyrosine kinases and its reversal by protein tyrosine phosphatases recruited by inhibitory BCR co-receptors. More specifically, a basal enzymatic activity of Lyn and Syk could account for random transient BCR-ITAM-phosphorylation which constitutes the origin of the tonic BCR survival signal. At the same time, full-blown receptor signaling is prevented by the activity of CD22-associated SH2-domain containing protein tyrosine phosphatase 1 (SHP-1). This model is in line with the effects of CD22- or SHP-1-deficiency on BCR-signaling (Cyster and Goodnow, 1995: ; Pani, et al., 1995), although the actual mechanism *in vivo* may be more complex and involve additional factors. BCR-stimulation with antigen would tip the balance between positive and negative signaling events within the complex towards productive phosphorylation. This could be achieved through the physical exclusion of inhibitory receptor components from the lipid-raft localized BCR-clusters and/or by transient inactivation of protein tyrosine phosphatases through the generation of reactive oxygen species. While this model makes a very coherent suggestion

regarding the initiation of tonic versus immunogenic BCR signaling, the nature of the downstream events continues to be unknown. Specifically, it remains to be determined how transient ITAM-phosphorylation is translated to cell survival versus the multitude of cellular changes in response to BCR-stimulation. This could be due to a quantitative difference in the signaling strength or a qualitative difference involving a distinct set of signaling factors or a combination of both. To date, any specific differences between tonic and ligand-induced BCR-signaling on a molecular level, qualitatively or quantitatively, have remained elusive.

1.2.2 The role of Syk in B cell biology

Syk was first purified as a tyrosine kinase activity from porcine spleen, hence the name for spleen tyrosine (Y) kinase (Kobayashi, et al., 1990: ; Sakai, et al., 1988: ; Taniguchi, et al., 1991). It forms a subgroup of cytoplasmic tyrosine kinases together with the closely related protein ZAP-70 (zeta-chain associated protein kinase, 70kD). Syk is expressed widely throughout the lymphoid and myeloid lineage as well as in some non-hematopoietic cell types including fibroblasts and epithelial cells (Turner, et al., 2000: ; Yanagi, et al., 2001).

Syk plays a critical role in signaling downstream of various receptors in different cell types. In B cells, Syk is rapidly activated upon cross-linking of the BCR (Hutchcroft, et al., 1991: ; Law, et al., 1994: ; Yamada, et al., 1993). Furthermore, BCR-induced signaling responses are abrogated in a Syk-deficient variant of the chicken B cell line DT40, indicating an indispensable function of Syk in BCR-mediated signal transduction (Takata, et al., 1994). In addition to its function in B cells, Syk plays a role in signaling from various types of Fc receptors through which immune cells bind and respond to soluble antibodies. Fc stands for “fragment, crystallizable” and corresponds to the constant region of an antibody molecule (Janeway, et al., 2001). In mast cells, Syk mediates the response to IgE via FcεRI and is required for degranulation and cytokine release (Costello, et al., 1996: ; Taylor, et al., 1995: ; Zhang, et al., 1996). Similarly, Syk is involved in antibody-dependent cell-mediated cytotoxicity in response to FcγRIII cross-linking in natural killer (NK) cells (Brumbaugh, et al., 1997: ; Colucci, et al., 1999) and FcγR-mediated phagocytosis in macrophages (Crowley, et al., 1997: ; Kiefer, et al., 1998). Syk is also expressed in double-negative thymocytes and a role for Syk in pre- T cell receptor (TCR) signaling has been suggested based on its ability to

interact with the phosphorylated TCRζ chain in thymocytes and Jurkat T cells (Chan, et al., 1994: ; Thome, et al., 1995: ; van Oers and Weiss, 1995). A major role for Syk in mature T cells is unlikely based on its low expression level (Chu, et al., 1999). However, enforced Syk-expression is able to restore the impaired T cell development ensuing from a lack of ZAP-70 in mice (Gong, et al., 1997), indicating that the functions of Syk and ZAP-70 may be partially interchangeable and differences may mainly be bestowed by a differential expression pattern. In support of this hypothesis, enforced expression of ZAP-70 restores BCR-signaling in Syk-deficient DT40 B cells (Kong, et al., 1995).

Both Syk and ZAP-70 are composed of an N-terminal tandem SH2-domain followed by a linker region and a C-terminal kinase domain (figure 3). The main difference between the two proteins is found in the linker region, which contains a 23 residues insertion in Syk as compared to ZAP-70. Interestingly, a deletion of the same region occurs in SykB which arises from alternative splicing of the full-length Syk transcript. This difference has functional consequences as full-length Syk displays a higher affinity to phosphorylated ITAMs and mediates more efficient signaling from the FcεRI receptor (Latour, et al., 1998).

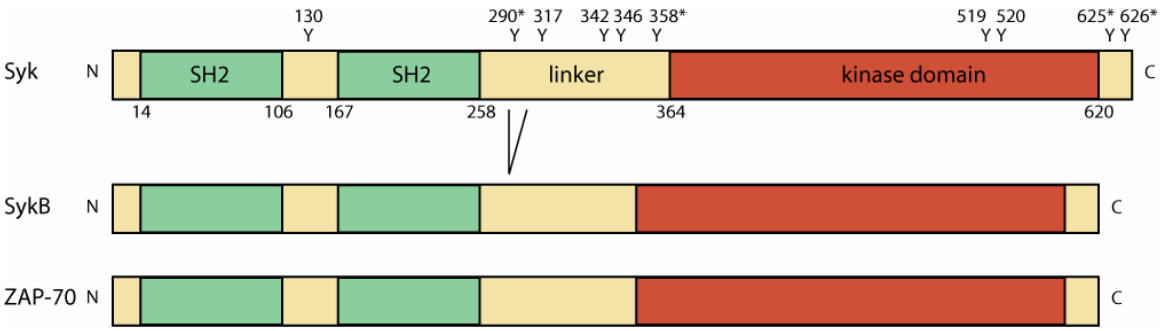


Figure 3: Domain structure of Syk, SykB and ZAP-70. The positions of the structural domains as well as of tyrosine residues are indicated. Residue numbering is based on the full-length murine Syk protein. Asterisks denote residues whose phosphorylation has only been observed *in vitro* (modified from Turner et. al. 2000).

Syk-activation downstream of the BCR, pre-TCR and Fc-receptors most likely occurs through a shared mechanism which depends on the ITAMs present in all these receptor complexes. Syk has the capacity to bind phosphorylated ITAMs via its tandem SH2-domain (Johnson, et al., 1995: ; Law, et al., 1994: ; Law, et al., 1993: ; Saouaf, et al., 1994). This induces a conformational

change in the protein which likely enables kinase activity, resulting in the phosphorylation of

downstream targets as well as Syk itself (Kimura, et al., 1996: ; Rowley, et al., 1995). Initial BCR ITAM-phosphorylation was originally believed to be accomplished by Src-family tyrosine kinases, although later evidence suggested that Syk-activation does not require prior Src-kinase activity and Syk itself is capable of ITAM-phosphorylation (Kurosaki, et al., 1994: ; Zoller, et al., 1997). The order of proximal signaling events downstream of the BCR is therefore not entirely clear and it is not known, how potential Syk-activity on BCR-ITAMs is regulated in the absence of stimulation. Finally, alternative ITAM-independent mechanisms of Syk activation may exist as Syk also plays in role in signaling downstream of non-ITAM receptors, for example integrins, although it is possible that such receptors require ITAM-containing adaptor molecules (Gao, et al., 1997: ; Mocsai, et al., 2006: ; Woodside, et al., 2001).

Syk contains multiple tyrosine residues whose phosphorylation has been observed *in vitro* and *in vivo* (Furlong, et al., 1997: ; Keshvara, et al., 1998). Upon BCR-cross linking, Syk-phosphorylation can be detected on Y130 located between the two SH2-domains, the linker region residues Y317, Y342 and Y346 as well as Y519 and Y520 within the kinase domain (figure 3). Phosphorylation of Y130 and the kinase domain tyrosines is accomplished through auto-phosphorylation. Y519 and Y520 autophosphorylation is essential to Syk catalytic activity whereas the modification of Y130 appears to play a role in modulating Syk binding to the BCR (Couture, et al., 1997: ; Keshvara, et al., 1997: ; Kurosaki, et al., 1995). Phosphorylation of the linker region tyrosines is presumably mediated by Lyn and serves important biological functions. On the one hand, it appears to play a role in the binding of Syk to downstream targets such as PLCγ1 and Vav (Deckert, et al., 1996: ; Law, et al., 1996). On the other hand, phosphorylation of Y317 is critical for Syk recognition by the ubiquitin-ligase Cbl (Casitas B-lineage lymphoma). Cbl-mediated proteolytic degradation of Syk constitutes an important mechanism in the down regulation of Syk-activity (Lupher, et al., 1998: ; Sohn, et al., 2003: ; Yankee, et al., 1999). In addition to the sites mentioned above, Syk-phosphorylation on Y290, Y358, Y624 and Y625 has been observed *in vitro* (Furlong, et al., 1997). The potential modification of the C-terminal residues Y624 and Y625 is particularly intriguing due to a potentially analogous mechanism in tyrosine kinases of the Src-family. Here, phosphorylation of C-terminal tyrosine residues by C-terminal Src kinase (Csk) triggers kinase inactivation (Nada, et al., 1991). Indeed, the conversion

of Syk C-terminal tyrosine residues to phenylalanines creates a gain-of-function Syk mutant (Zeitlmann, et al., 1998). Yet, neither a mechanism of Syk-inactivation through C-terminal phosphorylation nor a potential C-terminal Syk kinase has been described to date.

Syk-deficiency in mice results in perinatal lethality due to excessive hemorrhaging (Cheng, et al., 1995: ; Turner, et al., 1995). This is caused by a failure in the segregation of lymphatic and blood vessels during embryonic development (Abtahian, et al., 2003). The potential function of Syk in the immune system has been assessed through adoptive transfer of fetal liver which is the site of fetal hematopoiesis, from Syk-deficient embryos into immunodeficient hosts. The resulting chimeric mice exhibit a grossly normal T cell compartment and various functional deficits in myeloid cell types. The most dramatic effect is observed in the B cell compartment. Lack of Syk causes a severe block in early B cell development in the bone marrow at the transition from the pro B to the pre B stage, resulting in a near absence of B cells in the peripheral lymphoid organs. This demonstrates an obligatory role for Syk at the onset of B lymphopoiesis. A similar phenotype in mutant mice which are genetically unable to express a functional BCR can be overcome by the expression of a rearranged BCR-transgene, but this is not possible in the absence of Syk (Rajewsky, 1996); (Cornall, et al., 2000: ; Turner, et al., 1997). In the latter case, the fraction of IgM-positive cells in the bone marrow is increased by expression of a transgenic BCR. However, these cells remain phenotypically immature, are refractive to BCR-stimulation and are unable to exit the bone marrow and persist in the periphery. Similarly, extension of B cell survival by ectopic expression of B-cell lymphoma 2 (Bcl-2) does not rescue B cell maturation in the absence of Syk (Turner, et al., 1997). These results argue for an indispensable signaling function for Syk downstream of the BCR.

The early developmental block in the absence of Syk precludes an assessment of its function at later B cell stages including the survival, activation and differentiation of mature naive B cells. In an attempt to circumvent this problem, a conditional Syk knock-out was generated (Saijo, et al., 2003) and figure 4A). A targeting vector was constructed from genomic DNA of 129/Sv origin encompassing the first two exons of the murine Syk locus. The first exon was flanked with a single loxP site on the one side and with a loxP-flanked neomycin resistance cassette (neo^r) on the other. E14.1 embryonic stem (ES) cells were transfected with the targeting vector and screened for homologous recombination by Southern blotting of genomic DNA

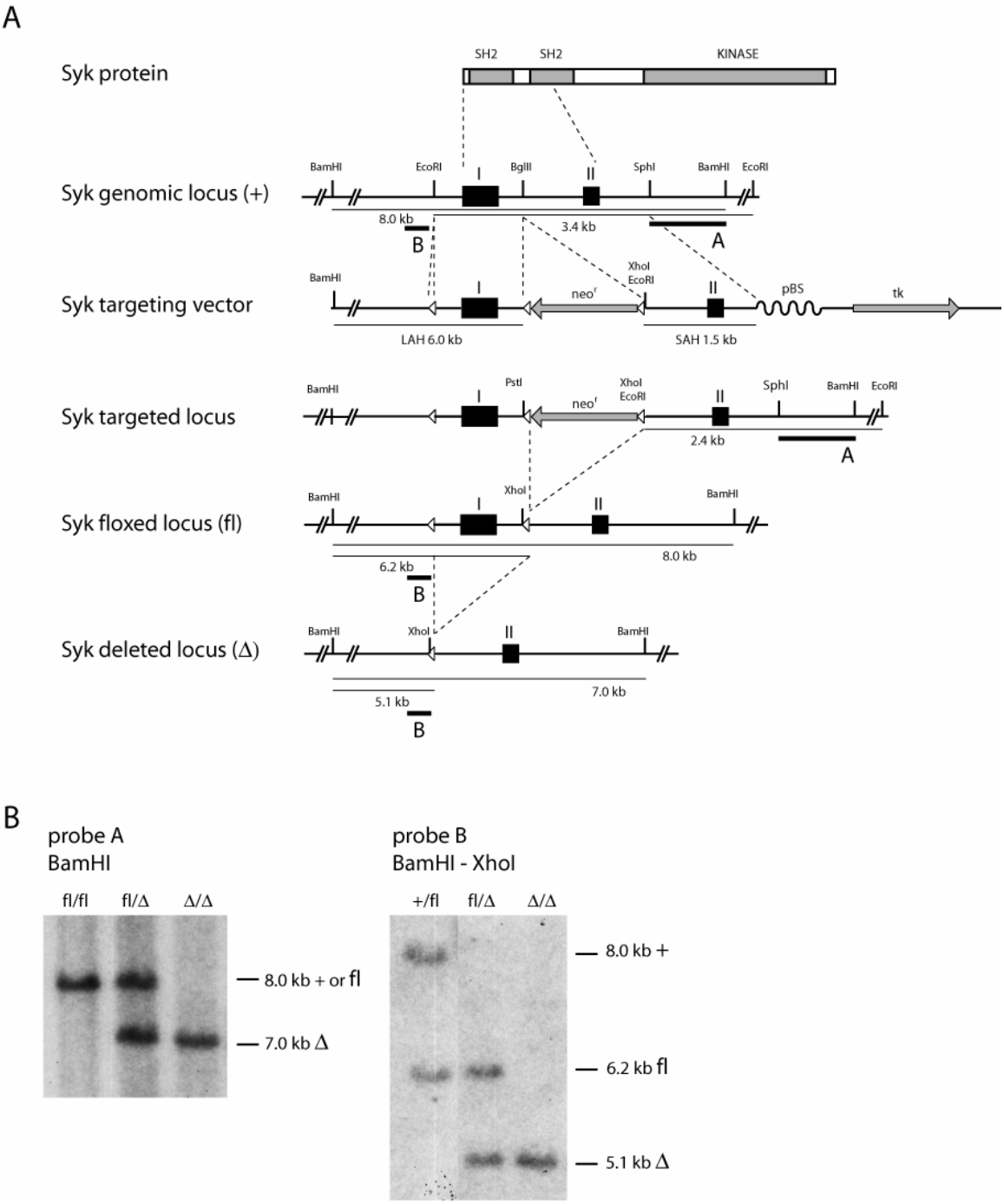


Figure 4: Conditional Syk inactivation in mice. A. Gene targeting strategy for conditional Syk inactivation. The domain structure of the Syk protein is shown together with part of the Syk genomic locus, the targeting vector and part of the Syk locus before and after Cre-mediated recombination. Black squares denote exons I and II. Open triangles represent loxP sites. Gray bars denote expression cassettes for the neomycin resistance (neo^r) and herpes simplex thymidine kinase (tk) genes with the arrows indicating the transcriptional orientation. Essential elements of the Southern blot strategies used in the characterization of the various loci forms, are indicated as follows: Probes A and B as thick black lines, genomic fragments as thin black lines

between endonuclease restriction sites. LAH = long arm of homology, SAH = short arm of homology, pBS = pBluescript. Modified from Saijo et. al., 2003. B. Southern blot analysis of genomic DNA isolated from mouse tail tips. left: floxed (fl, 8 kb) and deleted (Δ, 7 kb) alleles using BamHI restriction digestion and probe A; right: wild-type (+, 8 kb), floxed (fl, 6.2 kb) and deleted (Δ, 5.1 kb) alleles using BamHI-XhoI double digestion and probe B.

after EcoRI digestion using the external probe A. The neo^r cassette was removed from positive clones through transfection with a Cre-recombinase expression vector and newly neomycin-sensitive clones were screened for correct excision of only the neo^r-cassette by Southern blot using BamHI-XhoI double digestion and probe B. ES cell clones containing the correctly targeted locus were injected into C57BL/6 blastocysts and the resulting chimeras were bred to C57BL/6 mice for germline transmission of the floxed Syk allele. The floxed Syk allele is functionally indistinguishable from a wild-type allele (my unpublished observation) and can be converted to a null allele through Cre-recombinase mediated excision of the first exon which contains the translation start codon. Genotyping of mice or cells is feasible through two different Southern blot strategies: use of probe A on BamHI-digested genomic DNA distinguishes the deleted allele from the wild-type or floxed allele, whereas probe B on BamHI-XhoI double-digested genomic DNA separates all three different alleles (figure 4B). The spatiotemporal regulation of Syk inactivation is determined by the pattern of Cre-expression. Different mouse Cre-strains are available in which Cre-expression is inducible by an external signal or targeted to a specific cell type.

1.3 Mature B cell survival through BAFF signaling

1.3.1 The BAFF-related protein network

It was long believed that tonic BCR signaling is the principle or even sole factor in mature B cell maintenance until a cytokine was discovered which dramatically increased the survival of B cells *in vitro* and *in vivo* (Batten, et al., 2000: ; Do, et al., 2000: ; Gross, et al., 2000: ; Khare, et al., 2000: ; Mackay, et al., 1999: ; Moore, et al., 1999: ; Mukhopadhyay, et al., 1999: ; Rolink, et al., 2002: ; Schneider, et al., 1999: ; Shu, et al., 1999: ; Thompson, et al., 2000). BAFF, also known as BLyS, TALL-1, THANK, zTNF4 and TNFSF 13b, increases B cell viability in culture and causes B cell hyperplasia and

autoimmunity when ectopically expressed in mice. In contrast to potent B cell mitogenic stimuli such as BCR cross-linking, CD40-ligand or endotoxin, BAFF exclusively enhances B cell viability without inducing proliferation.

Together with a related protein called a proliferation-inducing ligand (APRIL) BAFF forms a subgroup within the tumor necrosis factor (TNF) family. As such, BAFF is synthesized as a type II transmembrane protein which homotrimerizes via its C-terminal TNF-homology domain (THD). Soluble BAFF is the result of cleavage by furin family proteases, releasing a 138 residues product from the full-length (309 residues) pre-curser (Schneider, et al., 1999). BAFF contains putative *N*-linked glycosylation sites in its extracellular domain and its sensitivity to treatment with peptide *N*-glycanase indicates BAFF-glycosylation *in vivo*. Mouse and human BAFF are 59% identical in their amino acid sequence, the main difference being a 31 residues insertion in mouse BAFF between the proteolytic cleavage site and the conserved THD-fold. Heterotrimers between BAFF and ARPIL are possible when the two proteins are co-expressed and have been found in the serum of patients with systemic rheumatic disease (Roschke, et al., 2002). A second BAFF isoform termed ΔBAFF arises from alternative splicing of the BAFF-transcript and can form multimers together with full-length BAFF. The biochemical properties of ΔBAFF vary significantly from the full-length protein in its poor release through protease-mediated cleavage and its decreased receptor affinity (Gavin, et al., 2003). Accordingly, ΔBAFF opposes full-length BAFF activity and cannot functionally substitute for loss of BAFF *in vivo* (Gavin, et al., 2005). BAFF-trimers can assemble into a 60-mer which forms a virus-like structure (Cachero, et al., 2006: ; Liu, et al., 2002). This form of BAFF is biologically active, although the significance of this particular assembly remains unknown (Zhukovsky, et al., 2004).

Three TNF receptor proteins have been found to bind BAFF although their ligand specificity partially overlaps with APRIL (figure 5). Transmembrane Activator and CAML Interactor (TACI) and B-Cell Maturation Antigen (BCMA) bind both BAFF and APRIL while BAFF is the sole ligand for BAFF receptor (BAFF-R) (reviewed in (Bodmer, et al., 2002: ; Bossen and Schneider, 2006: ; Mackay, et al., 2003). All three receptor proteins are untypical members of the TNF receptor family in that they are type III transmembrane proteins and their intracellular portion lacks a death domain. The extracellular domains contain one or more Cystein-Rich Domains (CRDs), the ligand binding unit of the receptor, although its canonical motif is only partially conserved in the case of BAFF-R. Additional complexity to this receptor-ligand system is afforded by

the differential affinity of certain members to proteogycans (see below and figure 5).

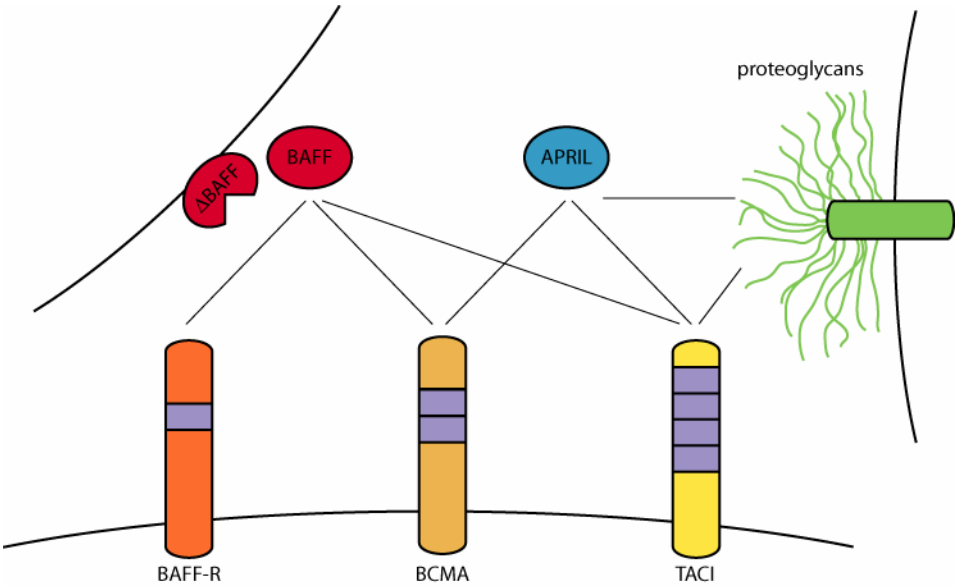


Figure 5: The BAFF protein family. Ligands BAFF, ΔBAFF and APRIL are shown together with the receptor proteins BAFF-R, BCMA and TACI as well as the proteoglycan moiety of other cell surface-expressed proteins. Boxes in the receptors denote cystein rich domains (CRDs). Ligand-receptor interactions are indicated by thin black lines. Note that the oligo- or multimeric nature of ligand and receptor complexes is not represented in this diagram.

1.3.2 The functions of BAFF and BAFF-R in B cell survival and beyond

The function of the BAFF-related protein network has been investigated using mouse models which lack individual components of the ligand-receptor system. In line with its dramatic impact on B cell viability, loss of BAFF diminishes the peripheral B cell compartment in mice. While early B cell development in the bone marrow proceeds largely unimpaired, a characteristic block ensues during peripheral B cell maturation in the spleen from the T1 to the T2 stage. Consequently, mature B cells are largely absent from the periphery of BAFF-deficient mice (Gross, et al., 2001: ; Schiemann, et al., 2001: ; Schneider, et al., 2001). A very similar phenotype, albeit somewhat less severe, is observed in the natural mouse mutant A/WySnJ (Lentz, et al., 1996: ; Miller and Hayes, 1991). This lead to the identification of BAFF-R as the principle receptor protein involved in BAFF-mediated B cell

survival. A/WySnJ mice harbor a transposon insertion in the BAFF-R locus which alters the C-terminal eight amino acids of the receptor and presumably impairs its signaling capacity (Thompson, et al., 2001: ; Yan, et al., 2001). Subsequent BAFF-R ablation by gene targeting provided additional support for the vital role of BAFF-R in BAFF-mediated survival of peripheral B cells from the T2 stage on (Sasaki, et al., 2004: ; Shulga-Morskaya, et al., 2004). This BAFF-dependency is not only restricted to conventional follicular B cells, but likely includes MZ B cells. The analysis of this question has been complicated by the fact that BAFF regulates the expression of CD21/35 and CD23 which are commonly used surface markers in the identification of splenic B cell subsets (Gorelik, et al., 2004). It appears that some MZ B cells are present in BAFF null and A/WySnJ mice, whereas none are detected in BAFF-R null mice and those expressing a TACI-Ig transgene (Amanna, et al., 2003: ; Gorelik, et al., 2004: ; Sasaki, et al., 2004: ; Tardivel, et al., 2004). Overall, these results indicate that some level of BAFF-signaling is required for MZ B lymphopoiesis or maintenance or both. This conclusion is also supported by an expansion of the MZ B cell compartment in BAFF-transgenic mice (Gross, et al., 2000: ; Khare, et al., 2000: ; Mackay, et al., 1999). Quite surprisingly, despite the near absence of mature B cells in the absence of BAFF signaling, it is not required for some secondary B cell immune responses including the formation of germinal centers (GCs) and BCR affinity maturation. Yet, it does contribute to GC stability and BCR class-switch recombination to selected isotypes (Kalled, 2006). Most reports have concluded that BAFF signaling does not have an impact on the B-1 subset of B cells, although one study documented a modulation of B-1 cell numbers by decrease or increase in BAFF (Gavin, et al., 2005). In addition to its most prominent role in B cell biology, BAFF modulates the T cell compartment in several ways (reviewed in (Mackay and Leung, 2006). BAFF can directly stimulate BAFF-R-expressing T cell subpopulations including activated and regulatory T cells. BAFF provides a direct co-stimulatory signal to anti-CD3-triggered T cells through an autocrine loop by which activated T cells synthesize BAFF and increase BAFF-R expression. BAFF-costimulation of T cells enhances cytokine release and their differentiation into effector T cells. *In vivo*, BAFF-influences T cell-mediated allograft rejection, although it is not established whether this is due to direct BAFF-signaling on T cells.

The essential function of BAFF in B cell homeostasis is revealed not only by lack of but also by excess BAFF-signaling. Ectopic BAFF-expression in mice leads to B cell hyperplasia, elevated serum Ig titers and the manifestation of

systemic autoimmunity (Gross, et al., 2000: ; Khare, et al., 2000: ; Mackay, et al., 1999). Correspondingly, elevated levels of BAFF have been measured in the serum of autoimmune-prone mice and in patients suffering form various autoimmune disorders including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and primary Sjogren's syndrome (pSS) (reviewed in (Mackay, et al., 2005). A mechanistic basis for BAFF's involvement in the negative selection of auto-reactive cells stems from the fact that the latter require higher BAFF-concentrations for survival than normal naive B cells (Lesley, et al., 2004: ; Thien, et al., 2004). Given that the amount of available BAFF is limited in a normal physiological context, this differential BAFF-requirement ensures the exclusion of auto-reactive cells from the stable peripheral B cell pool. Conversely, excess BAFF enables the persistence of B cells with a self-reactive receptor specificity in the periphery with the potential to cause tissue damage. This model fits very well with the observed clinical correlation between BAFF titers and autoimmune symptoms in humans and mice. Also in line with this model is the observation that ablation of two BAFF-regulated negative players in B cell survival, namely PKCδ and Bcl-2 interacting mediator of cell death (Bim), renders B cell survival BAFF-independent, ablates B cell tolerance and causes autoimmunity (see below).

The dual B cell pathologies resulting from insufficient as well as excess BAFF-signaling illustrate that the amount of biologically active BAFF has to be very tightly regulated to ensure the maintenance of a physiological peripheral B cell pool. BAFF-synthesis has been attributed to radiation-resistant stromal cells in the spleen, different myeloid cell types including monocytes, macrophages, neutrophils and dendritic cells, as well as activated T cells, follicular dendritic cells, astrocytes, osteoclasts and others (Craxton, et al., 2003: ; Gorelik, et al., 2003: ; Kalled, 2006: ; Litinskiy, et al., 2002: ; Nardelli, et al., 2001: ; Scapini, et al., 2005). While stromal cells could be a constitutive source of BAFF which is mainly responsible for the maintenance of physiological BAFF serum titers, BAFF-production by monocytes, macrophages, dendritic cells or neutrophils is externally regulated by other cytokines and could serve the modulation of BAFF-concentrations in specialized micro-environments such as inflammatory sites. Some instances of BAFF-production have been related to a pathological state, such as BAFF-synthesis by fibroblast-like synoviocytes in rheumatic joints, by ductal epithelial cells in the salivary gland of Sjogren's syndrome patients and by nurse-like cells in chronic lymphocytic leukemia (CLL) (Groom, et al., 2002: ; Ittah, et al., 2006: ; Nishio, et al., 2005: ; Ohata, et al., 2005). Despite the identification of numerous BAFF-producing cell types, the

mechanism accounting for the precise regulation of BAFF levels remains unknown.

In addition to the available amount of BAFF, the level of BAFF-R surface expression seems to impact the strength of BAFF-signaling. This is evidenced by the intermediate B cell phenotype of heterozygous A/WySnJ mice compared to homozygous A/WySnJ mice and the parental A/J strain and indicates a gene dosage effect (Harless, et al., 2001: ; Lentz, et al., 1996: ; Miller and Hayes, 1991).

1.3.3 The functions of TACI, BCMA and APRIL

Despite certain structural similarities between the ligands BAFF and APRIL as well as the receptors TACI, BCMA and BAFF-R, the biological functions of the individual proteins vary dramatically. As described above, mature B cell survival is predominantly accomplished by BAFF-binding to BAFF-R whereas a similar role for the other proteins is less likely based on the phenotype of the respective knock-out mice. Lack of BCMA does not have a discernable effect on mature B cell viability (Xu and Lam, 2001). Yet, it plays a role in the survival of long-lived plasma cells in the bone marrow (Avery, et al., 2003: ; O'Connor, et al., 2004).

The function of TACI in B cell biology appears to be fairly complex and may not be entirely conserved between mice and humans. On the one hand, TACI-deficiency in mice renders B cells hyper-responsive to mitogenic stimuli and leads to an SLE-like disorder, B cell hyperplasia and even spontaneous development of lymphoma in a significant fraction of mutant animals (Seshasayee, et al., 2003: ; von Bulow, et al., 2001: ; Yan, et al., 2001). These symptoms argue for a predominantly inhibitory role for TACI in B cell activation in mice. On the other hand, TACI is required for a proper immune response to type II T cell-independent antigens and plays a role in class switch recombination to IgG1 and IgA, a process which is promoted by both BAFF and APRIL through BAFF-R and TACI (Castigli, et al., 2004: ; Castigli, et al., 2005: ; Litinskiy, et al., 2002: ; Sakurai, et al., 2006). Furthermore, mutations in TACI are associated with an immunodeficiency syndrome in humans (Castigli, et al., 2005: ; Salzer, et al., 2005). Interestingly, in addition to the ligands BAFF and APRIL, TACI was shown to interact with the heparan sulfate moiety of the cell surface protein Syndecan-2 and this interaction induced TACI-mediated signaling (Bischof, et al., 2006: ; Sakurai, et al.,

2006). Collectively, TACI-signaling appears to effect B cell functions both negatively and positively. The precise impact may be related to the B cell maturation stage, the type of ligand or even the species.

APRIL-deficiency does not appear to influence the survival of mature B cells, but as mentioned above, it can contribute to class-switch recombination by signaling through TACI and promotes plasma cell survival through BCMA (Avery, et al., 2003: ; O'Connor, et al., 2004). Intriguingly, in addition to binding to the TNF-family receptors TACI and BCMA, APRIL displays an affinity for the glycosaminoglycan side chains of proteoglycans (Ingold, et al., 2005). APRIL has also been implicated in the survival of lymphatic malignancies including B-cell chronic lymphoid leukemia (B-CLL), multiple myeloma and non-Hodgkin's lymphoma (Dillon, et al., 2006).

In addition to their individual functions, APRIL, TACI and BCMA can have a dramatic effect on B cell survival by titrating the available amounts of biologically active BAFF and BAFF-R. For example, TACI- or BCMA-fusion proteins function as decoy BAFF receptors when injected in mice or expressed from a transgene, depleting the peripheral B cell compartment (Gross, et al., 2001: ; Pelletier, et al., 2003: ; Schneider, et al., 2001: ; Thompson, et al., 2000). Also, chimeric receptor proteins containing the intracellular portion of BAFF-R, TACI or BCMA, respectively, elicit partially overlapping outcomes in terms of cell survival and molecular responses when expressed and triggered in B cell lines (Craxton, et al., 2005). In this experimental system, all three proteins boost cell survival while BAFF-R and TACI but not BCMA cause Bim-phosphorylation and only BAFF-R induces NF-κB processing (see below). In summary, there appears to be an intricate web of interactions between the ligands BAFF and APRIL, the receptors BAFF-R, TACI and BCMA and possibly also the proteoglycan moieties of cell surface molecules such as members of the syndecan family. While individual functions have been assigned to specific contacts, the full scope of potential cross-communication may continue to unfold.

1.3.4 The molecular consequences of BAFF-signaling

Considering the enormous interest and the wealth of knowledge on the function of BAFF in B cell physiology and disease, the level of insight into its signaling mechanism is surprisingly modest. A search for proximal signaling factors which directly interact with BAFF-R has so far yielded a single protein,

TRAF3. A physical and functional association has been proposed between BAFF-R, TRAF3 and the adaptor protein NF-κB activator 1 (Act1) which dampens BAFF-induced cellular responses (Qian, et al., 2004: ; Xu and Shu, 2002). Despite limited knowledge on the direct proximal signaling events upon B cell stimulation with BAFF, several molecular changes have been observed which likely contribute to its B cell survival effect (figure 6). First, it was discovered that BAFF induces activation of the transcription factor NF-κB which is known to play a central role in lymphocyte survival. NF-κB in mammals comprises a family of five related proteins (p50, p52, p65, RelB and c-Rel) which function in transcriptional regulation as hetero- or homodimers (Bonizzi and Karin, 2004). p50 and p52 originate from the processing of precursor proteins p105 (NF-κB1) and p100 (NF-κB2), respectively. The mechanism of NF-κB activation has been well studied for many years and relies on the rapid IKK-mediated phosphorylation and subsequent proteolytic degradation of IκB proteins which allows for the nuclear translocation of transcriptionally active NF-κB dimers. An example for this type of NF-κB activation is B cell stimulation through its antigen receptor. By contrast, BAFF primarily elicits NF-κB activation through an alternative mechanism in which NF-κB2 p100 is processed to mature p52 and subsequently forms transcriptionally active dimers with RelB (Claudio, et al., 2002: ; Kayagaki, et al., 2002: ; Senftleben, et al., 2001: ; Xiao, et al., 2001). The transcriptional targets of BAFF-induced NF-κB activation are not well characterized. Upregulated expression of some pro-survival members of the Bcl-2 family has been observed in some instances but not in others (Claudio, et al., 2002: ; Hatada, et al., 2003: ; Hsu, et al., 2002: ; Lesley, et al., 2004: ; Thomas, et al., 2005: ; Trescol-Biemont, et al., 2004) and my own unpublished observations).

In addition to NF-κB activity, BAFF controls the subcellular localization of PKCδ. PKCδ is an important negative regulator of B cell viability as evidenced by B cell hyperplasia, autoimmunity and lack of B cell tolerance in PKCδ-deficient mice (Mecklenbrauker, et al., 2002). Furthermore, mature B cell survival becomes BAFF-independent in the absence of PKCδ (Mecklenbrauker, et al., 2004). The pro-apoptotic function of PKCδ is associated with its translocation to the nucleus and capacity for histone modification. BAFF promotes the cytoplasmic retention of PKCδ, thus containing its pro-apoptotic potential.

While the BAFF-induced expression of pro-survival Bcl-2 family members remains somewhat controversial, the pro-apoptotic Bcl-2 family member Bim is an undisputed target of BAFF-mediated regulation (Craxton, et al., 2005). A

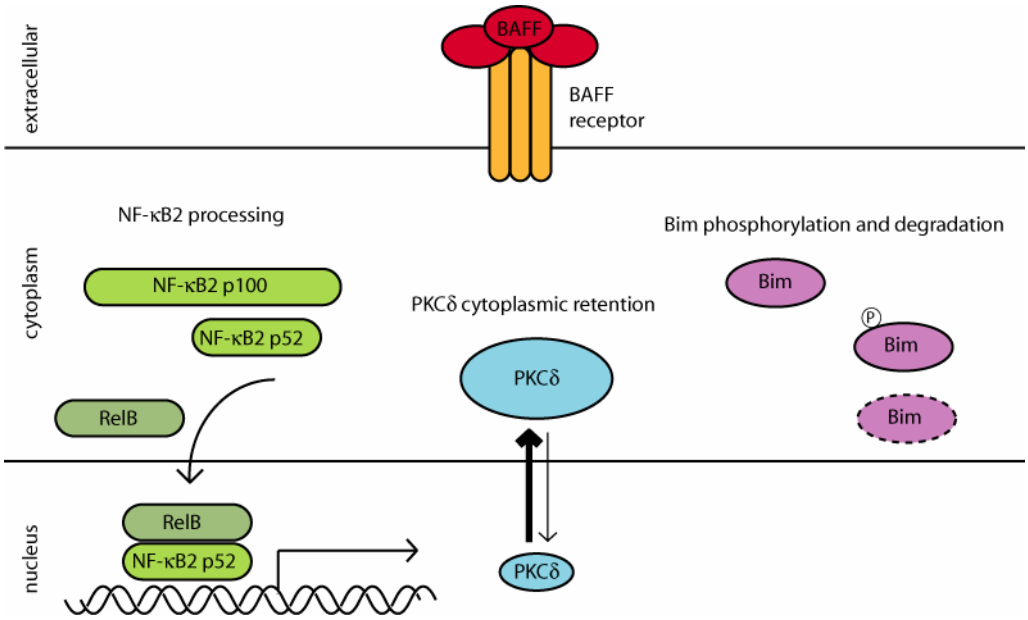


Figure 6: BAFF-induced changes in cellular signaling. BAFF binding to its receptor induces NF-κB activation by enhancing the processing of NF-κB2 p100 to mature p52 which regulates gene transcription in conjunction with RelB. BAFF-mediated cytoplasmic retention of PKCδ is indicated by the symbol and arrow size. Regulation of Bim by BAFF involves its phosphorylation and degradation.

key function for Bim in B cell homeostasis is demonstrated by elevated numbers of B cells and impaired deletion of auto-reactive cells in Bim-deficient mice as well as by the extended survival of Bim-deficient B cells *in vitro* (Bouillet, et al., 1999: ; Enders, et al., 2003). As in the case of PKCδ-deficiency, absence of Bim alleviates the BAFF-dependency of mature B cells for survival (Oliver, et al., 2006). BAFF-treatment also has a direct effect on Bim expression as a consequence of BCR-cross linking which it is able to suppress (Craxton, et al., 2005). This coincides well with the augmentation of BCR-induced proliferation and survival upon co-stimulation with BAFF. Also, BAFF promotes Erk-mediated phosphorylation of Bim which curbs its pro-apoptotic function.

The transcription factor c-Myb has been identified as another regulator of BAFF-mediated B cell survival (Thomas, et al., 2005). Lack of c-Myb reduces the frequency of mature peripheral B cells in mice and c-Myb-deficient B cells are partially refractive to BAFF-mediated responses, possibly due to suboptimal BAFF-R expression. Lack of c-Myb impairs BAFF-induced survival and cytoplasmic retention of PKCδ but not p100 processing. Impaired BAFF-mediated B cell survival and defective BAFF-R expression also occur in the

absence of the GTPases Rac1 and Rac2, suggesting that these proteins may play a role in the regulation of BAFF-signaling (Walmsley, et al., 2003).

BAFF-mediated effects on NF-κB activation, PKCδ localization and Bim expression are observed in a range of 12 to 24 hours after stimulation, raising the question, whether they represent direct BAFF-induced signaling events. Alternatively, diminished p100 processing, nuclear translocation of PKCδ and upregulation of Bim in the absence of BAFF could merely reflect early signs of cellular atrophy which are prevented by BAFF's effect on B cell viability. In either scenario, all the described molecular changes are most likely involved in enhancing B cell survival in the presence of BAFF. Indeed, artificial augmentation of B cell viability by ectopic expression of Bcl-2, Bcl-xL (Bcl2-like 1) or constitutively active NF-κB as well as removal of PKCδ is sufficient to restore peripheral B cell survival in BAFF-R-mutant mice (Amanna, et al., 2003: ; Gorelik, et al., 2004: ; Mecklenbrauker, et al., 2004: ; Rahman and Manser, 2004: ; Sasaki, et al., 2004: ; Sasaki, et al., 2006: ; Tardivel, et al., 2004).

1.4 Lymphocyte survival through growth factor signaling

1.4.1 The association between the trophic state of a cell and its viability

The supply of nutrients from the extracellular environment is indispensable to the viability, growth, division and differentiation of all cell types. At the same time, it is imperative for multicellular organisms to regulate these processes not only based on nutrient availability, but on additional extracellular cues whose concerted action ensures the proper development and function of the organism as a whole. Lymphocytes are a prime example for a cell type whose survival and expansion is, among others, dependent on extracellular nutrients as well as trophic cytokines or growth factors. The latter afford cells the ability to not only ingest nutrients, but to optimize nutrient metabolism for cellular growth and energy derivation. For example, stimulation with trophic cytokines promotes the cell surface expression of glucose and amino acid transporters, thus enabling cellular nutrient uptake (Edinger and Thompson, 2002). At the same time, it influences nutrient metabolism by regulating the activity of enzymes which control rate-limiting steps of glycolysis. Glycolytic glucose utilization is promoted through localization of hexokinase at the mitochondrial

membrane where access to intramitochondrial ATP enhance the enzyme's kinetics, while the activity of phosphofructokinase is sensitive to growth factor-dependent phosphorylation (Bertrand, et al., 1999: ; Deprez, et al., 1997: ; Marsin, et al., 2000: ; Robey and Hay, 2006). Glycolytic glucose-utilization and energy derivation feed back to mitochondrial respiration by supplying critical intermediates of the electron transport chain. Cellular deprivation from growth factors creates a shortage of electron transport substrates, leading to a drop in mitochondrial membrane potential and a subsequent closure of voltage-dependent anion channels, preventing the exchange of metabolites between mitochondria and the cytoplasm (Plas, et al., 2002: ; Plas and Thompson, 2002). Atrophy as a consequence of growth factor withdrawal in lymphoid and hematopoietic cells is characterized by impaired nutrient transporter expression and nutrient uptake, decreased glycolytic rates and a drop in the mitochondrial membrane potential (Barata, et al., 2004: ; Rathmell, et al., 2000: ; Vander Heiden, et al., 2001).

An important consequence of trophic cytokine mediated nutrient uptake is the cells' ability to maintain or increase their size. Cell growth is obviously a prerequisite for proliferative expansion and has to precede cell division unless daughter cells should become progressively smaller. At the same time, cell growth should not proceed beyond a certain checkpoint, unless in preparation for subsequent cell division. Demonstrating the trophic function of growth factors, cells deprived of nutrients or growth factors will become progressively smaller before they collapse (Plas, et al., 2001: ; Rathmell, et al., 2000). While constitutive ectopic expression of anti-apoptotic Bcl-2-family proteins prevents the death of such cells, it does not restore size maintenance, nutrient uptake or energy homeostasis. This indicates that cell survival mediated by Bcl-2-family proteins or growth factors constitute separate non-redundant mechanisms.

1.4.2 Growth factor signaling is transduced by the PI3K-Akt pathway

In contrast to Bcl-2-family members, components of the PI3K pathway are able to avert the decline in cellular metabolism as a consequence of growth factor deprivation, indicating that this pathway plays a vital role in growth factor signaling (Plas, et al., 2001). PI3K can act on lipids as well as proteins, but its best characterized and presumably most important function is the phosphorylation of inositol phospholipids on the D3-position of the inositol ring

to create, depending on the substrate, phosphatidylinositol-3,4-bisphosphate (PIP_2) and phosphatidylinositol-3,4,5-triphosphate (PIP_3) (Fruman, 2004: ; Okkenhaug and Vanhaesebroeck, 2003). The most common PI3K isoform in B cells belongs to the class IA subgroup and is composed of a catalytic subunit p110δ and a regulatory subunit p85α. PI3K-activation occurs by binding of the regulatory subunit's SH2-domain to phosphorylated tyrosine residues in upstream regulatory proteins which result from tyrosine kinase activation by growth factor stimulation. p85-binding of upstream activators induces a conformational change in the protein complex which allows for enzymatic activity of the constitutively associated catalytic subunit. PI3K-mediated PIP_3-accumulation at the plasma membrane creates a docking site for signaling proteins bearing a PH-domain. In terms of growth factor signaling, the most important of these is the oncogenic serine-threonine kinase Akt (figure 7).

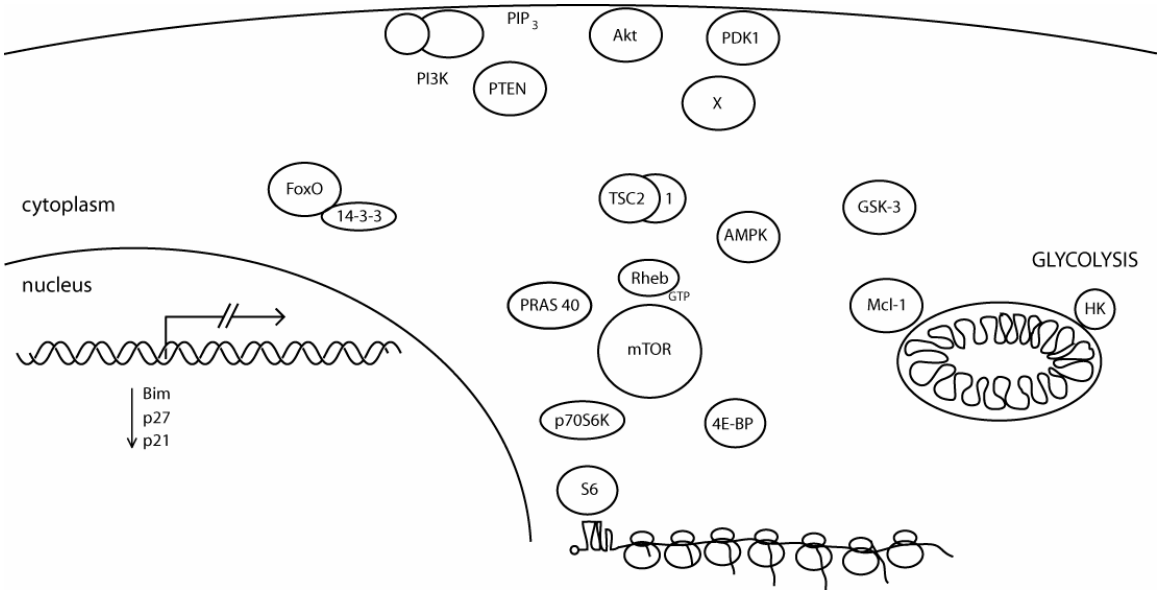


Figure 7: The PI3K-Akt signaling pathway. See text for details.

Akt, sometimes also called protein kinase B (PKB), is known as a master regulator of cell survival, growth and cell cycle progression (Massague, 2004: ; Plas and Thompson, 2005). It directly influences cell viability through down-regulation of Bcl-2-associated death promoter (Bad), a member of the pro-apoptotic BH3-only sub-family of Bcl-2 proteins (Datta, et al., 1997: ; del Peso, et al., 1997). A major executer of Akt's effect on cell survival, cell cycle progression and metabolism are members of the Forkhead box O (FoxO)-

family of transcription factors (Burgering and Kops, 2002). In a process which is evolutionary conserved from *Caenorhabditis elegans* through to mammals, FoxO transcription factors protect cells from oxidative stress and signal cell cycle exit under glucose limiting conditions. Apoptosis and cell cycle exit are promoted by FoxO proteins through upregulation of Bim and the cyclin-dependent kinase (Cdk) inibitors p21 and p27 (Seoane, et al., 2004: ; Stahl, et al., 2002). Collectively, FoxO-proteins antagonize growth factor-induced cellular responses. Accordingly, direct FoxO-phosphorylation by growth factor-activated Akt inhibits FoxO-mediated transcriptional regulation in the nucleus by creating a binding site for 14-3-3 proteins and subsequent sequestration of FoxO-proteins in the cytosol (Brunet, et al., 1999). In addition to this immediate mechanism of FoxO-inactivation, prolonged Akt-activity has the potential to permanently modify the cellular transcriptional program by targeting FoxO-proteins for proteolytic degradation (Plas and Thompson, 2003). In mature B cells, mitogenic stimulation leads to FoxO1-phosphorylation in a PI3K-dependent manner and over-expression of a mutant FoxO-protein which is resistant to Akt-mediated inactivation, causes cell cycle arrest and apoptosis concomitant with an induction of p27 and Bim in activated lymphocytes (Stahl, et al., 2002: ; Yusuf, et al., 2004).

One of the most important targets of Akt-signaling is the mammalian target of rapamycin (mTOR), a kinase of the phosphoinositide-3-kinase-related kinase (PIKK) family which also includes ATM (ataxia telangiectasia mutated), DNA-PK (DNA-dependent protein kinase) and others (Martin and Hall, 2005: ; Wullschleger, et al., 2006). Although mTOR has been a subject of close investigation for many years, its regulation and function appear to be incredibly complex and are still incompletely understood. mTOR exists in complex with accessory proteins raptor (mTORC1) or rictor (mTORC2). The two complexes serve distinct cellular functions and are further distinguished by their differential sensitivity to the immunosuppressant rapamycin (Bhaskar and Hay, 2007). While mTORC2 functions in actin-polymerization and is indifferent to rapamycin treatment, mTORC1 represents the “classic“ mTOR complex which acts in protein synthesis, metabolism and growth in a rapamycin-sensitive fashion. The following description of mTOR function and regulation will apply to mTORC1 unless otherwise stated. Under growth-favorable conditions, mTOR has a direct impact on protein synthesis through phosphorylation of ribosomal S6 kinase 1 (S6K1) and 4E binding protein 1 (4E-BP1) (Ruggero and Sonenberg, 2005). S6K1-mediated phosphorylation of ribosomal S6 protein is considered a hallmark of active protein synthesis,

whereas 4E-BP1 is an inhibitor of translational initiation through its sequestration of eukaryotic translation initiation factor 4E (eIF4E). Phosphorylation of 4E-BP1 disrupts its association with eIF4E, thus enabling the latter's participation in the translation initiation complex. mTOR-activity also contributes to cell growth by promoting ribosome biogenesis as well as the expression of many metabolically relevant genes (Martin and Hall, 2005: ; Peng, et al., 2002). Furthermore, mTOR negatively impacts protein degradation via autophagy (Lum, et al., 2005). Finally, organization of the cytoskeleton which is important in growth related events including establishment of cell polarity and cell division is regulated by mTORC2 (Jacinto, et al., 2004: ; Sarbassov, et al., 2004).

Given that the mTOR-dependent cellular events are overwhelmingly energy-consuming, it is probably as appropriate as it is necessary that mTOR functions as an integrator for a multitude of signaling inputs including growth factors, nutrients, energy and stress, all of which can impact mTOR-activity. The metabolic function of mTOR is dependent on the small G-protein Rheb (Ras homolog enriched in brain) which is subject to inactivation by the GTPase activating protein (GAP) Tuberous sclerosis 2 (TSC2) (Tee and Blenis, 2005). Mutations in the TSC2 gene are the leading cause for the disease Tuberous sclerosis, a hereditary condition characterized by the frequent appearance of benign tumors in multiple organs. Very recently, PRAS40 (proline-rich Akt substrate 40kD) has been identified as another major mTOR inhibitor (Sancak, et al., 2007: ; Vander Haar, et al., 2007). Under conditions unfavorable for mTOR-activity and growth, PRAS40 binds to mTOR and prevents its activation. One important characteristic of unfavorable growth conditions is a low ATP to AMP ratio which is directly sensed by the AMP-activated protein kinase (AMPK) (Hardie, 2005). AMPK-activity has a dual negative effect on mTOR-signaling: it enhances TSC2's GAP-activity through direct phosphorylation, thus constraining mTOR activity, while at the same time inhibiting activation of the mTOR targets 4E-BP1 and S6K. Growth factor-induced activation of the PI3K-Akt pathway has a profound effect on mTOR activity at multiple levels. First, Akt phosphorylates and inactivates TSC2, thus enabling Rheb·GTP-dependent mTOR activity. As has been discovered very recently, Akt also alleviates PRAS40-dependent suppression of mTOR activity (Kovacina, et al., 2003: ; Sancak, et al., 2007: ; Vander Haar, et al., 2007). Through a mechanism reminiscent of Akt-mediated FoxO-inactivation, phosphorylation of PRAS40 by Akt enhances its binding to 14-3-3 proteins, thus sequestering it away from mTOR. In addition to these two direct

mechanisms, Akt can relieve AMPK-mediated suppression of mTOR-activity through its effect on cellular energy homeostasis. Akt promotes the localization of hexokinase at the mitochondrial membrane which contributes to cellular energy supply as described above. mTOR activity is also sensitive to the cellular concentration of amino acids, in particular leucine, although the mechanistic basis for this phenomenon is still unresolved (Martin and Hall, 2005).

Another well-characterized target of Akt is glycogen synthase kinase 3 (GSK-3). In addition to its metabolic role in inactivating glycogen synthase, GSK-3 impacts cell cycle progression by regulating the protein stability of myc and cyclins D and E (Diehl, et al., 1998: ; Embi, et al., 1980: ; Sears, et al., 2000: ; Welcker, et al., 2003). Furthermore, GSK-3-dependent destabilization of the pro-survival Bcl-2 family protein Mcl-1 (myeloid cell leukemia sequence 1) compromises mitochondrial integrity, thus creating a direct link between GSK-3 activity and apoptosis (Maurer, et al., 2006). GSK-3-mediated inhibition of the transcription factor NFAT could be of particular importance in lymphocytes (Neal and Clipstone, 2001). GSK-3 is inactivated by direct phosphorylation by Akt (Cross, et al., 1995).

Growth factor-mediated Akt-activation occurs in a two-step mechanism. One prerequisite already mentioned is the binding of Akt's PH-domain to PIP_3, the product of PI3K, at the plasma membrane. Subsequent Akt-phosphorylation at T308 and S473 is essential to its catalytic activity. T308 is part of the activation loop within the kinase domain and is the target for 3-phosphoinositide-dependent kinase 1 (PDK1), whereas S473 resides in the hydrophobic motif C-terminal of the kinase domain and, most likely, more than one kinase mediates its phosphorylation in different contexts or cell types (Fayard, et al., 2005: ; Yang, et al., 2004). S473 phosphorylation of Akt stabilizes the active conformation and allows it to move from the plasma membrane to the cytosol and nucleus following activation at the plasma membrane (Andjelkovic, et al., 1999: ; Yang, et al., 2002).

Three Akt-isoforms exist in mammals whose functions appear to be at least partially non-redundant based on the phenotypes of individual knock-out mice. Lack of the ubiquitously expressed Akt1 increases neonatal lethality, retards embryonic growth and elevates susceptibility to apoptosis in several cell types (Chen, et al., 2001: ; Cho, et al., 2001: ; Yang, et al., 2003). While Akt1-deficiency does not impair glucose homeostasis, absence of Akt2 causes insulin resistance and a type-II diabetes like disease in addition to growth

retardation (Cho, et al., 2001: ; Garofalo, et al., 2003). This corresponds to the elevated expression level of Akt2 in insulin-responsive tissues such as liver and muscle. Combined deletion of Akt1 and Akt2 aggravates the neonatal lethality and growth retardation in addition to impairing skin, bone and muscle development (Peng, et al., 2003). By contrast, Akt3 is predominantly expressed in neuronal tissue and is required for normal size and cellularity of the brain (Easton, et al., 2005: ; Tschopp, et al., 2005). The phenotype of the B cell compartment in the absence of one or more Akt-isoforms has not been reported. However, an important role for the PI3K-Akt pathway in B cell biology can be inferred from the phenotype of p85α and p110δ-mutant mice which display impaired B lymphopoiesis in the bone marrow as well as a reduction in splenic B cell numbers (Clayton, et al., 2002: ; Fruman, et al., 1999: ; Jou, et al., 2002: ; Okkenhaug, et al., 2002: ; Suzuki, et al., 1999)

1.4.3 Association between the PI3K pathway and cell transformation

The cellular response to growth factor stimulation is strikingly reminiscent of the signaling and metabolic signature of transformed cells. As noted by Otto Warburg more than 80 years ago, glucose utilization in tumor cells leads to an increased aerobic glycolysis rate (Warburg, 1956: ; Warburg, et al., 1924) similar to the effect of growth factor stimulation on non-transformed cells. In fact, a characteristic feature of many malignancies is the ability to strive in the absence of growth factors. One way to achieve this growth factor independence is the constitutive activation of the PI3K-Akt pathway in the absence of an external signal. Indeed, Akt was first identified as a cellular proto-oncogene to the viral fusion protein v-Akt which encodes a constitutively membrane-targeted and active form (Bellacosa, et al., 1991). Amplification and overexpression of PI3K p110 or Akt occur in a subset of human cancers (Hennessy, et al., 2005). Moreover, one of the most important tumor suppressors, PTEN (phosphatase and tensin homolog deleted from chromosome 10), plays a vital role in Akt-regulation. PTEN is a 3-phosphoinositide phosphatase which antagonizes the function of PI3K (Maehama and Dixon, 1998). In this manner, it ensures that the presence of PIP_3 at the plasma membrane and subsequent Akt-activation is a transient event following growth factor stimulation (Stambolic, et al., 1998). Loss of PTEN is a very common feature of many human cancers (Sansal and Sellers, 2004). In mice, PTEN-haploinsufficiency in heterozygotes causes the

development of a wide range of tumors, which can be prevented by concomitant loss of Akt1 (Chen, et al., 2006: ; Di Cristofano, et al., 1998: ; Podsypanina, et al., 1999: ; Suzuki, et al., 1998). With regard to the lymphoid compartment, PTEN heterozygous mice develop a fatal autoimmune disorder characterized by lymphocyte hyper-proliferation and insensitivity to apoptosis (Di Cristofano, et al., 1999). A lethal lymphoproliferative disease also ensues from PTEN-inactivation targeted solely to the T cell compartment (Suzuki, et al., 2001).

In addition to the PI3K-Akt pathway, trophic signaling utilizes a second oncogenic kinase Pim-2. Expression of Pim-2 is induced by growth factor stimulation and, in analogy to constitutively active Akt, ectopic Pim-2 expression can compensate for growth factor deprivation in terms of cell survival and energy homeostasis (Fox, et al., 2003: ; Hammerman, et al., 2005). One molecular target of Pim-2 activity appears to be 4E-BP1, indicating a dual and potentially redundant mechanism of protein synthesis stimulation by growth factors.

1.5 The aims of this study

The broad aim of this study was to elucidate the mechanisms of mature B cell survival through two distinct pathways: signaling by the cytokine BAFF and tonic BCR signaling with an emphasis on the role of the protein tyrosine kinase Syk in this process. The results presented here characterize BAFF as a trophic growth factor for mature B cells which boosts B cell viability, growth and metabolism through activation of the PI3K-Akt pathway and Pim-2. Furthermore, the present study demonstrates an indispensable role for Syk in BCR signaling throughout B lymphopoiesis, including an obligatory function in tonic BCR survival signaling in mature resting B cells. Within the scope of this project, a novel method of Syk protein inactivation was investigated which shows potential for inducible kinase inactivation.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment and consumables

- Semidry blotters TE 77 and TE 70, Amersham
- Trans-Blot Cell tank transfer system, BIO-RAD
- miniVE Vertical Electrophoresis System, Amersham
- EPS 2A200 Power Supply, Amersham
- UV Transilluminator, Spectroline
- DNA-Engine Peltier Thermal Cycler, BIO-RAD
- Gel-Dryer and Vacuum Pump, BIO-RAD
- ProBlot Hybridization oven, Labnet
- BioPhotometer spectrophotometer, Eppendorf
- Image Master Video Documentation System, Pharmacia
- UV Stratalinker, Stratagene
- Bench-top refridgerated centrifuge, Eppendorf
- Microcentrifuge, Eppendorf
- Sorvall RC 5C Plus Superspeed centrifuge, Sorvall
- Optima L-90K Ultracentrifuge, Beckman Coulter
- UA-6 UV-detector, ISCO
- FACSCalibur, Becton Dickinson
- BD LSR flow cytometer
- BD FACSort flow cytometer
- ÄktaPrime, Amersham
- Innova incubator shaker, New Brunswick Scientific
- Heracell CO_2-incubator, Heraeus
- SterilGARD III Advance Biological Safety Cabinet, The Baker Company
- EdgeGARD Laminar-Flow Clean Bench, The Baker Company
- Nikon Eclipse microscope, Nikon
- Horizontal Electrophoresis System, C.B.S. Scientific
- Microwave, GE
- Waterbath, Precision
- Heat blocks, VWR
- Kodak X-OMAT 2000A Processor, Kodak

- Milli-Q Ultrapure Water Purification System, Millipore
- Orbitron Rotator II, Boekel Scientific
- Shaker, IKA Labortechnik
- Roto-Torque rotator, Cole-Parmer
- Sonifier 450 sonicator, Branson
- Vortex Genie 2, Fisher
- VERSAmax microplate reader, Molecular Devices

Chemicals were purchased from Sigma, Fisher or Fluka unless otherwise stated.

Plastic ware was purchased from Falcon, Corning or Sarstedt unless otherwise stated.

2.1.2 Standard buffers

10 x phosphate-buffered saline (PBS)

For 1l, dissolve 80g NaCl, 2g KCl, 11.5g Na_2HPO_4, 2g KH_2PO_4; pH to 7.2 for 1 x PBS

Hanks Balanced Salt Solution (BSS)

Prepared from 20 x stock solutions:

20 x BSS Stock 1: 20.0 g/l glucose, 1.2 g/l KH_2PO_4, 3.8 g/l Na_2HPO_4, 40ml/l 0.5% phenol red

20 x BSS Stock 2: 3.8 g/l $CaCl_2 \cdot 2H_2O$, 4.0 g/l KCl, 80 g/l NaCl, 4 g/l $MgCl_2 \cdot 6H_2O$, 1.0 g/l $MgSO_4$

TE

10 mM Tris-HCl pH 8.0, 1 mM EDTA

4 x DNA sample buffer for agarose gel electrophoresis

50% Tris/EDTA pH 8.0, 50% glycerol, orange G

50 x TAE

For 1l, dissolve 242g Tris-base, 57 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0

20 x SSC

For 1l, dissolve 175.25g NaCl, 88.25g Na Citrate; pH to 7.0

5 x Protein Sample Buffer for SDS-PAGE

250 mM Tris-HCl pH 6.8, 3.5 M β-mercaptoethanol, 10% SDS, 0.5% Bromphenolblue, 50% glycerol

10 x SDS-PAGE Running Buffer

For 1l, dissolve 30g Tris-base, 144g glycine, 5g SDS

10 x TBS

For 1l, dissolve 24.2g Tris-base, 80g NaCl; pH to 7.6. 1 x TBS containing 0.1% Tween-20 (TBS-T) was used for Western blotting.

2.1.3 Oligonucleotides

Oligonucleotides were purchased lyophilized from commercial vendors (Invitrogen or Fisher), reconstituted in ddH_2O to a 200 µM concentration and stored at -20ºC.

Table 1: Oligonucleotides used for mouse genotyping

Primer name	**Sequence**
Syk F	5’ - GCC CGT TCT GTG CCT ACT GG - 3’
Syk R	5’ - GCT GGT TCC CTT TTC CTT CC - 3’
Syk R2	5’ - TAG CTA ACC AAA CCC ACG GC - 3’
Mx Cre F	5’ - CAT GTG TCT TGG TGG GCT GAG - 3’
Mx Cre R	5’ - CGC ATA ACC AGT GAA ACA GCA T- 3’
CD19-Cre 1	5’ - AAC CAG TCA ACA CCC TTC C - 3’
CD19-Cre 2	5’ - CCA GAC TAG ATA CAG ACC AG- 3’
CD19-Cre 3	5’ - TCA GCT ACA CCA GAG ACG G- 3’

Table 2: PCR-programs used for mouse genotyping (WT = wild-type, fl = floxed, Δ = deleted)

Program	Annealing temperature	Annealing time (sec)	Extension time (sec)	Cycles	Expected product size
Syk 1	58°C	60	120	31	WT 1186 bp; fl 1428 bp; Δ 320 bp
Syk 2	58°C	30	40	29	WT 234 bp; fl 349 bp; Δ no band
Mx-Cre	58°C	60	120	35	WT no band; Mx-Cre 596 bp
CD19-Cre	59.5°C	60	120	33	WT 450 bp; Cre 715 bp

2.1.4 Antibodies

Table 3: Staining antibodies for flow cytometry

Antibody name	Company and catalogue number	Dilution
rat anti-mouse CD21/35-FITC clone 7G6	BD Pharmingen #553818	1:100
rat anti-mouse CD45R/B220-cychrome clone RA3-6B2	BD Pharmingen #553091	1:100
rat anti-mouse CD45R/B220-biotin clone RA3-6B2	BD Pharmingen #553086	1:100
rat anti-mouse CD43-FITC clone S7	BD Pharmingen #553270	1:100
rat anti-mouse CD25-biotin clone 7D4	BD Pharmingen #553070	1:100
rat anti-mouse CD90.2-PE clone 53-2.1	BD Pharmingen #553006	1:400
rat anti-mouse CD5-PE clone 53-7.3	BD Pharmingen #553023	1:100
rat anti-mouse CD19-FITC clone 1D3	BD Pharmingen #553785	1:100
rat anti-mouse IgD-FITC clone 11-26c.2a	BD Pharmingen #553439	1:100
Streptavidin-Phycoerythrin-Cy7	BD Pharmingen #557598	1:400
F(ab')2 fragment goat anti-mouse IgM-PE µ chain-specific	Jackson ImmunoResearch #115-116-075	1:100
F(ab')2 fragment goat anti-mouse IgM-FITC µ chain-specific	Jackson ImmunoResearch #115-097-020	1:200
Goat anti-mouse BAFF-R-biotin	R&D Systems #BAF1357	1:20

Table 4: Antibodies for Western Blotting; all antibodies were diluted in TBS-T containing 3% non-fat dry milk or 5% bovine serum albumin (BSA)

Antibody name	Company and catalogue number	Dilution
rabbit polyclonal anti-phospho-S6 ribosomal protein (S235/236)	Cell Signaling #2211	1:1000
rabbit polyclonal anti-S6 ribosomal protein	Cell Signaling #2212	1:1000
rabbit polyclonal anti-phospho-eIF4E (S209)	Cell Signaling #9741	1:1000
rabbit polyclonal anti-eIF4E	Cell Signaling #9742	1:1000
rabbit polyclonal anti-phospho-4E-BP1 (S65)	Cell Signaling #9451	1:1000
rabbit polyclonal anti-4E-BP1	Cell Signaling #9452	1:1000
rabbit polyclonal anti-phospho-Akt (S473)	Cell Signaling #9271	1:1000
rabbit monoclonal anti-phospho-Akt (T308) clone 244F9	Cell Signaling #4056	1:1000
rabbit polyclonal anti-phospho-TSC2 (S1462)	Cell Signaling #3611	1:1000
rabbit polyclonal anti-phospho-FoxO1 (S256)	Cell Signaling #9461	1:1000
rabbit polyclonal anti-FoxO1	Cell Signaling #9462	1:1000
rabbit polyclonal anti-phospho-GSK-3ß (S9)	Cell Signaling #9336	1:1000
rabbit polyclonal anti-p44/42 MAP Kinase (Erk)	Cell Signaling #9102	1:1000
rabbit polyclonal anti-phospho-PTEN (S380)	Cell Signaling #9551	1:1000
rabbit polyclonal anti-PTEN	Cell Signaling #9552	1:1000
rabbit polyclonal anti-phospho-PDK1 (S241)	Cell Signaling #3061	1:1000
rabbit polyclonal anti-PDK1	Cell Signaling #3062	1:1000
mouse monoclonal anti-Pim-2 clone 1D12	Santa Cruz #sc-13514	1:200
goat polyclonal anti-Akt1/2	Santa Cruz #sc-1619	1:1000
rabbit polyclonal anti-Tuberin (TSC2)	Santa Cruz #sc-893	1:500
rabbit polyclonal anti-PKCßII	Santa Cruz #sc-210	1:1000
rabbit polyclonal anti-PKCδ	Santa Cruz #sc-937	1:1000
goat polyclonal anti-Lamin B	Santa Cruz #sc-6216	1:1000
rabbit polyclonal anti-cyclin D2	Santa Cruz #sc-593	1:1000
rabbit polyclonal anti-cyclin E	Santa Cruz #sc-198	1:1000
rabbit polyclonal anti-Cdk4	Santa Cruz #sc-260	1:1000
goat polyclonal anti-Mcm3	Santa Cruz #sc-9849	1:1000
rabbit polyclonal anti-Mcm2	BD Pharmingen #559542	1:1000
rabbit polyclonal anti-Syk	Santa Cruz #sc-1077	1:1000
mouse monoclonal anti-phospho-tyrosine	Upstate #05-321	1:1000

clone 4G10		
rabbit polyclonal anti-GSK-3ß	Stressgen #KAP-ST002C	1:1000
rabbit polyclonal anti-p85a	Upstate #06-497	1:1000
mouse monoclonal anti-p110d clone AW103	Upstate #05-703	1:500
rabbit polyclonal anti-p52	Upstate #06-413	1:1000
rabbit polyclonal anti-Mcm2	BD Pharmingen #559542	1:1000
mouse monoclonal anti-Rb clone XZ55	BD Pharmingen #554144	1:1000
rat monoclonal anti-Ki67 clone TEC-3	Dako #M7249	1:1000
rabbit polyclonal anti-survivin	Chemicon #AB3611	1:500
rat monoclonal anti-Bim clone 14A8	Chemicon #MAB17001	1:500
mouse monoclonal anti-α-Tubulin clone DM1A	Sigma #T6199	1:2000
mouse monoclonal anti-FLAG clone M1	Sigma #F3040	1:1000
rabbit polyclonal anti-Lyn	gift from Cliff Lowell	1:1000
donkey anti-rabbit IgG HRP-linked	Amersham #NA934	1:10000
sheep anti-mouse IgG HRP-linked	Amersham #NA931	1:10000
goat anti-rat IgG HRP-linked	Jackson ImmunoResearch #112-035-167	1:10000
rabbit anti-goat IgG HRP-linked	Sigma #A5420	1:10000

2.2 Methods

2.2.1 Molecular Biology

2.2.1.1 Bacterial transformation

Heat shock competent *Escherichia coli* (*E. Coli*) DH5α were mixed with approximately 10 ng of plasmid DNA or 1µl of a ligation reaction, incubated for 15 min on ice and heat-shocked for 90 sec in a 42ºC water bath. After cooling on ice for 2 min and addition of 1 ml LB medium, the transformation reaction was incubated for 30 min at 37ºC under agitation and plated on LB agar containing appropriate selection antibiotics. Single colonies were picked after 12 to 16 hrs of incubation at 37ºC.

2.2.1.2 Plasmid DNA purification

For Mini-Preps, bacteria from a single colony were grown under agitation for 12 to 16 hrs in 2 ml LB medium containing the appropriate selection antibiotic. Larger culture volumes (50 ml to 100 ml) were used for Midi and Maxi-Preps. Plasmid DNA was purified using Qiagen Kits according to the manufacturer's protocols.

2.2.1.3 Restriction digests

DNA was digested with restriction enzymes from New England Biolabs according to the manufacturer's protocols.

2.2.1.4 DNA sequencing

DNA sequencing was performed by commercial vendors (Genewiz) and the DNA sequencing core facility at The Rockefeller University.

2.2.1.5 Agarose gel electrophoresis

DNA was separated on 0.5% to 2% agarose gels in TAE running buffer.

2.2.1.6 Labeling of hybridization probes

Gel-purified restriction fragments or PCR-products were radioactively labeled using 25 – 50 ng DNA, 50 µCi ^{32}P α-dCTP (Amersham) and Ready-To-Go

DNA Labeling beads (-dCTP) (Amersham) and purified using ProbeQuant G-50 micro columns (Amersham) according to the manufacturer's instructions.

2.2.1.7 Southern blot

Approximately 10 µg of genomic DNA was digested for 12 to 16 hrs with the appropriate restriction enzyme and resolved on a 0.6% agarose gel. The gel was equilibrated in transfer buffer (0.4 M NaOH, 0.6 M NaCl) for 20 min and DNA was transferred onto a nitrocellulose membrane (Hybond N+, Amersham) by capillary transfer. Following equilibration in neutralization buffer (0.5 M Tris-HCl pH 7.0, 1M NaCl), DNA was fixed to the membrane by UV cross-linking (UV Stratalinker, Stratagene) and the membrane was blocked in hybridization buffer (50 mM Tris-HCl pH 7.5, 1 M NaCl, 1% SDS, 10% dextran sulfate, 300 µg/ml sonicated salmon sperm DNA) for 4 hrs at 65ºC. The incubation was continued over night following the addition of the radioactively labeled DNA probe. The membrane was washed several times in 0.5 – 2 x SSC, 0.1 – 1% SDS and exposed for autoradiography.

2.2.1.8 Genomic DNA purification

Mouse tail tips were digested over night at 55ºC in 500 µl tail lysis buffer containing 10 mM Tris-HCl pH 8.5, 200 mM NaCl, 5 mM EDTA, 0.2% SDS and 100 µg/ml Proteinase K (Roche). Debris was removed by centrifugation at 15,000 x g for 10 min and DNA was precipitated from the supernatant by addition of 1 ml 100% EtOH. The precipitate was pelleted through centrifugation at 15,000 x g for 1 min, washed in 70% EtOH, air-dried and dissolved in 200 µl TE buffer.

2.2.1.9 Polymerase chain reaction (PCR)

PCR was used routinely for genotyping of mouse mutant strains. 1 µl of genomic tail DNA was used as template in a 20 µl PCR reaction containing 2 µl 10 x Klentherm PCR buffer (670 mM Tris-HCl pH 9.1, 160 mM $(NH_3)_2SO_4$, 35 mM $MgCl_2$, 1.5 mg/ml BSA), 0.2 µl 25 mM dNTPs (Amersham), 0.5 µl 20 µM Primer Mix and 1 U Taq DNA polymerase (Sigma). Primer sequences and PCR programs are listed in tables 1 and 2. PCR products were resolved on 1 – 2% agarose gels.

For cloning purposes, other DNA polymerase including Pfu (Stratagene) and

KOD (Novagen) were used according to the manufacturers' protocols.

2.2.1.10 RNA isolation

Total RNA was isolated from 1 x 10^5 – 10^6 B cells using Trizol (Gibco) according to the manufacturer's protocol.

2.2.1.11 Reverse transcriptase (RT)-PCR and cloning of the mouse Syk cDNA

First strand cDNA synthesis was performed on 100 ng of total RNA using oligo (dT) priming and Superscript II (Invitrogen) according to the manufacturer's instructions. Full-length Syk cDNA was amplified from the first-strand reaction using the Expand Long Template PCR System (Roche) and primers 5'-agaactctgaaggggtgc-3' (forward) and 5'-cttgggaatccgtggtgt-3' (reverse). The PCR-product was gel-purified (QIAquick Gel Extraction Kit, Qiagen) and cloned into the pCR2.1 vector using TA cloning (TA Cloning Kit, Invitrogen). Insert-containing colonies were identified by blue-white selection and sequenced. Point-mutations were repaired using the QuickChange XL Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions.

2.2.2 Cellular Methods

2.2.2.1 Preparation of murine lymphocyte suspensions

Mice were sacrificed on dry ice (CO_2). Spleen, thymus and lymph nodes were removed and single cell suspensions were made in BSS by pressing individual organs through a wire mesh. Bone marrow cells were isolated by flushing femur, tibia and ilium of both hind legs with BSS. Peritoneal cavity cells were collected by rinsing the peritoneum with 10 ml BSS. Where necessary, erythrocytes were lyzed by incubating cells for 1 min in ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Live cells numbers were determined using Trypan Blue (Gibco) exclusion in a Neubauer counting chamber.

2.2.2.2 Lymphocyte purification by Magnetic Cell Sorting (MACS)

Mature B lymphocytes were purified from murine spleen and lymph nodes by MACS in a two-step protocol. First, non-B cells were depleted using anti-CD43 antibody coupled magnetic beads. Mature B cells were further purified by enrichment of CD62L-positive cells. Cells were kept in BSS throughout the purification procedure. Antibody-coupled magnetic beads (Miltenyi) were used in a 1 in 10 dilution and MACS LD and LS columns (Miltenyi) were used. Purification purity was controlled by cell surface staining and FACS (fluorescence-activated cell sorting) analysis and was routinely above 95%.

2.2.2.3 Cell surface staining and flow cytometry

For surface staining, 5 x 10^5 to 1 x 10^6 cells were washed once in PBS/BSA (1 x PBS, 0.5% BSA, 0.02% NaN_3) and resuspended in 10 µl of the appropriate antibody dilution in PBS/BSA in a 96-well U-bottom plate. After 20 min of incubation at 4 ºC, cells were washed in PBS/BSA and, where necessary, stained with secondary antibody. Staining antibodies and dilutions are listed in table 3. Cells were analyzed on a flow cytometer (FACSCalibur, BD) using CellQuest (BD) software. Dead cells were excluded from the analysis through addition of 10 nM TO-PRO-3 (Invitrogen) prior to acquisition and subsequent gating on the TO-PRO-3^- cell population.

2.2.2.4 Primary B cell culture

Purified B cells were cultured in RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS, HyClone), 2 mM L-glutamine, 50 µM β-mercaptoethanol and 100 U/ml penicillin/streptomycin (all Gibco) at 37ºC in a humidified 7.5% CO_2-incubator. For analysis of BAFF-induced signaling, cells were pre-incubated in medium plus the above supplements for 12 hrs to reduce the background caused by exposure to endogenous BAFF *in vivo* prior to the cell preparation (after (Claudio, et al., 2002). Cells were stimulated with 25 ng/ml recombinant mouse BAFF (R&D Systems), 1.3 µg/ml $F(ab')_2$ fragment goat anti-mouse IgM (Jackson ImmunoResearch Laboratories) and 5 µg/ml lipopolysaccharide (LPS, Sigma). LY294002 (Calbiochem) was used at a 10 µM concentration.

2.2.2.5 Analysis of B cell proliferation using CFSE labeling

5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Molecular Probes) is a colorless, cell-permeable compound which is processed to fluorescent carboxyfluorescein succinimidyl ester (CFSE) inside the cell. The latter is highly reactive and forms protein adducts which are retained inside the cell and split equally onto daughter cells following division such that cell division can be tracked by FACS analysis as a sequential decline in fluorescence. For CFSE labeling, purified B cells were washed extensively in plain RPMI and incubated with 5 µM CFSE for 10 min at 37ºC at a concentration of 1 x 10^7 cells per 1 ml. Labeling was stopped by addition of complete B cell culture medium. Cells were washed twice in complete medium and plated with the indicated stimuli for 3 days. At the time of analysis, cells were surface-stained for the pan-B cell marker B220 and analyzed by flow-cytometry. CFSE-dilution was monitored on live $B220^+$ cells.

2.2.2.6 Analysis of B cell proliferation using bromodeoxyuridine (BrdU) labeling

Purified B lymphocytes were cultured in the presence of 10 µM BrdU (BD Biosciences). Cells were fixed and stained using the FITC BrdU Flow Kit (BD Biosciences) and analyzed by flow cytometry.

2.2.2.7 Measurement of mitochondrial membrane potential

Purified B cells were cultured with 100 nM of the potentiometric dye tetramethylrhodamine ethyl ester (TMRE, Invitrogen). 50 µM carbonyl cyanide m-chlorophenyl hydrazone (CCCP, Sigma-Aldrich) was added simultaneously to control samples as an uncoupler of the mitochondrial respiratory chain. Following 30 min of incubation at 37 ºC in a CO_2-incubator, cells were analyzed by flow cytometry.

2.2.2.8 Metabolic labeling of primary B cells

Purified B cells were cultured in labeling medium composed of methionine- and cysteine-free RPMI 1640 (Sigma), 10% FBS (Hyclone, dialyzed over night in PBS), 2 mM L-glutamine, 50 µM β-mercaptoethanol, 100 U/ml penicillin/streptomycin (all Gibco) and 500 µCi/ml Trans ^{35}S-label (ICN). Labeling was continued for 6 hrs at 37ºC in a humidified 7.5% CO_2-incubator,

after which cells were washed extensively first in normal complete medium not containing radioactivity and then in PBS prior to lysis in a buffer containing 350 mM NaCl, 20 mM HEPES/NaOH pH 7.9, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM EGTA, 1% NP-40, 20% glycerol, 10 µM NaF, 2 mM Na_3VO_4 and protease inhibitor cocktail (Sigma). Cell extracts were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. The gel was treated with EN3HANCE (PerkinElmer) according to the manufacturer's instructions, dried and exposed for autoradiography.

2.2.2.9 Cell line culture

DT40 cells were cultured in RPMI 1640 (Gibco) containing 10% FBS (HyClone), 2 mM L-glutamine, 50 µM β-mercaptoethanol, 100 U/ml penicillin/streptomycin (all Gibco) and 1% chicken serum (Sigma). Wild-type and Syk-deficient (Takata, et al., 1994) lines were a kind gift of Ed Clark. 2 µg/ml of mouse anti-chicken IgM antibody (clone M-4, Southern Biotechnology) was used for stimulation.

293GP cells (Clontech) and HEK 293 (ATCC) were maintained in DMEM (Gibco) containing 10% FBS (HyClone), 1 mM sodium pyruvate and 100 U/ml penicillin/streptomycin (both Gibco).

2.2.2.10 Calcium-phosphate mediated transient transfection of HEK 293 cells

HEK 293 cells were transfected with mammalian expression vectors for various Syk-constructs and TEV-protease. All inserts were cloned into pcDNA3.1 (Invitrogen) using standard molecular biology procedures. In brief, the full-length Syk cDNA was PCR amplified from the original cloning vector pCR2.1 (see 2.2.1.11) using primers 5'-cgggatcccagacatggcgggaagtgctgtg-3' (forward) and 5'-cggaattccgttagaggtcttcctcagaaatcaacttctgctcgccggagccgttaac cacgtcgtagtag-3' (reverse) with the reverse primer containing a C-terminal Myc-tag. The PCR-product was cloned into pcDNA3.1 using BamHI and EcoRI restriction sites. Syk mutants X1 through X6 were generated from the wild-type pcDNA3.1-Syk template by site directed mutagenesis using the Quickchange XL Site-directed mutagenesis kit (Stratagene) and the following mutagenesis primers:

X1 forward: 5'-gtggctgtgaaaatcctgaagaacgaggccggcagcggcgagaacctgtacttcca

gtccgcgggcagcaacgacccggctttgaaggacgagctgctg-3’

X1 reverse: 5’-cagcagctcgtccttcaaagccgggtcgttgctgcccgcggactggaagtacaggttc tcgccgctgccggcctcgttcttcaggattttcacagccac-3’

X2 forward: 5’-tcctggatgctggtgatggagatggcggagggcagcggcgagaacctgtacttccag tccgcgggcagcctggggccgctcaacaagtacctgcagcag-3’

X2 reverse: 5’-ctgctgcaggtacttgttgagcggccccaggctgcccgcggactggaagtacaggttc tcgccgctgccctccgccatctccatcaccagcatccagga-3’

X3 forward: 5’-agcgaagtgaccgccatgctggagaaaggaggcagcggcgagaacctgtacttcc agtccgcgggcagcgagcggatggggtgccctgcaggatgcccg-3’

X3 reverse: 5’-cgggcatcctgcagggcaccccatccgctcgctgcccgcggactggaagtacaggtt ctcgccgctgcctcctttctccagcatggcggtcacttcgct-3’

X4 forward: 5’-gatttcggtctttccaaagccctgcgtgctggcagcggcgagaacctgtacttccagtc cgcgggcagcgatgaaaactactacaaggcccagacccac-3’

X4 reverse: 5’-gtgggtctgggccttgtagtagttttcatcgctgcccgcggactggaagtacaggttctc gccgctgccagcacgcagggctttggaaagaccgaaatc-3’

X5 forward: 5’-gcgttctcctatgggcagaagccctacagaggcagcggcgagaacctgtacttccag tccgcgggcagcgggatgaaagggagcgaagtgaccgccatg-3’

X5 reverse: 5’-catggcggtcacttcgctcccctttcatcccgctgcccgcggactggaagtacaggttctc gccgctgcctctgtagggcttctgcccataggagaacgc-3’

X6 forward: 5’-ctcaacaagtacctgcagcagaacaggcacggcagcggcgagaacctgtacttcc agtccgcgggcagcattaaggataagaacatcatagagctggtt-3’

X6 reverse: 5’-aaccagctctatgatgttcttatccttaatgctgcccgcggactggaagtacaggttctcg ccgctgccgtgcctgttctgctgcaggtacttgttgag-3’

Correct cloning and successful mutagenesis was confirmed by sequencing in all cases.

For the TEV-protease expression construct, TEV was PCR-amplified from the TEV-containing vector pGEX2T-27k (kind gift of Michael Ehrmann) using primers 5’-cgcggatccctgtttaagggaccacg-3’ (forward) and 5’-ccggaattccgagtacaccaattcattc-3’ (reverse) and cloned in frame into a previously constructed pcDNA3.1 plasmid containing an N-terminal triple FLAG-tag using BamHI and EcoRI restriction sites. Correct cloning was confirmed by sequencing.

For transient expression of the above DNA constructs in HEK 293 cells, a total of 12 µg DNA in a 150 µl volume was mixed with an equal volume of 0.5

M $CaCl_2$ and added drop wise under vortexing to a 2 x HEPES-buffered saline solution (HBS) containing 0.28 M NaCl, 0.05 M HEPES and 1.5 mM Na_2HPO_4. The mixture was incubated for 30 min at room temperature and spread onto cells plated in a 10 cm dish 24 hrs previously. Cells were analyzed 24 hrs to 48 hrs following transfection.

2.2.2.11 Calcium flux analysis

The calcium-sensitive dye Indo-1 was used to measure BCR-induced Calcium-flux in DT40 cells. When excited at 360 nm, Indo-1 emits light at 485 nm in the Ca^{2+}-free state and at 404 nm in the Ca^{2+}-bound state. Indo-I light emission was recorded at 395 nm (violet) and 530 nm (blue) and the violet/blue ratio was used as readout for the relative intracellular calcium concentration. 5 x 10^6 DT40 cells were loaded with 1.25 nM Indo-I (Invitrogen) at 37ºC for 30 min. Cells were acquired on a BD LSR FACS machine equipped with a UV-laser. A baseline of the Ca^{2+}-bound/ Ca^{2+}-free ratio was acquired for 20 sec followed by addition of 2 µg/ml anti-chicken IgM antibody and further acquisition for 200 sec.

2.2.2.12 Retroviral tranduction of DT40 cells

The wild-type Syk cDNA and its mutants X1 through X6 were excised from the respective pcDNA3.1 plasmids (see 2.2.2.10) using BamHI and EcoRI restriction sites and inserted into the pMIG retroviral vector (kind gift of Luk Van Parijs) using BglII and EcoRI restriction sites. The packaging cell line 293GP (Clontech) which stably expresses the *gag* and *pol* genes of the Moloney murine leukemia virus was co-transfected with the retroviral constructs in combination with a packaging plasmid (pVSV-G, Clontech) encoding the pantropic envelope protein VSV-G derived from vesicular stomatitis virus using Lipofectamine 2000. Viral supernatant was collected 72 hrs and 96 hrs after transfection, filtered and stored at -80ºC. Wild-type and Syk-deficient DT40 cells were infected with viral supernatant diluted 1:2 in culture medium and containing 8 µg/ml polybrene. 72 hrs post-infection, single GFP-positive cells were sorted into 96-well U-bottom tissue culture plates on a FACSort (BD). Clones were grown for 14 days and analyzed for GFP-expression by FACS and Syk-expression by Western blot. Selected positive clones for each construct were expanded and used for further analysis.

2.2.3 Biochemical Methods

2.2.3.1 Preparation of cell lysates

For routine cytoplasmic extracts, cells were washed once in ice-cold PBS and lyzed in a buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 10% glycerol, 1% NP-40, 10 µM NaF and 2 mM Na_3VO_4 as phosphatase inhibitors and protease inhibitor cocktail (Sigma). After 15 min rotation at 4ºC, nuclei were pelleted by centrifugation at 15,000 x g for 10 minutes. The resulting supernatant was either immediately used for Western blot or immunoprecipitation or stored at -80ºC for later use. For whole cell lysates, the lysis buffer contained 350 mM NaCl, 20 mM HEPES/NaOH pH7.9, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM EGTA, 1% NP-40, 20% glycerol, 10 µM NaF, 2 mM Na_3VO_4 and protease inhibitor cocktail (Sigma).

2.2.3.2 Western blot

The protein concentration of cellular extracts was measured using the D_C Protein Assay (BIO-RAD) and a VERSAmax microplate reader (Molecular Devices). Equal protein amounts were loaded onto SDS polyacrylamide gels after boiling for 5 min in 1 x sample buffer. Commercial protein standard mixtures (BIO-RAD) were used as size markers. Proteins were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 8% to 15% gels and transferred onto polyvinylidenediflouride (PVDF) membranes (Immobilon P, Millipore) using standard procedures (Sambrook, et al., 1989). Protein transfer was routinely confirmed by reversible staining of the membrane with Ponceau S solution (Sigma). Membranes were blocked in 1 x TBS, 0.1% Tween (TBS-T) containing 3% non-fat dry milk (Carnation) and incubated over night at 4ºC with primary antibody diluted in blocking buffer or TBS-T containing 5% bovine serum albumin (BSA, Roche). Antibodies and dilutions are listed in table 4. Membranes were washed 3 times for 5 min in TBS-T and incubated for 1 hr with the appropriate horseradish peroxidase-coupled secondary antibody diluted in blocking buffer. After repeated washes in TBS-T, proteins were detected using chemiluminescent substrates. Depending on the signal strength, ECL (Amersham) or more sensitive substrates (SuperSignal West Dura Extended Duration Substrate and SuperSignal West Femto Maximum Sensitivity Substrate, both from Pierce) were used. Signal quantification was done using NIH Image 1.63 software.

2.2.3.3 Western blot stripping

Membranes were agitated in 63 mM Tris-HCl pH 6.7, 2% SDS, 200 mM β-mercaptoethanol for 30 min in a 50ºC water bath. Complete removal of primary antibodies was confirmed by developing membranes after incubation with secondary antibody.

2.2.3.4 Immunoprecipitation

Cell lysates were pre-cleared with non-specific immunoglobulin of the same species as the antibody used for immunoprecipitation and protein A or protein G sepharose (Amersham) for 1 hr at 4ºC. Protein A was used for rabbit antisera, protein G was used for mouse and goat antisera. Pre-cleared lysates were incubated with primary antibody over night at 4ºC followed by addition of protein A or protein G sepharose for 2 hrs at 4ºC or incubated with primary antibody directly coupled to protein A agarose over night. Immunoprecipitates were washed 3 – 5 times in lysis buffer. Bound proteins were eluted by boiling samples in 1 x SDS sample buffer and subjected to SDS-PAGE.

2.2.3.5 Subcellular fractionation

Fractionation of primary mouse B cells into cytoplasmic and nuclear extracts was done as described (Mecklenbrauker, et al., 2004). Briefly, cells were lyzed in a cytoplasmic lysis buffer containing 320 mM sucrose, 10 mM Tris-HCl pH 8.0, 3 mM $CaCl_2$, 2 mM Mg acetate, 0.1 mM EDTA, 0.5% NP-40 and fresh protease and phosphatase inhibitors. Nuclei were pelleted through centrifugation at 500 x g for 5 min, washed once in wash buffer (as cytoplasmic lysis buffer, but without addition of NP-40) and lyzed in nuclear lysis buffer containing 500 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% NP-40, 10% glycerol and fresh protease and phosphatase inhibitors. After sonication, debris was removed through centrifugation at 13,000 rpm for 15 min. Cytosolic and nuclear extracts were stored at -80ºC for Western blot analysis. Fractionation purity was assessed by the presence of tubulin as cytoplasmic and lamin B as nuclear markers, respectively.

For preparation of cytoplasmic and membrane extracts, cells were disrupted by hypotonic lysis for 15 min on ice in a buffer containing 10 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM Mg Cl_2, 0.1 mM EDTA and fresh protease and phosphatase inhibitors. Nuclei were removed by centrifugation at 400 x g for

10 min. The supernatant was then centrifuged at 95,000 x g for 1 hr in a SW55Ti rotor (Beckman Coulter). The supernatant was removed and saved as the cytoplasmic fraction. The pellet containing the membrane fraction was resolved in 1 x SDS sample buffer and aliquots of cytoplasmic and membrane fractions were subjected to Western blot analysis. Fractionation purity was assessed using tubulin as cytoplasmic and Lyn as membrane markers, respectively.

2.2.3.6 Lipid raft isolation

Lipid rafts were isolated as described (Dobenecker, et al., 2005). Briefly, cells were lyzed in TNNE buffer containing 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100 and fresh protease and phosphatase inhibitors and mixed with iodixanol (Optiprep, Axis-Shield) to a final concentration of 40% in TNNE buffer. Samples were overlaid with 30% iodixanol and TNNE buffer to create an iodixanol step gradient and were centrifuged at 60,000 rpm for 3 hrs in a Sorvall TH-660 rotor. 500 µl fractions were collected with lipid rafts localizing to the top fraction. Aliquots of each fraction were used for Western blot analysis. The purity of the preparation was assessed by the presence of Lyn and the lipid raft marker ganglioside GM1 which was detected using horseradish peroxidase-coupled cholera toxin B subunit (Sigma).

2.2.3.7 Purification of polysomal RNA

Polysome purification was performed after Stefani et. al. (Stefani, et al., 2004). All chemicals and reagents were of molecular biology grade purity and nuclease-free. B cells were incubated with 100 µg/ml cycloheximide (CHX, Sigma) for 15 min, washed three times in PBS containing CHX and lyzed in 10 mM HEPES-KOH pH 7.4, 150 mM KOH, 5 mM $MgCl_2$, 1% NP-40, 0.5 mM DTT, 100 µg/ml CHX, 200 U/ml RNAsin (Promega), 100 U/ml SUPERase (Ambion) and EDTA-free protease inhibitor cocktail (Roche). Nuclei were removed from the extracts by centrifugation at 2000 x g for 10 min. An aliquot of the lysate was saved for preparation of total RNA. The remaining lysate was loaded onto a 20% - 50% w/w linear density sucrose gradient in 10 mM HEPES-KOH pH 7.4, 150 mM KCl and 5mM $MgCl_2$. Gradients were centrifuged for 2 hrs at 40,000 x g at 4ºC in a SW41 rotor (Beckman Coulter). Fractions of 0.7 ml volume were collected with continuous monitoring at 254

nm using an ISCO UA-6 UV-detector. Polyribosome-containing fractions were identified based on the UV-trace and, in trial experiments, on the presence of S6 ribosomal protein in the fractions after TCA-precipitation and SDS-PAGE. For RNA-isolation, polysome-containing fractions were pooled and RNA was isolated from total and polysomal samples using Trizol LS (Invitrogen) according to the manufacturer's protocol.

2.2.3.8 Purification of recombinant TEV-protease

The plasmid pTPSN (Lucast, et al., 2001) containing TEV S219N fused to a C-terminal Hexa-His-tag in the bacterial expression vector pET15b (Novagen), was obtained from Jennifer Doudna. The PTD-sequences were inserted in frame into an N-terminal NcoI restriction site using the following oligonucleotides:

TAT forward: 5'-catggggtacggtcgcaagaaacgtcgccaacgtcgccgtggcagcggcgc-3'

TAT reverse: 5'-catggcgccgctgccacggcgacgttggcgacgtttcttgcgaccgtaccc-3'

MTS forward: 5'-catgggggcagccgttcttctcccctgttcttcttgccgcacccggcagcggcgc-3'

MTS reverse: 5'-catggcgccgctgccgggtgcggcaagaagaacaggggaagaacggctgccc c-3'

Pen forward: 5'-catggggcgccagatcaaaatttggttccagaatcgtcgcatgaaatggaagaaag gcagcggcgc-3'

Pen reverse: 5'-catggcgccgctgcctttcttccatttcatgcgacgattctggaaccaaattttgatctgg cgccc-3'.

Oligonucleotides were annealed, digested with NcoI and cloned into the NcoI site of pTPSN. A plasmid containing TAT-NES-TEV was constructed by PCR-amplification of TEV from pTPSN using the primers 5'-ataccatggggtacggtcgcaagaaacgtcgccaacgtcgccgtggcagcggcgccttgcagaagaag ctggaggagctggagcttgacgctgaaagcttgtttaag-3' (forward) and 5'-cgggctttgttagcagcc-3' (reverse). The PCR-product was digested with NcoI and BamHI and inserted into the NcoI-BamHI-opened pTPSN vector. All constructs were confirmed by DNA-sequencing prior to application.

TEV-protease was purified in a denaturing preparation following the procedure of Lucast et. al. (Lucast, et al., 2001). *E. coli* BL21 (DE3) cells (Novagen) were transformed with plasmid DNA and overnight cultures were grown from single colonies in LB medium containing 100 µM ampicillin (LB Amp). 2 l LB Amp were inoculated with the overnight culture in a 1 in 10

dilution and incubated at 37ºC under constant agitation. At an OD of 1.0, protein expression was induced by addition of 400 µM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) and incubation continued for another 5 hrs. Cells were collected by centrifugation at 4000 rpm for 30 min at 4ºC, resuspended in 50 mM Tris-HCl pH 8.0, 300 mM NaCl and protease inhibitor cocktail (Roche) and stored at -80ºC. Upon thawing, cells were disrupted by addition of 500 µg/ml lysozyme, 200 µg/ml DNase I, 20 mM $MgSO_4$ and 2 mM $CaCl_2$, agitation for 30 min at 4ºC followed by addition of 1% Triton X-100. Bacterial inclusion bodies were pelleted by centrifugation at 10,000 g for 20 min at 4ºC and protein was extracted from the pellet by overnight incubation in 6 M guanidine-HCl, 100 mM NaH_2PO_4, 10 mM Tris-HCl pH 8.0 at 4ºC under gentle agitation. The protein extract was cleared by centrifugation at 17,000 rpm for 30 min at 4ºC.

A pre-charged Ni Sepharose column (HisTrap HP affinity column, Amersham) was equilibrated in a binding buffer containing 8 M Urea, 100 mM NaH_2PO_4 and 10 mM Tris-HCl pH 8.0. The protein extract was loaded onto the column and the protein content was monitored by UV-absorption at 280 nm in an Äkta Prime (Amersham). Column-bound proteins were eluted using 8 M Urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl pH 4.5. Protein-containing fractions confirmed by SDS-PAGE and Coomassie-staining were pooled and dialyzed overnight against 100 mM Tris-HCl pH 8.5, 500 mM NaCl, 5 mM DTT, 0.5 mM EDTA, 50% glycerol at 4ºC in dialysis tubing with a 6 – 8000 Dalton MWCO (Spectrapor). Refolded protein was cleared by centrifugation at 17,000 rpm for 30 min at 4ºC and concentrated in Amicon Ultra-15 filters with a 5000 Dalton MWCO. The protein was further purified on a HiTrap Desalting column (Amersham) pre-equilibrated in 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20% glycerol, 50 mM β-mercaptoethanol. Protein-containing fractions whose purity had been confirmed by SDS-PAGE and Coomassie-staining were pooled and concentrated as above to a maximum concentration of approximately 2 mg/ml. Aliquots were stored at -80ºC.

2.2.3.9 Measurement of TEV activity in vitro

The catalytic activity of the purified PTD-TEVs was measured in an *in vitro* assay using a recombinant substrate protein (kind gift of André Hoelz) which contains a TEV target site and whose size changes from 36 to 33 kD upon TEV-mediated cleavage. Assays were carried out in a reaction buffer containing 500 mM NaCl, 10 mM Tris-HCl pH 8.0, 5 mM DTT, 20 µg substrate

protein and 10 µg recombinant TEV for 30 min at room temperature. Conversion of the substrate protein was visualized by SDS-PAGE and Coomassie-staining.

2.2.3.10 FITC-labeling of recombinant TEV

Purified TEV or BSA was fluorescently labeled using the EZ-label FITC Protein Labeling Kit (Pierce) according to manufacturer's instructions. Briefly, proteins were first dialyzed against PBS and then against a borate buffer supplied by the manufacturer to remove Tris from the protein preparation. The protein was then incubated with a 24-fold molar access of fluorescein isothiocyanate (FITC) for 2 hrs in the dark. Unincorporated FITC was removed from the labeling reaction by dialysis against PBS. Labeled proteins were stored at 4ºC.

2.2.4 Other Methods

2.2.4.1 Gene expression analysis

RNA quality analysis, labeling and hybridization as well as raw data processing and normalization were performed by the Genomics Core Laboratory at the Memorial Sloan-Kettering Cancer Center.

Quality of RNA was confirmed before labeling by analyzing 20 - 50 ng of each sample using the RNA 6000 NanoAssay and a Bioanalyzer 2100 (Agilent Technologies). All samples had a 28S/18S ribosomal peak ratio of 1.8 – 2 and were considered suitable for labeling. 2 µg of total RNA was used for cDNA synthesis using an oligo(dT)-T7 primer and the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen). Synthesis, linear amplification and labeling of cRNA were accomplished by transcription *in vitro* using the MessageAmp aRNA Kit (Ambion) and biotinylated nucleotides (Enzo Diagnostics). 10 µg of labeled and fragmented cRNA were hybridized to the mouse genome MOE430 2.0 array (Affymetrix), which interrogates ~39,000 transcripts at 45°C for 16 hrs. Automated washing and staining were performed using the Fluidics Station 400 (Affymetrix), according to the manufacturer's protocols. Finally, chips were scanned with a high numerical aperture and flying objective lens in the scanner (GS3000; Affymetrix). Raw expression data were analyzed using Microarray Analysis software (version 5.1; Affymetrix). Data were normalized to a target intensity of 500 to account for differences in global chip intensity.

The complete set of data is available at the GEO public repository and can be accessed at http://www.ncbi.nlm.nih.gov/geo under accession no. GSE5985.

Genes which were differentially expressed between unstimulated and BAFF-treated cells, were initially identified using GeneChip Operating Software (GCOS, Affymetrix). 377 transcripts showed a change of two-fold or more and were selected for further analysis. Approximately three quarters of these genes were up-regulated upon BAFF stimulation, whereas one quarter was down-regulated. For a biological interpretation of the differentially regulated genes, the GoMiner gene ontology (GO) tool was used (Zeeberg, et al., 2003). GO is a genome project that describes gene products in terms of their associated biological processes, cellular components, and molecular functions (Ashburner, et al., 2000). GO categories are organized in directed acyclic graphs, a kind of hierarchy in which one category can have more than one "parent". GoMiner identifies GO categories that are over- or underrepresented in lists of genes of interest, such as differentially expressed genes from a microarray experiment, and calculates statistical significance as the one-sided nominal unadjusted p-value from Fisher's exact test. Only those biological processes for which the p-value was below <0.001 were included in the analysis.

2.2.4.2 Protein identification by mass-spectrometry

Protein identification by mass-spectrometry was performed by Hediye Erdjument-Bromage and Paul Tempst of the Molecular Biology Program at the Memorial Sloan-Kettering Cancer Center.

Gel-resolved proteins were digested with trypsin, batch purified on a reversed-phase microtip, and resulting peptide pools were individually analyzed by matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-reTOF) mass spectrometry (MS; ultraflex TOF/TOF; Bruker Daltronics Inc.) for peptide mass fingerprinting (PMF), as previously described (Sebastiaan Winkler, et al., 2002). Selected peptide ions (m/z) were taken to search a nonredundant (NR) protein database (3,245,378 entries on 28 January 2006; National Center for Biotechnology Information) using the PeptideSearch algorithm (developed by Matthias Mann, Max-Planck-Institute for Biochemistry, Martinsried, Germany; an updated version of this program is currently available as PepSea from Applied Biosystems/MDS SCIEX). A molecular mass range up to twice the apparent molecular weight (as estimated from electrophoretic relative mobility) was covered, with a mass

accuracy restriction of <35 ppm and a maximum of one missed cleavage site allowed per peptide. To confirm PMF results with scores ≤40, mass spectrometric sequencing of selected peptides was done by MALDI-TOF/TOF (MS/MS) analysis on the same prepared samples using the ultraflex instrument in "LIFT" mode. Fragment ion spectra were taken to search the NR protein database using the Mascot MS/MS ion search program (version 2.0.04 for Microsoft Windows; Matrix Science Ltd.) (Perkins, et al., 1999). Any tentative confirmation (Mascot score ≥30) of a PMF result thus obtained was verified by comparing the computer-generated fragment ion series of the predicted tryptic peptide with the experimental MS/MS data.

2.2.5 Mice

Mice were housed in the Laboratory Animal Research Center at the Rockefeller University under specific pathogen-free conditions and were analyzed at an age between 8 and 12 weeks. Animal protocols were approved by the Institutional Animal Care and Use Committee at the Rockefeller University.

Conditional Syk knockout mice were generated and kindly provided by Christian Schmedt (Saijo, et al., 2003). Cre-recombinase-bearing strains CMV-Cre (Schwenk, et al., 1995), CD19-Cre (Rickert, et al., 1997) and Mx-Cre (Kuhn, et al., 1995) were used for inactivation of the floxed Syk-allele in mice. All Syk mice from these crosses were on a mixed genetic background. PKCβ-deficient mice were generated by Michael Leitges (Leitges, et al., 1996) and used on a C57BL/6 background (Saijo, et al., 2002). MyD88-mutant (Kawai, et al., 1999) and ROSA-GFP mice (Mao, et al., 2001) have been described. C57BL/6 mice were obtained from commercial vendors (Charles River Laboratories, Taconic, The Jackson Laboratory) and used as a source of wild-type B cells.

3 Results

3.1 Part 1: BAFF acts as a survival and growth factor on B cells

3.1.1 BAFF promotes B cell growth and metabolic fitness

To test whether BAFF promotes cellular responses which are characteristic for growth factors besides survival, I measured B cell size, mitochondrial membrane potential and protein synthesis in naive versus BAFF-stimulated B cells. To this end, B cells were cultured in the presence of BAFF for up to 72 hrs and B cell viability and size was measured by FACS analysis at selected time points. BAFF-treatment enhances the survival of B cells as expected and this is accompanied by an increase in the cells' size (figure 8A and B). This cell growth is not caused by a potential contamination of the BAFF-preparation with endotoxin as evidenced by the identical response of MyD88-deficient B cells which are resistant to LPS stimulation (Kawai, et al., 1999) and figure 8C). The electrostatic potential across the inner mitochondrial membrane ($\Delta\Psi_m$) reflects the efficiency of electron transport along the respiratory chain and can be used as an indicator of the cellular metabolic rate. Mitochondrial membrane potential was measured in naive and BAFF-treated cells by flow-cytometry following incubation with the potentiometric fluorescent dye TMRE which incorporates into mitochondria in a $\Delta\Psi_m$-dependent manner (Edinger and Thompson, 2002). BAFF-stimulated cells have an elevated mitochondrial membrane potential compared to non-treated cells, whereas the $\Delta\Psi_m$ base-line, determined by co-incubation with the respiratory uncoupler CCCP, is identical in both types of cells (figure 8D). Together, these results show that BAFF promotes both cell growth and energy homeostasis in B cells in addition to its survival effect.

BAFF-induced cell growth corresponds to a raise in the cellular protein content and heightened protein synthesis, as judged from the quantification of protein concentrations in B cell lysates prepared from naive and BAFF-treated B cells as well as from the increased incorporation of ^{35}S-labeled amino acids into BAFF-stimulated B cells (figure 8E and F). This is reflected on a molecular level by the phosphorylation state of key translational regulatory proteins. First, BAFF-treatment leads to phosphorylation of S6 ribosomal protein, an indicator of active protein synthesis (figure 8G). Furthermore, BAFF facilitates the initiation of translation by activating eIF4E in a dual fashion. BAFF-stimulation induces phosphorylation of eIF4E itself, but also of

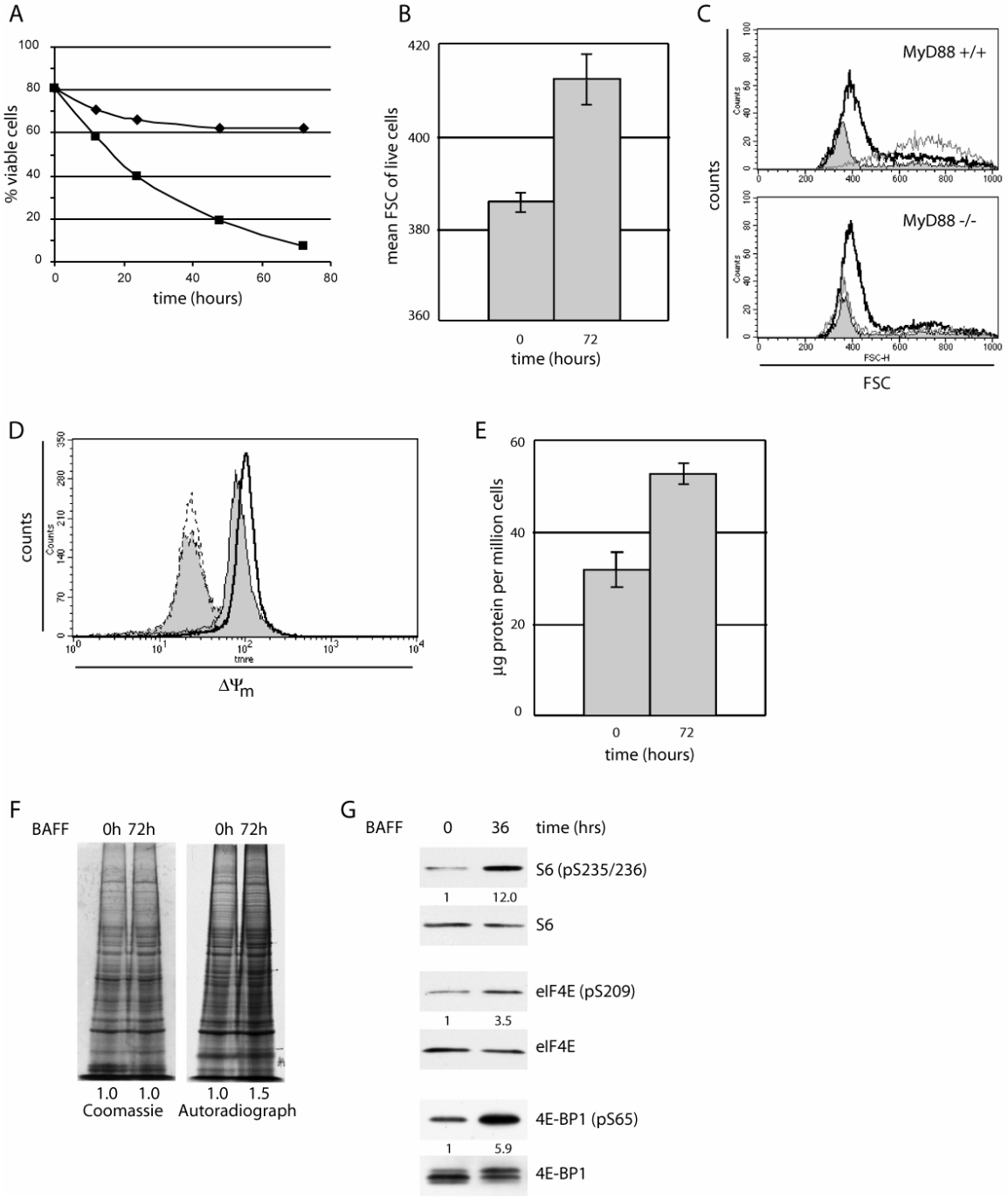


Figure 8: BAFF acts as a growth factor on B cells. A. Mature B cells were incubated in culture medium in the absence (squares) or presence (diamonds) of BAFF for three days. Cell viability based on exclusion of TOPRO-3 was quantified by FACS at selected time points. B. Mature B cells were incubated in culture medium in the presence of BAFF and the cell size was measured by FACS analysis of the forward scatter (FSC). Error bars represent the standard deviation based on three independent experiments. C. Mature B cells from MyD88 +/+ and MyD88 -/- mice were incubated in culture medium (filled gray area) or in the presence of BAFF (thick black line) or LPS (thin grey line). Cell size was measured by FACS analysis of the forward scatter (FSC) after 72

hours of culture. D. Mitochondrial membrane potential ($\Delta\Psi_m$) was measured by TMRE labeling. Histograms show TMRE fluorescence of B cells incubated in the absence (filled area, thin lines) or presence of BAFF (clear area, thick line). In control samples (dashed lines), the membrane potential was dissipated by addition of the uncoupling agent CCCP. E. Protein amount per million B cells. Error bars represent the standard deviation based on three independent experiments. F. Protein synthesis was measured by metabolic labeling of mature B cells using ^{35}S-methionine and -cysteine. Radioactivity incorporation was visualized by autoradiography (right) of cellular protein extracts stained with Coomassie Brilliant Blue (left). Numbers represent the relative signal intensity after quantification of individual lanes. G. BAFF activates components of the translational machinery. Purified B cells were incubated with BAFF for the indicated time and phosphorylation of proteins that control translation was measured by Western blot analysis of S6 (pS235/236), eIF4E (pS209) and 4E-BP1 (pS65). Membranes were stripped and re-probed with non-phospho-specific antibodies against the respective proteins. Numbers represent the fold change of the phospho-specific signal normalized to the non-phospho-specific signal.

its inhibitor 4E-BP1 which frees eIF4E to participate in the translation initiation complex. Collectively, BAFF induces changes to B cell morphology, energy homeostasis and protein synthesis which are characteristic features of canonical growth factor signaling. In addition to looking for specific growth factor-induced events, BAFF-mediated cellular changes were characterized using an unbiased approach in which the gene expression profile was compared between BAFF-treated and naive B cells. In an attempt to more specifically identify those genes whose protein expression correlates with BAFF stimulation, polyribosomes, whose associated RNA represents the fraction of actively translated transcripts within the cell, were isolated from cell lysates prior to RNA isolation. Hybridization of the reverse-transcribed samples to Affymetrix arrays which allow for probing of 34,000 well characterized mouse genes, yielded 377 transcripts which were differentially regulated by at least two-fold in BAFF-treated cells. Of those, approximately three quarters were upregulated while one quarter was downregulated. The complete set of data is available at the GEO public repository and can be accessed at http://www.ncbi.nlm.nih.gov/geo under accession no. GSE5985. In order to assess how these transcriptional changes would impact cellular physiology, we determined the biological processes in which the differentially regulated genes are involved. This was accomplished in an unbiased fashion using annotations from the Gene Ontology (GO) Consortium

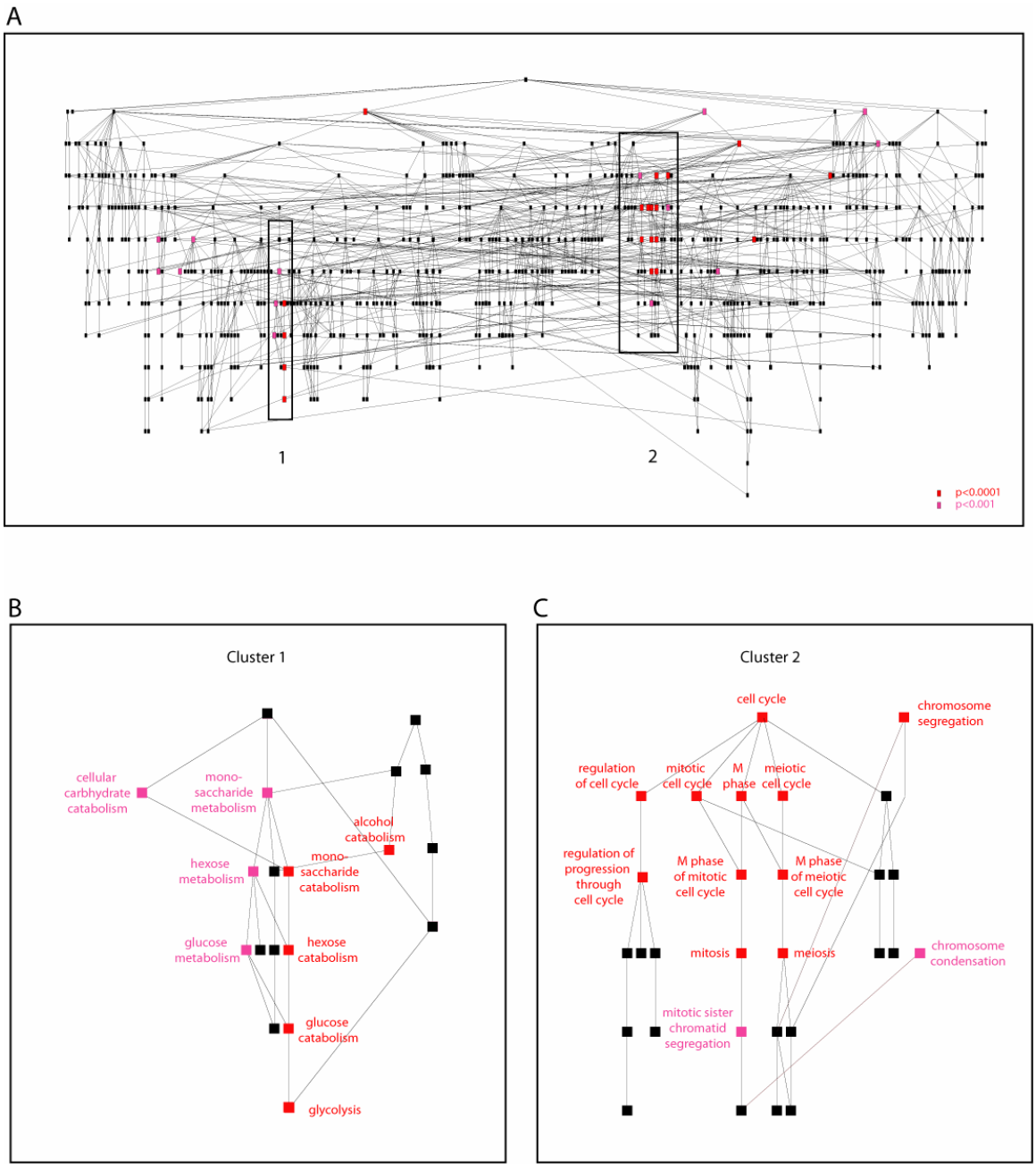


Figure 9: BAFF induces selective changes in gene transcription. The expression pattern and levels of polysome-associated mRNAs in unstimulated and BAFF-treated B cells were determined by Affymetrix microarray analysis. The biological significance of BAFF-induced changes in gene expression was then analyzed using the Gene Ontology tool GOminer. GO categories representing biological processes are displayed as a directed acyclic graph and those over-represented in BAFF-stimulated cells are marked. Colors indicate a statistical significance of p<0.001 (light red) or p<0.0001 (dark red) A. Two prominent clusters of over-represented GO categories are framed and displayed in B and C. Labeling represents GO "Biological Process" category names. Gene identities for the displayed GO categories are listed in table 5 (see Appendix).

(Ashburner, et al., 2000). GO represents an extensive pool of databases which aim to describe gene products in terms of, among others, the biological processes which they are involved in. The relationship between individual processes is illustrated by a directed acyclic graph which, similar to a hierarchy, organizes processes from broad and less specific to very specialized. To determine which biological processes were induced in BAFF-stimulated B cells, the GO tool GOMiner was used (Zeeberg, et al., 2003). This program identifies GO biological processes which are over- or underrepresented in a statistically significant manner in lists of genes of interest, in this case, genes differentially regulated by BAFF in B cells. In this way, 33 biological processes were found to be stimulated in BAFF-treated B cells (figure 9A). Approximately two thirds of those fell into either one of two clusters which center around the biological processes glycolysis and cell cycle, respectively (figure 9B and C). The individual genes which belong to these clusters are listed in table 5 (see Appendix).Both of these BAFF-regulated clusters are classic targets of growth factor regulation. BAFF-induced upregulation of glycolytic enzymes in combination with its previously observed effect on mitochondrial respiration argue for a complex function of BAFF in the maintenance of cellular energy homeostasis. The profound effect of BAFF on cell cycle regulation is again a typical feature of growth factor signaling. However, it is surprising in a sense that BAFF by itself is known not to elicit B cell division (Schneider, et al., 1999). Indeed, BAFF fails to induce DNA-replication, judging from a lack of BrdU-incorporation and B cell proliferation as measured by CFSE-dilution (figure 10 A and B). However, on a molecular level, the levels of a plethora of cell cycle regulatory proteins, including cyclin D2, cyclin E, cyclin-dependent kinase (Cdk) 4, minichromosome maintenance deficient (Mcm) 2 and 3, Survivin and the commonly used proliferation marker Ki67 are increased by BAFF-stimulation (figure 10C). In accordance with the increased expression of Cdk4 and cyclin D, BAFF enhances phosphorylation of the Cdk4 target retinoblastoma (Rb), a prerequisite for the expression of E2F-regulated genes and S-phase entry. This profile of cell cycle regulatory protein changes is indicative of cell cycle progression from G1 to S phase, but does not match the cellular physiological effect of BAFF. One way to explain this discrepancy is a potential BAFF-independent regulation of Cdk-activity by the inhibitor proteins p27 and p18 (Huang, et al., 2004). Overall, BAFF appears to promote B cell exit from the resting state G0 into G1, but stops short of inducing cell cycle progression beyond the G1 to S checkpoint. This effect of BAFF positions B cells in a state

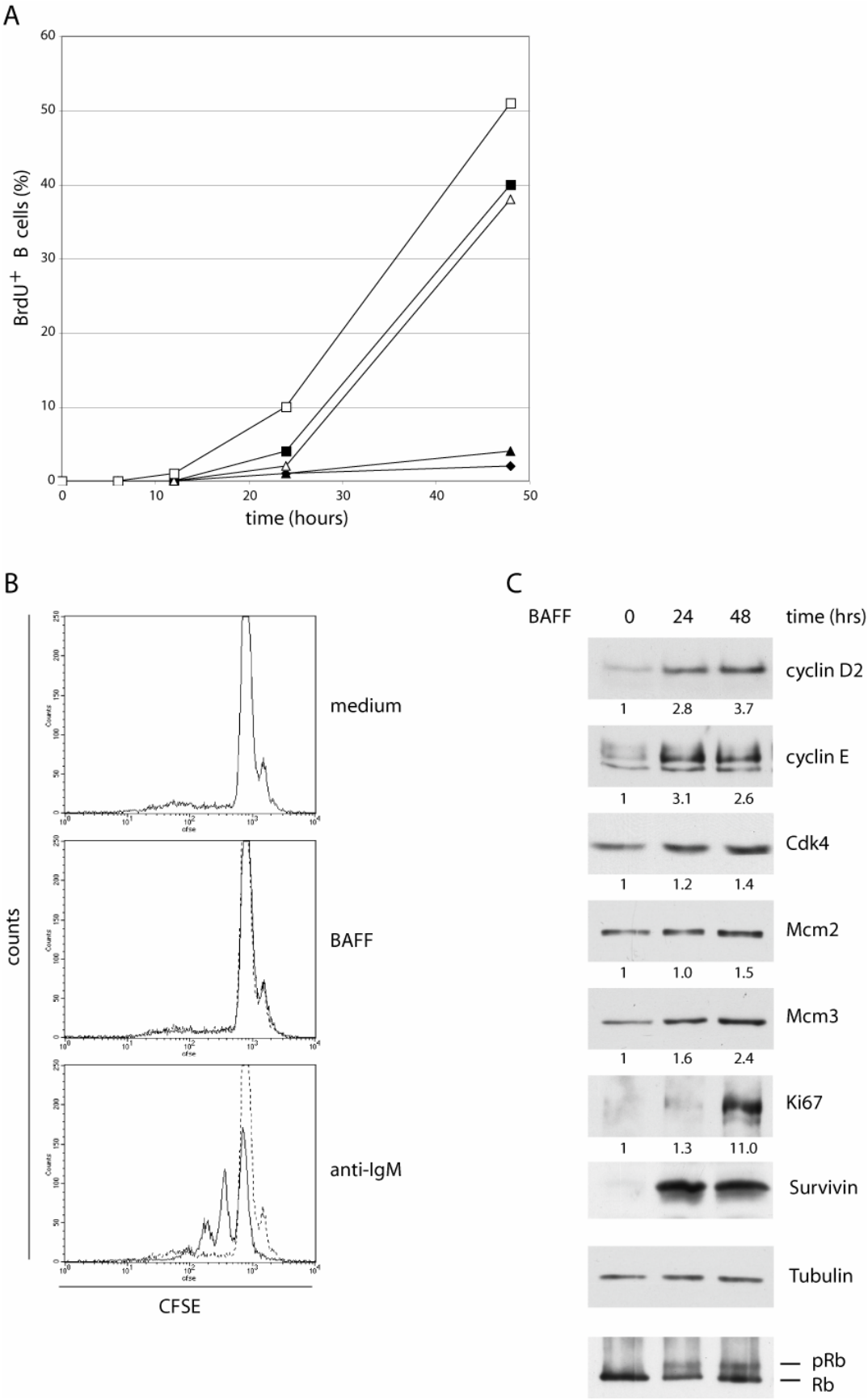


Figure 10: BAFF induces up-regulation of cell cycle-controlling proteins, but not cell division. A. B cell entry into S-phase *in vitro* was measured by BrdU-incorporation.

Frequencies of BrdU$^+$ B cells incubated in medium alone (diamonds) or in the presence of BAFF (filled triangles), of anti-IgM (filled squares), of BAFF and anti-IgM (open triangles) or after pre-incubation with BAFF for 24 hrs followed by anti-IgM treatment for the indicated time (open squares) are shown. B. Mature B cells were labeled with CFSE, incubated for 3 days in medium alone or in the presence of BAFF or anti-IgM and the fluorescence of live B220$^+$ B cells was measured by FACS. A reduction in CFSE fluorescence is proportional to the number of cell divisions. The position of the medium peak (dashed line) is included in BAFF and anti-IgM plots. C. Purified B cells were incubated with BAFF for the indicated time and the expression levels of cell cycle regulatory proteins cyclin D2, cyclin E, Cdk4, Mcm2, Mcm3, Ki67, Survivin and Rb following BAFF-stimulation were measured by Western blotting. Protein loading was controlled by tubulin expression level analysis. Numbers represent the fold induction normalized to the tubulin signal. Hyper-phosphorylated Rb (pRb) is identified by a migratory shift.

were they remain resting but highly responsive to a mitogenic signal and prepares them for an immediate proliferative response in the event of antigen encounter. This is in line with the observed co-stimulatory effect of BAFF in conjunction with mitogenic stimulation (Schneider, et al., 1999). In further support of this model, incubation of B cells with BAFF prior to their challenge with anti-IgM accelerates their entry into S phase (figure 10A, open squares).In summary, a BAFF-mediated effect on B cell cell growth, protein synthesis, cellular energy derivation and cell cycle progression is evident both from selectively testing the sensitivity of these processes to BAFF as well as from analyzing the gene expression profile of BAFF-treated B cells in an unbiased fashion, demonstrating that the effect of BAFF on B cells exceeds mere survival.

3.1.2 BAFF activates the PI3K-Akt signaling pathway

Having established that the effect of BAFF on B cells mirrors many growth factor-induced responses, it was plausible to look for potential activation of canonical growth factor induced signaling pathways by BAFF. As explained in detail in the previous section, cell survival, growth, energy homeostasis, metabolism and cell cycle progression are all subject to regulation by the PI3K-Akt pathway.

Activation of Akt can be inferred from its phosphorylation state at S473 and

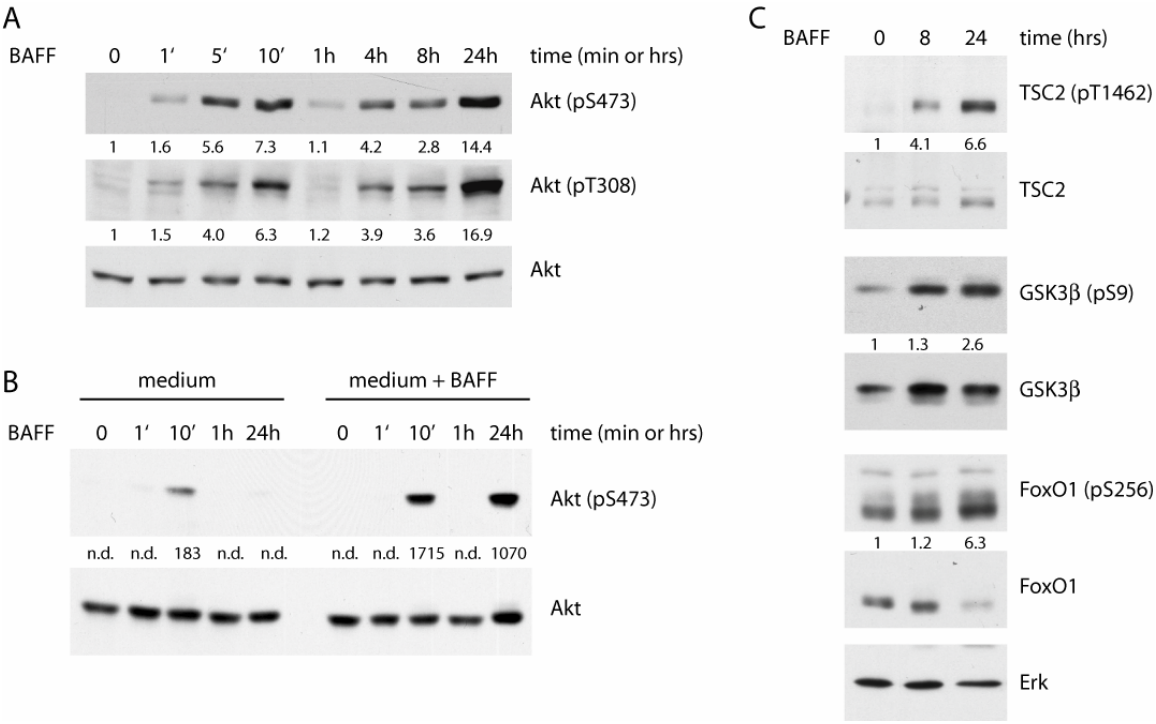


Figure 11: BAFF activates the Akt signal transduction pathway. A. BAFF-induced Akt phosphorylation on S473 and T308 was measured by immunoblot analysis using the respective phospho-specific antibody (top panel). Akt expression was controlled by reprobing with a non-phospho-specific Akt antibody. Numbers represent the fold change of the phospho-specific signal normalized to the non-phospho-specific signal. B. Akt-phosphorylation at S473 in response to BAFF (right) or control agent (left) was measured and quantified as in A. C. BAFF-induced phosphorylation of TSC2 at T1462, of GSK-3β at S9 and of FoxO1 at S256 were measured by sequential immunoblot analysis. The amount of tested proteins was measured by re-probing of immunoblots with non-phospho-specific antibodies against the respective proteins. Quantification was done as in A. Erk expression is provided as a protein loading control.

T308 and both residues are phosphorylated in B cells upon stimulation with BAFF. Akt-activity reaches a first peak early on at approximately 10 minutes of BAFF-treatment and, after a temporary decline, reaches a maximum at 24 hours (figure 11A). Mock stimulation of cells with medium not containing the stimulating agent results in weak Akt-phosphorylation after 10 minutes, indicating that a fraction of the BAFF-induced signal at this time point is of unspecific nature (figure 11B).Akt exerts its effect on cell growth, protein synthesis, metabolism, survival and cell cycle progression through direct inactivation of the downstream targets TSC2, GSK-3 and FoxO transcription factors. As expected, BAFF leads to phosphorylation of TSC2, GSK-3β and

FoxO1 at their respective Akt target sites (figure 11C). Furthermore, prolonged BAFF-treatment diminishes FoxO1 protein levels, which is a characteristic consequence of prolonged Akt-activity.

Akt-activation is a multi-step process initiated by its binding to PIP_3 at the plasma membrane, which mediates a conformational change in the protein and allows for its phosphorylation (figure 12A). This sequence of events is suggested by the fact that inhibition of PI3K activity using the specific pharmacological inhibitor LY294002 abrogates Akt-phosphorylation (Vanhaesebroeck and Alessi, 2000) and figure 12B).

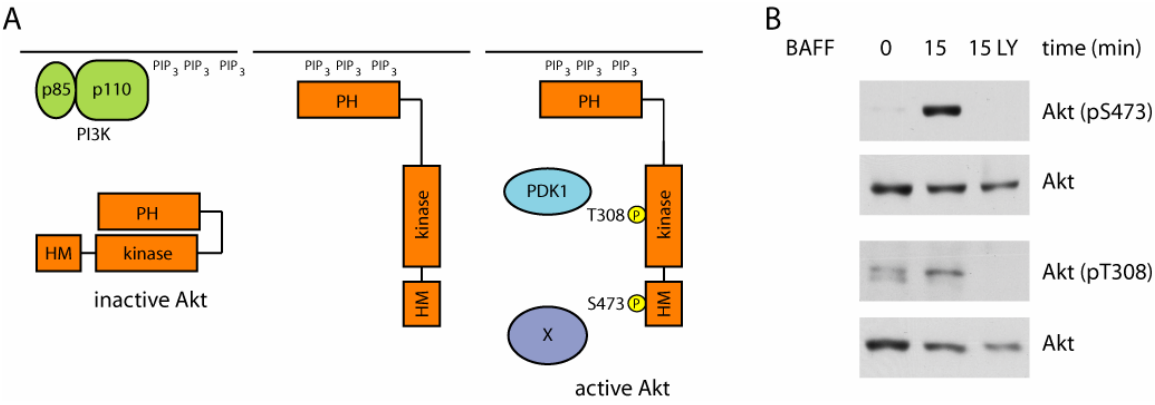


Figure 12: Multi-step mechanism of Akt-activation. A. Inactive Akt is recruited from the cytoplasm to the plasma membrane through binding to the PI3K-product PIP_3. A conformational change allows for PDK1-mediated phosphorylation of T308 within the kinase domain as well as for phosphorylation of S473 within the C-terminal hydrophobic motif (HM) by an as yet not conclusively defined kinase X. B. Pharmacological PI3K-inhibition abrogates Akt-phosphorylation. Mature B cells were stimulated with BAFF in the absence or presence of LY294002 and Akt-phosphorylation was analyzed as in figure 11.

The PIP_3 content within the plasma membrane is reversibly regulated through its generation by PI3K and its break-down by PTEN. In order to clarify the mechanism of BAFF-induced Akt-activation, the potential activation of PI3K or inactivation of PTEN were analyzed. In B cells, the main isoform of PI3K consists of the regulatory subunit p85α and the catalytic subunit p110δ. Immunoprecipitation of p85α from untreated and BAFF-stimulated B cells reveals co-precipitation of an approximately 110 kD protein which was inducibly tyrosine-phosphorylated (figure 13A). A protein band which co-migrated with phospho-p110 was subjected to mass-spectrometric analysis

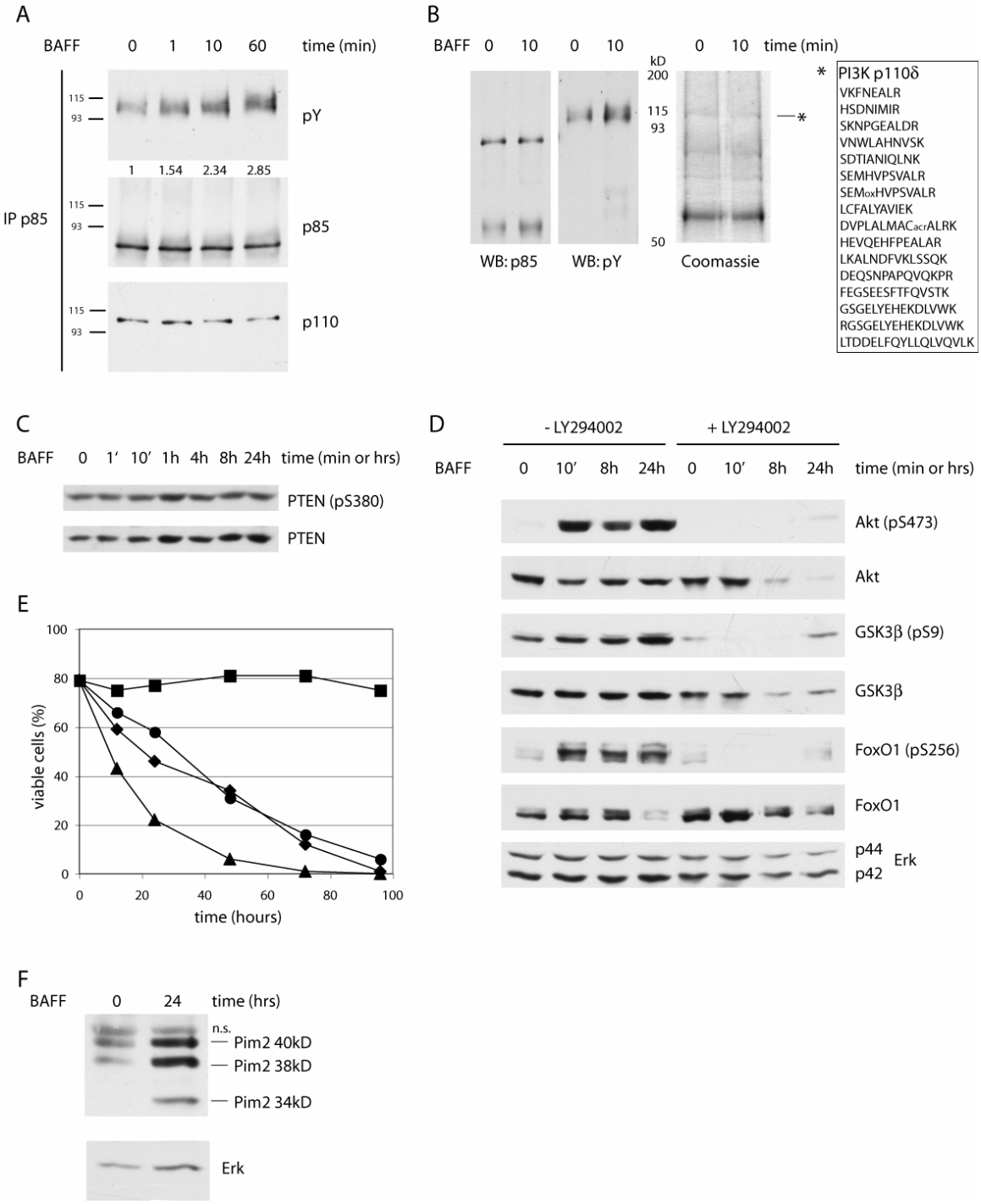


Figure 13: PI3K controls BAFF-induced Akt-activation and B cell survival. A. BAFF induces phosphorylation of p110. The p85 subunit was immunoprecipitated from extracts of BAFF treated cells and tyrosine phosphorylated proteins in immunoprecipitates were analyzed by Western blotting using anti-phosphotyrosine antibody. The amounts of p85 and p110 in the immunoprecipitates were measured by Western blot analysis. B. p85-immunoprecipitates from BAFF-treated and control B cells were resolved by SDS-PAGE and visualized by Coomassie Brilliant Blue staining (right). A protein band corresponding in size to phospho-p110 (*) was subjected to

mass-spectrometric analysis and identified as PI3K p110δ. The peptides used for protein identification are listed. Aliquots of the immunoprecipitates were resolved on a parallel gel and examined for p110 phosphorylation as in A (left). C. BAFF does not induce changes in PTEN-phosphorylation. Phosphorylation of PTEN in extracts of BAFF-treated cells was measured by immunoblotting with phospho-Ser380 PTEN antibody. The immunoblot was re-probed with a non-phospho-specific PTEN antibody. D. PI3K inhibition suppresses BAFF-induced Akt-, GSK-3β- and FoxO1-phsophorylation. B cells were stimulated with BAFF in the absence or presence of LY294002 and protein phosphorylation was measured as described in figure 11. E. Inhibition of PI3K reduces the survival of BAFF-treated cells. B cells were cultured in medium alone (circles) or in the presence of BAFF (squares), LY294002 (triangles) or BAFF together with LY294002 (diamonds). The frequencies of viable B cells were determined by FACS analysis. F. BAFF induces expression of Pim-2. The positions of three different Pim-2 isoforms are indicated. n.s. denotes non-specific. Erk expression is provided as a protein loading control in D and F.

and found to represent p110δ (figure 13B), suggesting that BAFF promotes tyrosine phosphorylation of the PI3K catalytic subunit. A similar phenomenon has previously been observed in B cell lines in response to stimulation through the BCR where it was interpreted as a sign of PI3K activation (Craxton, et al., 1999: ; Kuwahara, et al., 1996). It therefore seems that BAFF promotes PI3K-activity which is likely to account for its stimulatory effect on Akt. Alternatively, BAFF could impact the activity of the phosphoinositide phosphatase and Akt-inhibitor PTEN. However, PTEN activity, as judged by the phosphorylation state of the inhibitory residue serine 380 (Birle, et al., 2002), does not appear to be altered by BAFF (figure 13C). In further support of BAFF-mediated Akt-regulation through PI3K activation, the phosphorylation of Akt-target proteins GSK-3β and FoxO1 is abrogated by suppression of PI3K-activity using the pharmacological inhibitor LY294002 (figure 13D). Under the same conditions, BAFF-mediated B cell survival is severely impaired (figure 13E), illustrating the importance of the PI3K-Akt pathway for BAFF-mediated B cell survival.

In summary, BAFF elicits molecular changes to B cell signaling proteins which mirror growth factor-mediated activation of the PI3K-Akt pathway. In addition to this pathway, a second oncogenic kinase, Pim-2, has a role in trophic signal transduction and exerts partially overlapping functions with Akt. BAFF not only activates the PI3K pathway, but also induces expression of Pim-2

(figure 13F). Both Akt- and Pim-2 can contribute to 4E-BP1-phosphorylation and thus exert translational control.

3.1.3 PKCβ controls BAFF-mediated Akt-activation

Akt-binding to the PI3K-product PIP_3 at the plasma membrane is merely the first, albeit indispensable, step towards its full activation. Membrane translocation allows for Akt-phosphorylation at two essential sites, T308 and S473. In all models studied to date, PDK1 conducts Akt T308 phosphorylation (Vanhaesebroeck and Alessi, 2000). PDK1 itself is targeted to the plasma membrane in a similar fashion as Akt through its PH-domain, although it appears to have constitutive enzymatic activity. Therefore, Akt-phosphorylation by PDK1 is likely regulated by differential accessibility of T308 through Akt-membrane recruitment and conformational change. In support of this model, PDK1 activity, as judged by phosphorylation of the activating residue S241 is constitutive and not altered in response to BAFF (figure 14A).

In contrast to PDK1-mediated T308 phosphorylation, the kinase responsible for the phosphorylation of S473 has eluded conclusive identification despite substantial efforts. Among the kinase implicated in Akt S473-phosphorylation in different contexts and cell types are Akt itself, PDK1, integrin-linked kinase 1 (ILK1), mitogen activated protein kinase activated protein kinase 2 (MAPKAP-K2), PKCβ, DNA-PK, ATM and mTORC2 (Alessi, et al., 1996: ; Balendran, et al., 1999: ; Feng, et al., 2004: ; Kawakami, et al., 2004: ; Persad, et al., 2001: ; Sarbassov, et al., 2005: ; Toker and Newton, 2000: ; Viniegra, et al., 2005). In search for the enzyme which regulates BAFF-induced Akt S473-phosphorylation, PKCβ seemed a good candidate for several reasons. First, PKCβ is capable of phosphorylating Akt at S473 but not T308 *in vitro* (Kawakami, et al., 2004). Second, a wealth of information demonstrates an important function for PKCβ in B cell biology. Lack of PKCβ in mice results in an Xid-like immunodeficiency syndrome and reduces the frequency of splenic B cells (Leitges, et al., 1996). Furthermore, PKCβ-deficient B cells have prominent defects in survival and antigen-induced proliferation which has been at least partially attributed to defective BCR-induced NF-κB activation (Saijo, et al., 2002: ; Su, et al., 2002). In order to assess, whether PKCβ could play a potential role in BAFF-dependent Akt-phosphorylation, we investigated the effect of BAFF on PKCβ-activity. PKCβ belongs to the subfamily of conventional PKCs whose activation requires both

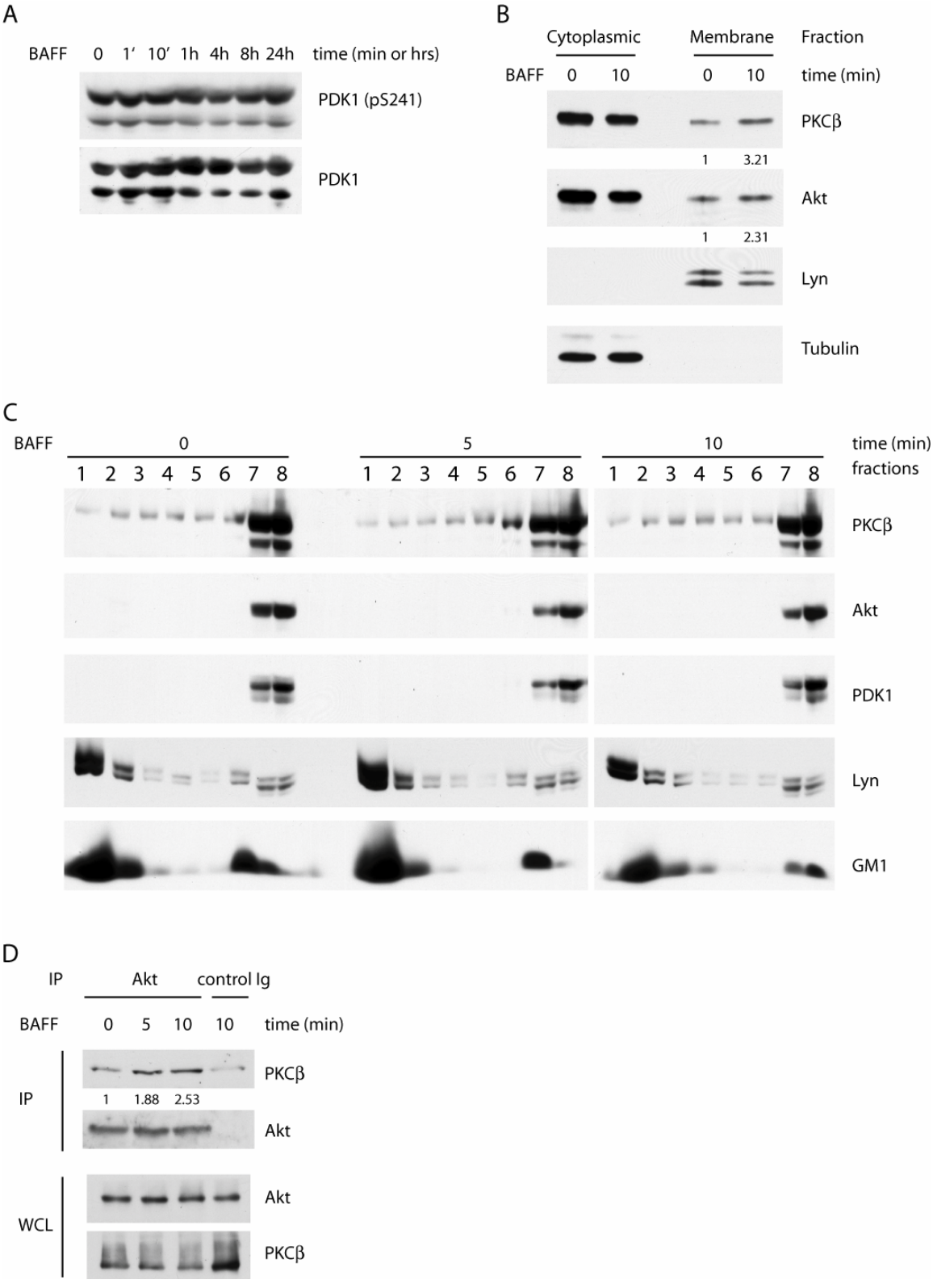


Figure 14: BAFF controls PKCβ-activity and association with Akt. A. BAFF does not induce changes in PDK1-phosphorylation. Phosphorylation of PDK1 in extracts of BAFF treated cells was measured by immunoblotting with phospho-Ser241 PDK1 antibody. The immunoblot was re-probed with a non-phospho-specific PDK1 antibody. B. BAFF induces membrane translocation of PKCβ. B cells were stimulated with BAFF

followed by the preparation of cytoplasmic and membrane protein extracts. PKCβ and Akt content in the extracts was measured by Western blotting. Fraction purity and protein loading was controlled by Western blotting using antibodies against tubulin and Lyn. Numbers represent the fold change of PKCβ and Akt contents in the membrane fractions normalized to the amount of Lyn. C. BAFF does not induce the translocation of PKCβ, Akt and PDK1 into lipid rafts. Extracts prepared from BAFF-stimulated B cells were subjected to high-speed centrifugation for purification of lipid rafts. Gradient fractions were collected and subjected to Western blot analysis using antibodies against PKCβ, Akt, PDK1 and Lyn. Expression of the lipid raft marker GM1 was detected using horseradish-peroxidase-coupled Cholera Toxin B subunit. D. BAFF induces PKCβ-Akt association. B cells were stimulated with BAFF and protein extracts were treated with Akt antibody or control serum. The amounts of PKCβ or Akt in immunoprecipitates (IP) or whole cell lysates (WCL) were measured by Western blot analysis. Amounts of PKCβ in Akt-IPs were quantified as fold change over the corresponding Akt signal.

Calcium-ions and DAG. The latter is a plasma membrane-resident product of PLC-activity and membrane recruitment of DAG-sensitive PKC-isoforms including PKCβ is typically used as an indicator of their activation (Newton, 2001: ; Parker and Murray-Rust, 2004). In naive B cells, PKCβ resides predominantly in the cytoplasm, while a small proportion is associated with the plasma membrane (figure 14B). BAFF-treatment increases the fraction of membrane-localized PKCβ, indicating BAFF-dependent activation of PKCβ. At the same time, Akt is enriched in the membrane fraction to a similar extend.Translocation of PKCβ into lipid rafts, a specialized ganglioside-rich plasma membrane subcompartment, is a critical event in BCR-induced NF-κB activation in B cells (Su, et al., 2002). Although a role of lipid rafts in BAFF signaling has not been reported, their reputation as important signaling platforms (Pierce, 2002) in combination with BAFF-induced enrichment of PKCβ at the plasma membrane prompted further investigation into a potential BAFF-dependent raft-association of PKCβ. However, an inducible recruitment of PKCβ into lipid rafts of BAFF-stimulated B cells is not observed (figure 14C). Supporting a raft-independent mechanism of BAFF-induced signaling, no association between rafts and Akt or PDK1 can be detected.

Concomitant BAFF-mediated activation of Akt and PKCβ as well as PKCβ-dependent Akt-phosphorylation *in vitro* suggest a functional correlation of the two proteins in BAFF-signaling. In further support of this hypothesis, BAFF-

treatment increases the physical association of Akt with PKCβ as judged from co-immunoprecipitation of PKCβ with anti-Akt serum (figure 14D).

In order to demonstrate the functional significance of these correlative observations, the phosphorylation state of Akt was determined in BAFF-treated B cells from PKCβ-deficient mice. BAFF-induced Akt-phosphorylation at S473, but not T308, is diminished compared to wild-type cells in the absence of PKCβ (figure 15A). This result, in conjunction with the phosphorylation of S473 but not T308 of Akt by PKCβ *in vitro* (Kawakami, et al., 2004), strongly suggests that PKCβ contributes to BAFF-induced Akt-activation through direct phosphorylation of the S473 residue. Because the previous results had indicated the contribution of a non-stimulus-specific

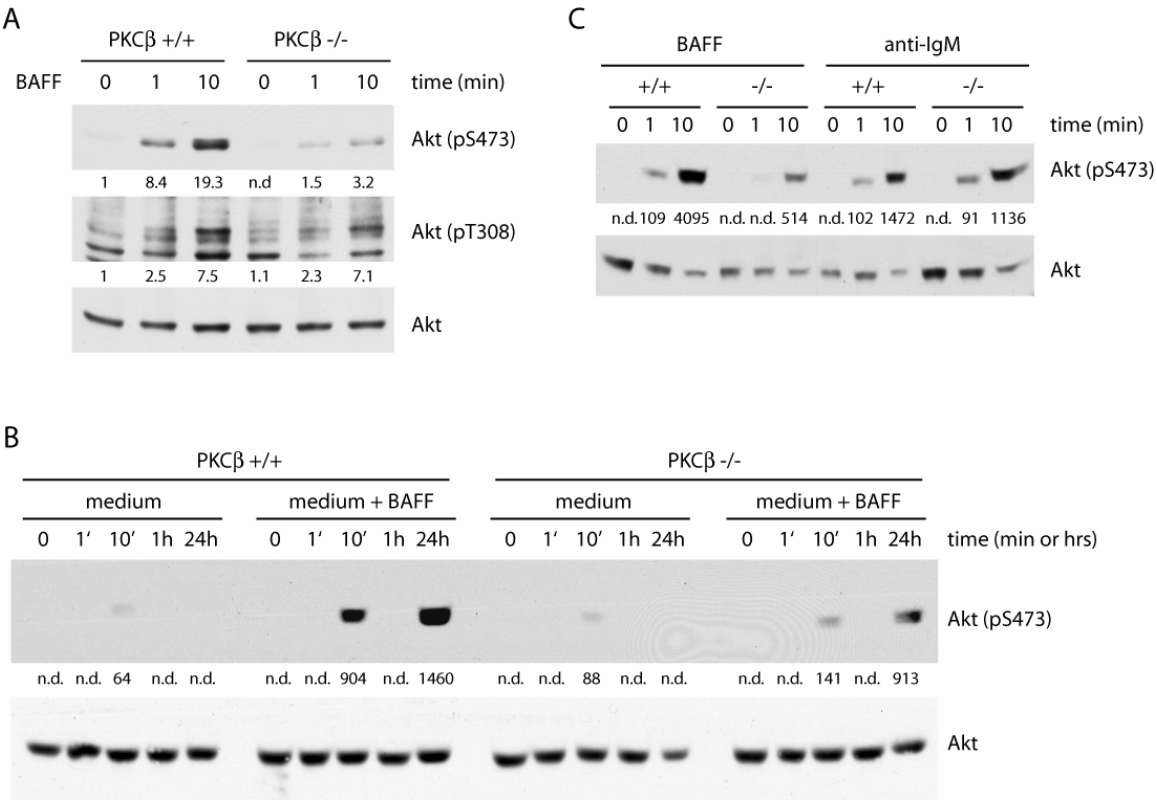


Figure 15: PKCβ controls BAFF-induced Akt phosphorylation. A. Akt phosphorylation levels in protein extracts derived from unstimulated and BAFF-treated B cells of PKCβ +/+ and PKCβ-deficient mice were measured and quantified as described in figure 11. B. Mature B cells from PKCβ-sufficient (right) and -deficient (left) mice were stimulated with BAFF or control medium and Akt-phosphorylation at S473 was analyzed and quantified as in A. C. B cells from PKCβ +/+ and PKCβ-deficient B cells were stimulated with BAFF or anti-IgM antibody as indicated. Akt phosphorylation at S473 was measured and quantified as in A.

component to BAFF-induced Akt-phosphorylation after 10 minutes, it was tested whether this could be the origin of the residual Akt-phosphorylation in PKCβ-deficient B cells. Mock-stimulation induces unspecific Akt-phosphorylation to the same extend in PKCβ-sufficient and -deficient cells (figure 15B). BAFF-treatment enhances this signal dramatically in wild- type cells and to a much lesser extend in PKCβ-null cells, suggesting that alternative kinases are in principle capable of Akt S473-phosphorylation following stimulation with BAFF. Akt-activation in PKCβ-deficient B cells has initially been described as normal (Kawakami, et al., 2004), prompting a differential analysis of Akt-activation in BAFF- versus BCR-stimulated B cells. Whereas lack of PKCβ has a strong impact on BAFF induced Akt-phosphorylation, the response to stimulation with anti-IgM is not impaired (figure 15C). This result illustrates the complexity of distinct Akt-activation mechanisms in which cell type and context appear to be the key determinants. It also highlights the specificity of the mechanism responsible for BAFF-mediated Akt phosphorylation.

3.1.4 Other BAFF-induced signaling events are PKCβ-independent

Given the significance of PKCβ in BAFF-induced Akt-activation, it is conceivable that PKCβ plays a universal role in BAFF signaling or that PKCβ acts specifically in Akt-activation. To test for a general function of PKCβ in BAFF-mediated responses, known molecular targets of BAFF signaling were examined in PKCβ-deficient B cells. Support for a potential role of PKCβ in BAFF-dependent NF-κB activity comes from the fact that it is known to play a critical role in NF-κB activation in BCR-stimulated B cells (Saijo, et al., 2002: ; Su, et al., 2002). However, as explained in detail in the previous section, NF-κB activation in response to antigenic or BAFF-stimulation occurs through distinct mechanisms. The canonical pathway downstream of BCR cross-linking relies on the IKK-mediated degradation of IκB-proteins, whereas BAFF promotes the processing of the NF-κB2 protein p100 to mature p52, increasing the fraction of transcriptionally competent p52/RelB dimers in the nucleus. Possibly due to these very different mechanisms, lack of PKCβ does not appear to have a discernable effect on BAFF-induced p100 processing to p52 (figure 16A).

Another BAFF-mediated survival mechanism concerns the subcellular localization of PKCδ. Unlike PKCβ, PKCδ is a member of the novel subfamily

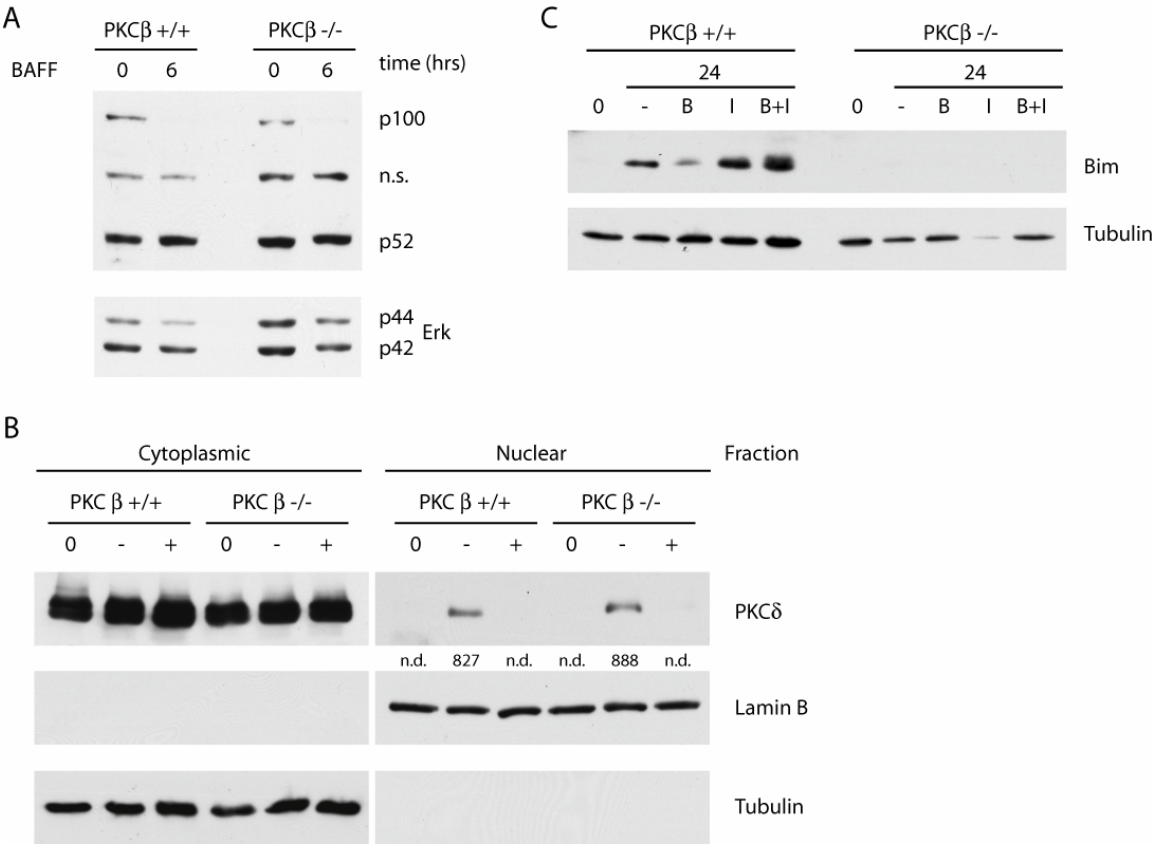


Figure 16: A. Unaltered BAFF-induced NF-κB2/p100 processing in the absence of PKCβ. The presence of p100 and p52 was determined by Western blot analysis using an antibody against NF-κB2. Protein loading was controlled by Erk (p44/p42) expression. B. Unaltered BAFF-induced cytoplasmic retention of PKCδ in the absence of PKCβ. Cytoplasmic and nuclear extracts were prepared from PKCβ +/+ and PKCβ-deficient B cells at time zero (0) and after 24 hrs of incubation in the absence (-) or presence (+) of BAFF. PKCδ expression was assessed by Western blotting. Fraction purity and protein loading were controlled by Western blotting using antibodies against tubulin and lamin B. C. Protein extracts were prepared from cells at time zero (0) or after 24 hrs of incubation in medium (-), with BAFF (B), with anti-IgM (I) or with BAFF and anti-IgM combined (B+I) and subjected to Western blot analysis using an antibody against Bim. Protein loading was controlled using anti-tubulin antibody.

of PKCs whose activity is sensitive to DAG but independent of Calcium ions. PKCδ also plays an important role in the regulation of B cell survival, but, where PKCβ- deficiency facilitates B cell death, lack of PKCδ is associated with exaggerated B cell survival (Mecklenbrauker, et al., 2002). The pro-apoptotic function of PKCδ in B cells is linked to its translocation to the nucleus where it causes histone 2B phosphorylation on serine 14, an early marker of apoptosis (Ajiro, 2000: ; Cheung, et al., 2003: ; Mecklenbrauker, et

al., 2004). In fresh *ex vivo* isolated B cells, PKCδ is exclusively cytoplasmic, but the fraction of nuclear PKCδ increases upon their *in vitro* culture concomitant with a decline in cell viability (Mecklenbrauker, et al., 2002). BAFF is able to contain the pro-apoptotic function of PKCδ by suppressing its nuclear accumulation. Lack of PKCβ does not alter the cytoplasmic retention of PKCδ in BAFF-treated B cells (figure 16B), indicating that this process occurs PKCβ-independently.

Finally, BAFF has been shown to influence the expression of Bim (Craxton, et al., 2005). As a member of the BH3-only subfamily of Bcl-2-proteins, Bim is pro-apoptotic and is strongly induced by anti-IgM stimulation in the absence of additional survival stimuli. In this context, co-stimulation with BAFF promotes Bim-phosphorylation which is a step towards its subsequent degradation. Remarkably, under conditions which normally induce Bim-expression in B cells, PKCβ-deficient B cells do not appear to upregulate Bim (figure 16C). This is surprising since a higher rather than lower content of pro-apoptotic Bim would be expected in cells harboring a survival defect. However, mutant B cell death is particularly rapid under conditions which provoke Bim-expression in a normal context. Thus, when cell extracts were prepared for the experiment shown in figure 16C, only 7% of PKCβ-deficient cells were alive 24 hours after anti-IgM stimulation, compared to 42% wild-type cells. The combined presence of BAFF improves the survival only moderately (19% live cells in PKCβ null cells versus 66% in wild-type). The exaggerated cell death and resulting drop in protein content may therefore in fact prevent Bim-detection. This precludes a conclusive analysis of whether BAFF-mediated Bim regulation requires PKCβ. Yet, it seems clear that elevated Bim-expression does not account for the diminished viability of PKCβ-deficient B cells in the absence or presence of BAFF.

In summary, it appears that PKCβ-deficiency specifically impairs Akt-activation in response to BAFF while other BAFF-induced signaling events proceed unperturbed or are not applicable in this particular cellular context.

3.1.5 PKCβ-deficiency impairs BAFF-induced cellular responses and causes a defect in peripheral B cell maturation

Given the requirement of PKCβ for certain but not all aspects of BAFF-induced signaling, the question arises, if and how BAFF-mediated cellular responses are affected by PKCβ-deficiency. To address this matter, wild-type

and PKCβ-null B cells were cultured in the absence or presence of BAFF and cell survival and growth were monitored over a period of four days (figure 17A). As expected from previous analysis (Saijo, et al., 2002), PKCβ-deficient B cells die more rapidly than wild-type cells in the absence of stimuli. Addition of BAFF significantly enhances the viability of both types of B cell, but does not fully restore the survival of PKCβ-deficient cells. Especially at later time points, the proportion of live PKCβ-null cells is significantly lower than in wild-type cultures. In addition to a partial defect in BAFF-mediated survival, PKCβ-deficient B cells are largely refractive to BAFF-induced cell growth (figure 17B). Neither growth nor viability of PKCβ-deficient cells is restored to wild-type levels using up to ten-fold higher BAFF concentrations (figure 17A and B). The survival defect of PKCβ-deficient cells therefore appears to differ mechanistically from that of auto-reactive cells whose susceptibility to cell death can be overcome by a higher BAFF-dose (Lesley, et al., 2004: ; Thien, et al., 2004). Along the same lines, flow-cytometric analysis of splenic B cells reveals that surface expression of BAFF-R is indistinguishable between PKCβ-sufficient and -deficient cells (figure 17C). In summary, these results support the notion that PKCβ-deficient cells harbor an intrinsic defect in BAFF-induced signaling.

Given the partial refractiveness of PKCβ-deficient B cells to BAFF-mediated signaling *in vitro*, it is important to establish whether this has an impact on the B cell compartment *in vivo*. As detailed previously, lack of BAFF-signaling in mice is characterized by a severe reduction in peripheral B cell numbers, with the remaining cells belonging for the most part to the IgM^{hi} $CD21/35^{lo}$ T1 stage and poor maturation to the IgM^{hi} $CD21/35^{hi}$ T2 and IgM^{int} $CD21/35^{hi}$ mature stage. While alteration of the B cell compartment in the absence of PKCβ is less dramatic, it display characteristic features indicative of a suboptimal BAFF-function. First, splenic B cell numbers in PKCβ knockout mice are reduced to approximately 70% of wild-type figures (Leitges, et al., 1996) compared to 10 - 20% in the absence of BAFF survival signaling (Lentz, et al., 1996: ; Sasaki, et al., 2004: ; Schiemann, et al., 2001: ; Shulga-Morskaya, et al., 2004). Second, flow-cytometric analysis of splenocytes reveals that the ratio between immature and mature B cells is shifted in PKCβ-deficient mice such that there is an enrichment of IgM^{hi} IgD^{lo} cells with a concomitant reduction in IgM^{lo} IgD^{hi} cells (figure 17D). Closer inspection of transitional B cell stages using the maturation marker CD21/35 shows that this coincides with a higher frequency of T1 cells over a reduction in T2 and mature cells. Collectively, the phenotype of the B cell compartment in PKCβ-

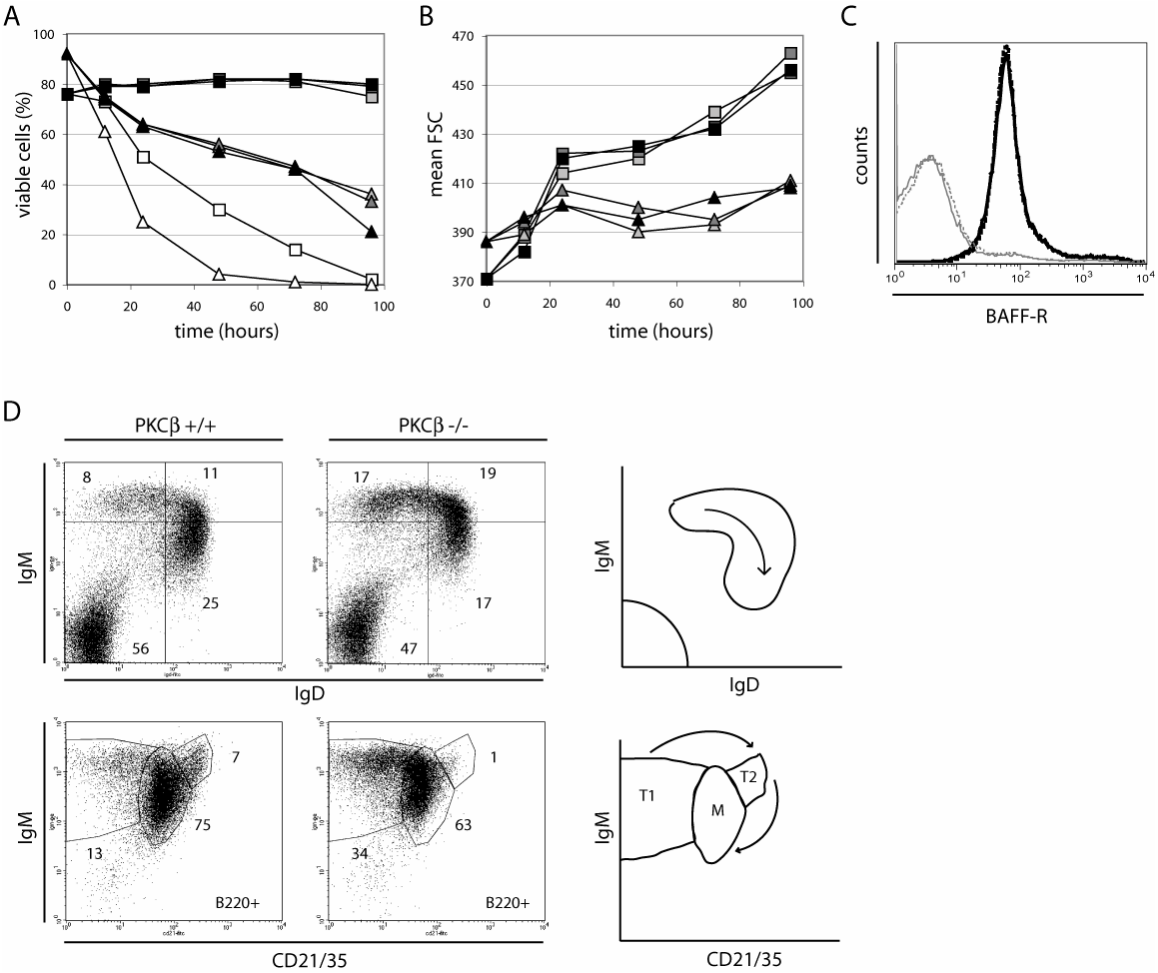


Figure 17: BAFF-induced survival of PKCβ-deficient B cells is impaired *in vitro* and *in vivo*. A. B cells from PKCβ +/+ (squares) and PKCβ-deficient (triangles) B cells were cultured in medium alone (white symbols) or in the presence of 25 ng/ml (light grey symbols), 100 ng/ml (dark grey symbols) or 250 ng/ml (black symbols) of BAFF and the frequencies of viable B cells were measured by FACS analysis. B. The cell size of live BAFF-treated PKCβ +/+ (squares) and PKCβ-deficient (triangles) B cells was measured by FACS-analysis of the forward scatter (FSC). Colors represent BAFF-concentrations as in A. C. BAFF-R expression on splenic B220^{+} cells from PKCβ +/+ (solid black line) and PKCβ-deficient (dashed black line) was measured by FACS. Thin grey lines represent staining with an isotype control antibody. D. Maturity of splenic B cells from PKCβ +/+ (left) and PKCβ-deficient (right) mice was assessed by expression of the surface markers IgD versus IgM (upper panel) and CD21/35 versus IgM (lower panel). Only live lymphocytes are shown and the lower panel is gated on B220^{+} cells. Numbers in the upper panel represent frequencies of cells in the respective quadrants. Gates in the lower panel and numbers correspond to B cell developmental stages T1 (IgM^{hi} CD21/35^{lo}), T2 (IgM^{hi} CD21/35^{hi}) and mature (M, IgM^{int} CD21/35^{int}).

deficient mice is indicative of a partially compromised ability to utilize BAFF-

mediated survival signals and this correlates well with the responses of PKCβ-null B cells to BAFF-stimulation *in vitro*. It also illustrates the physiological importance of BAFF-mediated Akt-activation to mature B cell homeostasis and suggests that this mechanism acts in concert with other BAFF-induced signaling events including NF-κB-activation, control of PKCδ subcellular localization and Bim-expression to regulate BAFF-dependent B cell responses.

3.2 Part 2: The role of Syk in B cell survival

3.2.1 Arrested B cell development in Syk-deficient mice

In previously published reports, Syk inactivation in mice through conventional gene targeting had resulted in perinatal lethality. Chimeric mice, generated by adoptive transfer of mutant fetal liver cells into immunodeficient hosts, had revealed a requirement for Syk during early B cell development at the transition from the pro B to the pre B stage. This has precluded an assessment of the role of Syk in mature B cells. In order to overcome this problem, a conditional Syk knockout was generated in which Cre-mediated recombination results in gene inactivation. A brief overview of the gene targeting procedure, conducted by Christian Schmedt, has been given previously. More detailed information is published elsewhere (Saijo, et al., 2003).

In order to create a Syk null allele (Syk Δ), mice with floxed Syk loci (Syk fl/fl) were bred to a CMV-Cre strain which mediates deletion in the germ line (Schwenk, et al., 1995). Surprisingly, intercrosses of Syk fl/Δ mice occasionally gave rise to viable Syk-deficient mice (Syk Δ/Δ), albeit at a very low frequency. Such mice generally have a reduced live span and profound internal hemorrhaging (data not shown), as expected from the deficits in vascular development in Syk-deficient embryos (Abtahian, et al., 2003). Yet, those mice surviving to adulthood allowed for an assessment of the B cell compartment in Syk null mice. Lymphocyte subpopulations were analyzed by surface staining of single cell suspensions from lymphoid organs including bone marrow, spleen and peritoneum. Flow-cytometric analysis of B lymphopoiesis in the bone marrow reveals an enrichment of $B220^+$ $CD43^+$ $CD25^-$ pro B cells, concomitant with a severe reduction of $B220^+$ $CD43^-$ $CD25^+$ pre B, $B220^+$ IgM^{lo} immature B and $B220^+$ IgM^{hi} recirculating B cells in Syk null mice (figure 18A). Consequently, B cells, as defined by the pan-B cell marker B220, are essentially absent from the spleens, lymph nodes and peripheral blood of mutant mice (figure 18B and data not shown). Furthermore, the peritoneal cavity of Syk-null mice is devoid of B cells from either the B-1 or the conventional B-2 lineage and this applies to both B-1a and B-1b cells, two B-1 cells subsets distinguished by the surface marker CD5 (figure 18C).

Collectively, Syk appears to be indispensable during development of both B-1 and B-2 lineage B cells. The latter is specifically affected early on at the transition from the pro to the pre B stage. By and large, the B cell

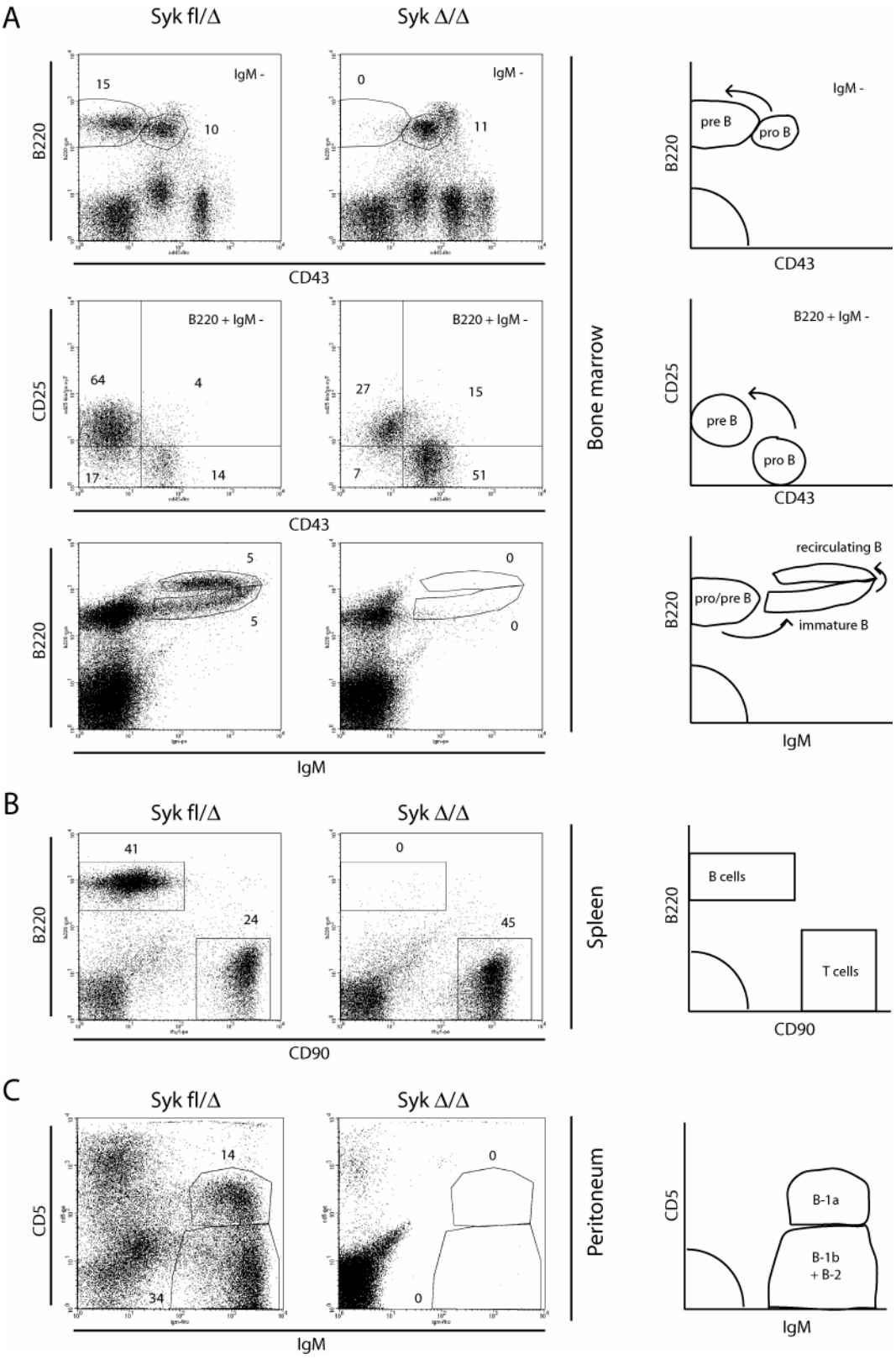


Figure 18: B cell development in Syk-deficient mice. A. Bone marrow cells were isolated from Syk-deficient (right) and control (left) mice and analyzed for surface expression of B220, CD43, CD25 and IgM by flow cytometry. B. The frequency of B220+ B cells and CD90^{+} T cells in splenocytes from Syk-deficient (right) and control (left)

mice. C. The frequency of B-1a (IgM^+, $CD5^+$) and B-1b/B-2 (IgM^+, $CD5^-$) lineage cells in the peritoneal cavity of Syk-deficient (right) and control (left) mice. Numbers in A, B and C represent the frequency of cells within the indicated gates which correspond to the different cell populations outlined in the diagrams. Only live lymphocytes are shown and plots in A are gated on IgM^- (top) and $B220^+$ IgM^- (middle) cells.

compartment in Syk Δ/Δ mice concurs with the phenotype reported for Syk-mutant fetal liver chimeras (Cheng, et al., 1995: ; Turner, et al., 1995).

3.2.2 Aberrant B cell development in the absence of Syk is a B cell intrinsic phenomenon

To address whether arrested B cell development in Syk-deficient mice is caused by a B cell intrinsic defect or whether Syk-deficiency in other cell types contributes to the phenotype, Syk floxed mice were crossed to CD19-Cre mice (Rickert, et al., 1997). In this strain, the Cre-recombinase coding sequence is inserted into the CD19 locus and is expressed under the control of the CD19 promoter in place of the CD19 gene. In order to prevent any potential phenotypic effects due to CD19-deficiency, all mice used in the subsequent experiments retained at least one wild-type CD19 allele (CD19 +/+ or CD19 +/Cre) and CD19 +/Cre mice are referred to as CD19-Cre positive. CD19 is a B cell specific marker whose expression begins at the late pro B stage. B lineage specific Cre-mediated recombination in CD19-Cre mice sets in at the pro B stage and its efficiency increases up to the mature B cell stage (Schwenk, et al., 1997). Analysis of the B cell compartment in different lymphoid organs by surface staining and flow-cytometric analysis of single cell suspensions reveals a reduction in the frequency of pre B, immature B and recirculating B cells in the bone marrow of Syk fl/Δ CD19-Cre mice compared to Syk wild-type (Syk +/fl) and heterozygous (Syk +/fl CD19-Cre, Syk fl/Δ) mice (figure 19A). Peripheral B cells in the spleen of Syk fl/Δ CD19-Cre mice are reduced to approximately a third of wild-type frequencies (figure 19B), consistent with a decline in total splenic B cell numbers to a similar extend (figure 19C). This phenotype is reminiscent albeit significantly milder than that of Syk-deficient mice. Two reasons could potentially account for the discrepancy: Lack of Syk in non-B cells could indeed be responsible for the aggravated B cell phenotype of Syk null mice or, more trivially, Cre-recombinase expression under control of the CD19 promoter might be

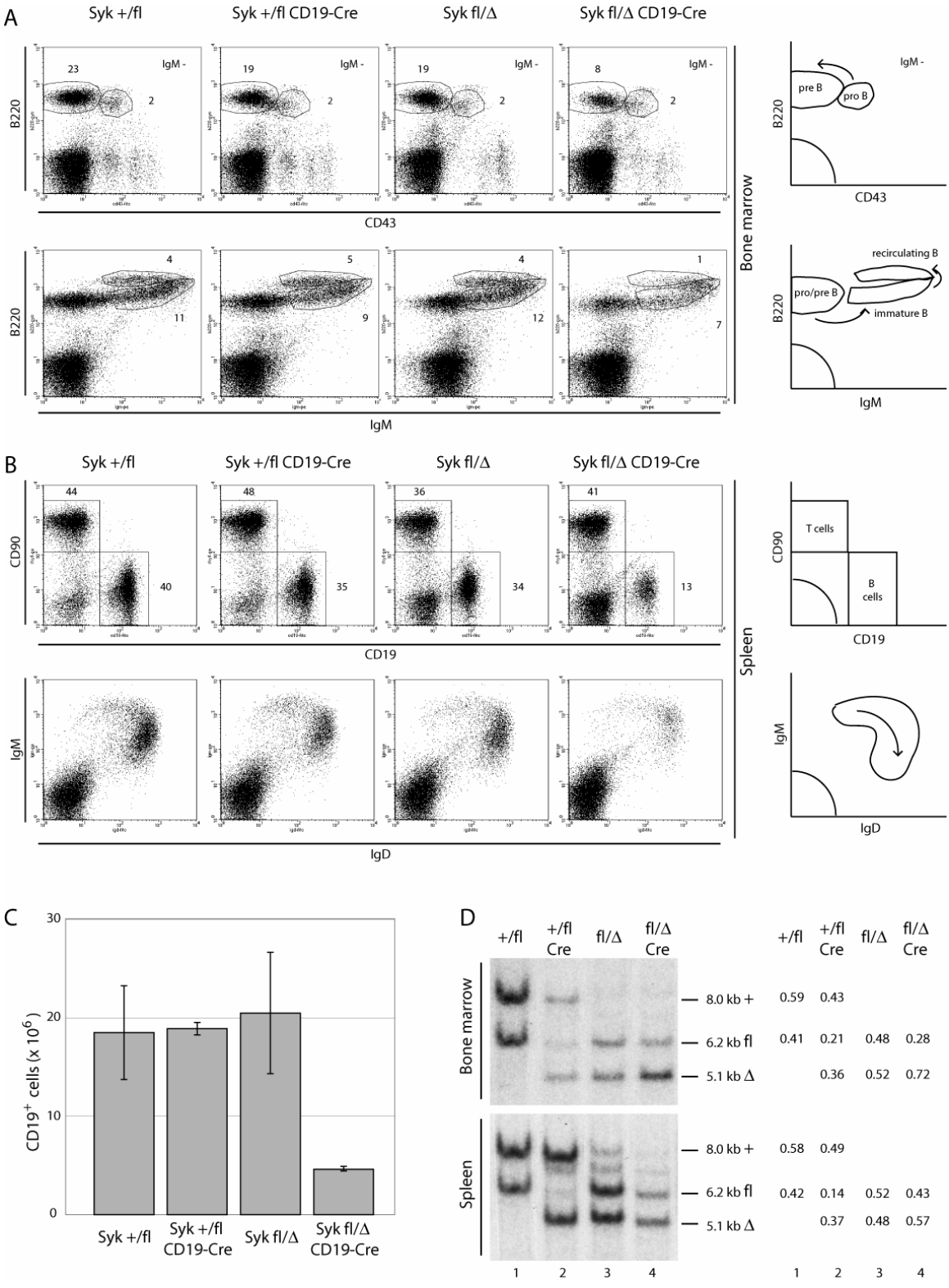


Figure 19: B cell development upon conditional Syk-inactivation in response to CD19-expression. Cells were isolated from Syk +/fl and Syk fl/Δ mice harboring two wild-type CD19-alleles or one wild-type and one CD19-Cre allele. A. B cell development in the bone marrow was analyzed as in figure 18 based on surface expression of B220, CD43 and IgM. B. Splenocytes were analyzed by flow cytometry for surface expression of

CD90, CD19, IgM and IgD. C. Absolute numbers of $CD19^+$ cells in the spleen. Error bars represent the standard deviation based on two independent experiments. D. Efficiency of CD19-Cre-mediated Syk-deletion. Syk-deletion was measured by Southern blot analysis of purified B cells isolated from bone marrow (top) and spleen (bottom). The positions of the wild-type (+), floxed (fl) and deleted (Δ) alleles are indicated. Numbers represent the relative signal intensity as a fraction of 1.0 for each lane. Numbers in A and B represent the frequency of cells within the indicated gates which correspond to the different cell populations outlined in the diagrams. Only live lymphocytes are shown and the top panel in A is gated on IgM^- cells.

insufficient to ensure complete Syk-inactivation. In the latter case, the majority of B cells present in Syk fl/Δ CD19-Cre mice would retain the floxed Syk allele and therefore essentially be wild-type cells. This scenario is not unlikely given that escape from Syk-deletion would afford the respective cells an enormous advantage in the chance for further developmental progression and survival, whereas Syk-deletion would be expected to cause their demise. The possibility of incomplete CD19-Cre mediated Syk-deletion was assessed by genotyping of purified B cells derived from bone marrow and spleen using a Southern blot strategy, which distinguishes between the wild-type (+), the floxed (fl) and the deleted (Δ) Syk allele. CD19-Cre mediated Syk-deletion works well in principle as evidenced by the near complete conversion of the floxed to the deleted Syk allele in those cells which retain the Cre-insensitive wild-type allele (figure 19D, compare lanes 1 and 2). In contrast, the floxed allele of Syk fl/Δ CD19-Cre mice is only marginally altered by CD19-Cre-mediated recombination (figure 19D, compare lanes 3 and 4). The latter observation is more pronounced in B cells isolated from the spleen (figure 19D bottom) than from the bone marrow (figure 19D top), indicating that the latter contains a higher frequency of Syk-deleted cells. The most likely explanation for incomplete Syk-deletion in splenic B cells from Syk fl/Δ CD19-Cre mice is that CD19-Cre-mediated Syk deletion does not work 100% effectively and that Syk-deleted cells disappear rapidly and thus cannot be detected by flow-cytometry, resulting in a gross enrichment of the fraction of cells which escape CD19-Cre-mediated recombination. In summary, Syk-inactivation beginning at the late pro B stage effects B cell development in a similar manner as complete absence of Syk in mice. The considerable phenotypic difference between Syk null and Syk fl/Δ CD19-Cre mice results from incomplete CD19-Cre mediated Syk inactivation and counter-selection of Syk-deleted cells such that the sizable B cell population of Syk fl/Δ CD19-Cre

mice essentially consists of wild-type cells. This experiment demonstrates that the requirement for Syk during early B lymphopoiesis is indeed a B cell intrinsic phenomenon. Furthermore, it shows that Syk-expression is indispensable for development and survival beyond the late pro B cell stage.

3.2.3 Syk is indispensable for mature B cell survival

While some informative conclusions could be made from CD19-Cre mediated Syk-inactivation, it turned out not to be suitable for determining the function of Syk in mature B cells, since it did not rescue the developmental block at the pro to pre B transition caused by Syk-deficiency. To overcome this drawback, a different Cre-strain was used in which Cre-recombinase is expressed transgenically under control of the Mx-promoter (Kuhn, et al., 1995). The Mx-protein is part of the cellular antiviral response and, as such, its expression is strongly induced by type I interferons (IFNs) α and β. Exogenous administration of type I interferons or the double-stranded RNA analogue polyinosinic acid - polycytidylic acid (poly(I)-poly(C)) which is a potent interferon inducing agent, can be used to induce transient expression of Cre-recombinase in mice harboring the Mx-Cre transgene. This system offers the advantage of external manipulation of Cre-expression at any desired time point. However, Mx-controlled Cre-expression will not discriminate between different cell types such that Cre-mediated gene inactivation will in principle occur ubiquitously. This method has previously been used successfully to inducibly ablate surface BCR-expression and has demonstrated the dependency of mature resting B cells on tonic BCR-signaling (Lam, et al., 1997), suggesting Mx-Cre as a suitable system to assess the requirement for Syk in peripheral B cells. One important technical difference between the two cases, however, is the intracellular localization of Syk-protein in contrast to the surface-expressed BCR. This precludes a convenient detection of deleted cells by flow-cytometry. Yet, from the previous experience with CD19-Cre mice, it is evident that the distinction between Syk-retaining and Syk-deleted cells is vital for the appropriate interpretation of any data. To allow for straightforward detection of Syk-inactivation on a single cell level by flow-cytometry, the ROSA-GFP allele was introduced into Syk Mx-Cre mice by interbreeding. In the ROSA-GFP strain, a novel gene-cassette is inserted into the ROSA26 locus, a ubiquitously expressed genomic region of unknown function (Mao, et al., 2001). The novel sequence is composed of the green fluorescent protein (GFP) coding region, which is preceded by a loxP-flanked

stop-cassette, such that Cre-mediated recombination in ROSA-GFP mice will result in GFP-expression. Assuming that Cre-mediated deletion at the Syk- and the ROSA26-locus occurs with equal efficiency, Syk-deficient cells can be identified as GFP-positive by flow-cytometry in mice harboring the correct combination of alleles.

Trial experiments were conducted to optimize the time between the induction of Cre-mediated recombination and analysis. Assuming a function of Syk in tonic BCR survival signaling, the expectation would be that B cells collapse upon loss of Syk. In order to maximize the chances of detecting Syk-deficient cells *in vivo*, it would be desirable to analyze B cell populations immediately after the induction of deletion. However, administration of the inducing agent interferon or poly(I)-poly(C) by itself elicits an immune response which transiently perturbs the B cell compartment. Based on the results of trial experiments, mice were analyzed on the seventh day after receiving a single dose of 300 µg poly(I)-poly(C) by intraperitoneal (i.p.) injection. Flow-cytometric analysis reveals that the $B220^+$ B cell compartment in the bone marrow is overwhelmingly GFP^+ in both Syk +/fl ROSA-GFP and Syk fl/Δ ROSA-GFP mice expressing Mx-Cre compared to those not carrying the Mx-Cre transgene (figure 20A, bottom row). In accordance, analysis of the bone marrow-resident B cell subpopulations shows a familiar pattern of changes associated with loss of Syk in Syk fl/Δ Mx-Cre ROSA-GFP mice, including enrichment of the pro B fraction versus decline in the populations of pre, immature and recirculating B cells (figure 20A, top and middle rows). Together, these observations argue for efficient Cre-mediated recombination at both the Syk- and the ROSA26-locus in the bone marrow upon induction of Cre-expression. Analysis of GFP-expression in Syk +/fl Mx-Cre ROSA-GFP mice reveals that Mx-Cre-mediated recombination is less efficient in the spleen than in the bone marrow, with only approximately 20% of splenic B cells becoming GFP^+ (figure 20B, bottom row). Despite the modest extend of Mx-Cre-mediated recombination in the spleen, it is important to note the difference in the frequency of GFP^+ B cells in the presence or absence of a wild-type Cre-insensitive Syk allele. In contrast to Syk +/fl Mx-Cre ROSA-GFP mice, a GFP^+ B cell population is largely absent in the spleen of Syk fl/Δ Mx-Cre ROSA-GFP mice. This is not due to differences in deletion efficiency between individual mice, as the frequency of GFP^+ non-B cells is not negatively affected by the absence of a wild-type Syk allele. This result indicates that B cells are unable to sustain survival upon loss of Syk and is in line with the lower frequency of $B220^+$ B cells in spleens of Syk fl/Δ Mx-Cre

ROSA-GFP mice (figure 20B, top row).

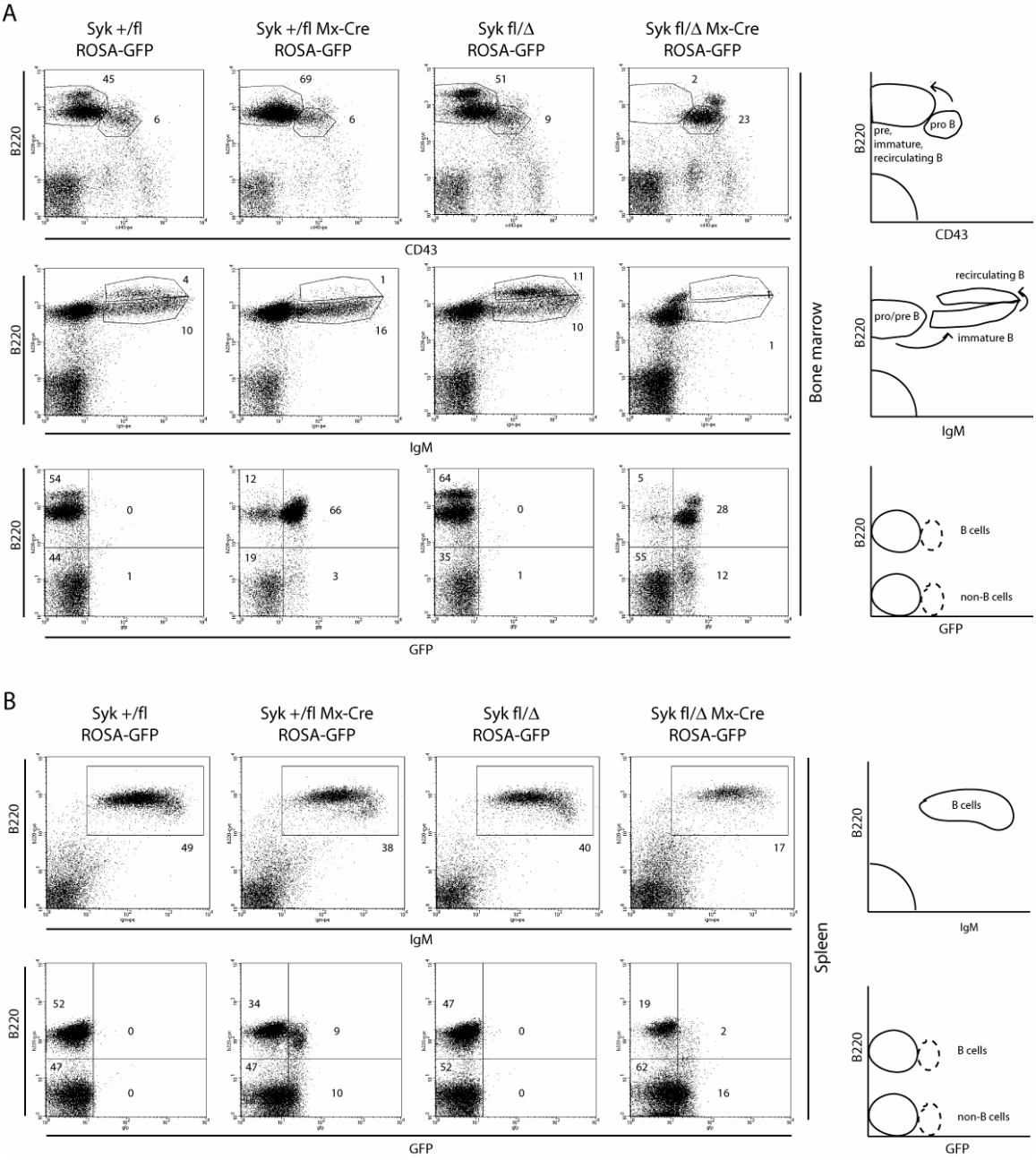


Figure 20: B cell development upon induction of Mx-Cre mediated recombination in mice harboring Cre-sensitive Syk and GFP-alleles as described in the text. A. B cell development in the bone marrow was analyzed as in figure 18 based on surface expression of B220, CD43 and IgM (top two panels). The efficiency of Syk-deletion is proportional to the fraction of GFP$^+$ cells (lower panel). B. The frequency of B cells was measured in splenocytes by expression analysis of the surface markers B220 and IgM (top panel). The efficiency of Syk-deletion is proportional to the fraction of GFP$^+$ cells (lower panel). Numbers in A and B represent the frequency of cells within the indicated gates or quadrants which correspond to the different cell populations outlined in the diagrams. Only live lymphocytes are shown.

In conclusion, Syk-deficiency appears to be incompatible with mature B cell survival supporting the hypothesis that Syk is a critical mediator of the tonic BCR survival signal.

3.2.4 Development of an experimental system for inducible Syk-ablation *in vitro*

The hypothesis that Syk is a mediator of the tonic survival signal initiating from the BCR-complex, traditionally originates from its function in B cell development and the activation of its kinase activity upon BCR-ligation. The result of the previous experiment additionally demonstrates its indispensability for mature resting B cell survival. Yet, none of the above observations offer any potential mechanistic explanation as to how Syk exerts its survival function during tonic BCR signaling. In quiescent B cells, Syk is either catalytically inactive or its activity is below the detection limit, complicating the search for potential downstream targets of Syk which translate the tonic signal to the actual executers of cell survival (Hutchcroft, et al., 1991: ; Law, et al., 1994: ; Yamada, et al., 1993). One way to possibly identify such factors could be to closely follow the course of cellular and molecular changes immediately following Syk-ablation. The feasibility of this approach, however, is hampered by technical limitations, which are unavoidable features of the standard procedure for gene inactivation based on Cre-recombinase. First, the above experiments in which Syk was inactivated by Cre-expression in mice, have demonstrated that the generation of Syk-deficient B cells *in vivo* is not a feasible approach as peripheral B cells either disappear or escape deletion. Second, for the purpose of examining the early stages of the cellular response to Syk removal, it would be necessary to induce Syk deletion *in vitro*. In the past, two different strategies have been employed to deliver Cre-recombinase into live cells *in vitro*, namely infection with a Cre-recombinase-expressing virus or cell transduction with a membrane-permeable Cre-protein. In lymphocytes, retroviral transduction is most commonly used to achieve Cre-mediated deletion of floxed alleles. However, this method works only on dividing cells which precludes an application on resting B cells. More recently, the ability of lentiviruses which constitute a subfamily within the retroviridae, to integrate into the genome of non-dividing cells has been exploited to deliver Cre-recombinase into cell and tissue types which had previously been unsusceptible to retroviral transduction (Naldini, et al., 1996). However,

successful use of this technology in lymphocytes has not been widely reported and an effort to establish this procedure for the purpose of Syk-deletion yielded good results in cell lines and stimulated, proliferating but not quiescent *ex vivo* isolated B cells (data not shown). An alternative to viral infection is the use of a Cre-recombinase fusion protein which contains a signal peptide that enables its translocation from the extracellular medium into live cells. This peptide is derived from the TAT protein of HIV, hence the name TAT-Cre denoting the fusion protein. TAT-Cre has been successfully employed for conditional gene inactivation in embryonic stem cells, fibroblasts and splenocytes (Peitz, et al., 2002). Yet, no application on resting B cells has been reported to date. In trial experiments using B cells isolated from ROSA-GFP mice, TAT-Cre-mediated recombination turned out to be highly ineffective as judged by GFP-expression in B cells unless stimulated with a mitogen (data not shown). Collectively, these standard Cre-based approaches did not appear suited to achieve rapid Syk-inactivation in resting B cells.

Another concern associated with Cre-mediated recombination in B cells *in vitro* is the time frame. Unless cultured in the presence of some form of stimulus, B cell viability rapidly declines over the course of only a few days. On the other hand, the Cre-based methods of gene inactivation described above usually take 24 hours or more to show an effect on the DNA level and possibly even longer on the protein level, depending on the stability of the target. This lengthy time frame would interfere with B cell viability *in vitro* in general and especially with the objective of examining early cellular changes specifically related to Syk-removal. Prompted by these restrictions, I sought to develop a different system for rapid inducible Syk inactivation. In the strategy I followed, the goal is to abruptly disrupt Syk function through its cleavage by a target-specific protease. One protease with a highly conserved target sequence is the NIa-protease derived from Tobacco Etch Virus (TEV). TEV-protease specifically recognizes the target sequence ENLYFQS with cleavage occurring between the glutamine and the serine (Carrington and Dougherty, 1988: ; Dougherty, et al., 1989). Database searching using BLAST did not detect this sequence as a natural component of the mouse proteome (data not shown). TEV-protease has a long-standing history in biochemistry, where it is commonly used to remove affinity tags from recombinant proteins (Lucast, et al., 2001). Beyond its *in vitro* application, TEV-protease has already been ectopically expressed in eukaryotic cells where it is active without showing obvious signs of cytotoxicity (Uhlmann, et al., 2000). All these factors made TEV my protease of choice for inducible Syk inactivation. The aim was to

create a modified version of Syk which contains the TEV-protease target site, but otherwise behaves like the wild-type protein. Introduction of TEV-protease should lead to Syk cleavage and its instant inactivation (figure 21A).

The first step towards the development of such a system was to clone the full-length Syk cDNA from a B cell RNA library. Details of this procedure are given in the methods section. Subsequently, a 39 base pair insertion was introduced at strategic sites within the Syk coding sequence through site-directed mutagenesis. The insertion encodes the seven amino acid TEV target sequence flanked by a three amino acid linker on either side (figure 21B). All insertion sites were located within the kinase domain with the intention that TEV-mediated cleavage should instantly abolish Syk catalytic activity. While the molecular structure of Syk has yet to be determined, crystal structures from several different kinase domains are available. Sequence alignments of these kinase domains with that of Syk revealed that the EGFR kinase domain shares the highest sequence homology to Syk's (41% identity, for comparison: the kinase domain of the closely related ZAP-70 shares 63% identity to Syk (data not shown). Given this large extend of homology as well as the generally high degree of structural conservation between the kinase domains of different proteins in the active state (Huse and Kuriyan, 2002), Syk insertion sites were chosen based on the EGFR-structure (Stamos, et al., 2002). Sites were selected for easy accessibility to the protease while avoiding critical catalytic features like the activation loop (figure 21C). Based on these criteria, six different Syk-mutants (X1 through 6) were generated which contain the TEV-target sequence at different positions within the Syk kinase domain (figure 21B).

To confirm whether the modified kinases were susceptible to TEV-mediated cleavage as anticipated, both the different Syk versions as well as TEV-protease were each cloned into a mammalian expression vector and transiently expressed in HEK 293 cells. Gross phenotypic examination did not reveal any discernable effect of TEV-expression on cell viability. Cell lysates were analyzed by SDS-PAGE and Western blotting using an N-terminal anti-Syk serum and anti-FLAG-tag antibody to detect FLAG-TEV (figure 21D). Full-length wild-type as well as modified Syk migrates at approximately 72 kD in the absence of TEV. Faster migrating fragments appear upon co-expression of the Syk-mutants, but not the wild-type protein, with TEV-protease. The size of the fragments corresponds to the calculated size of the respective N-terminal Syk cleavage products. To exclude the possibility that TEV-mediated Syk-cleavage could occur in cell extracts following cell

disruption, iodoacetamide was added to the lysis buffer at concentrations

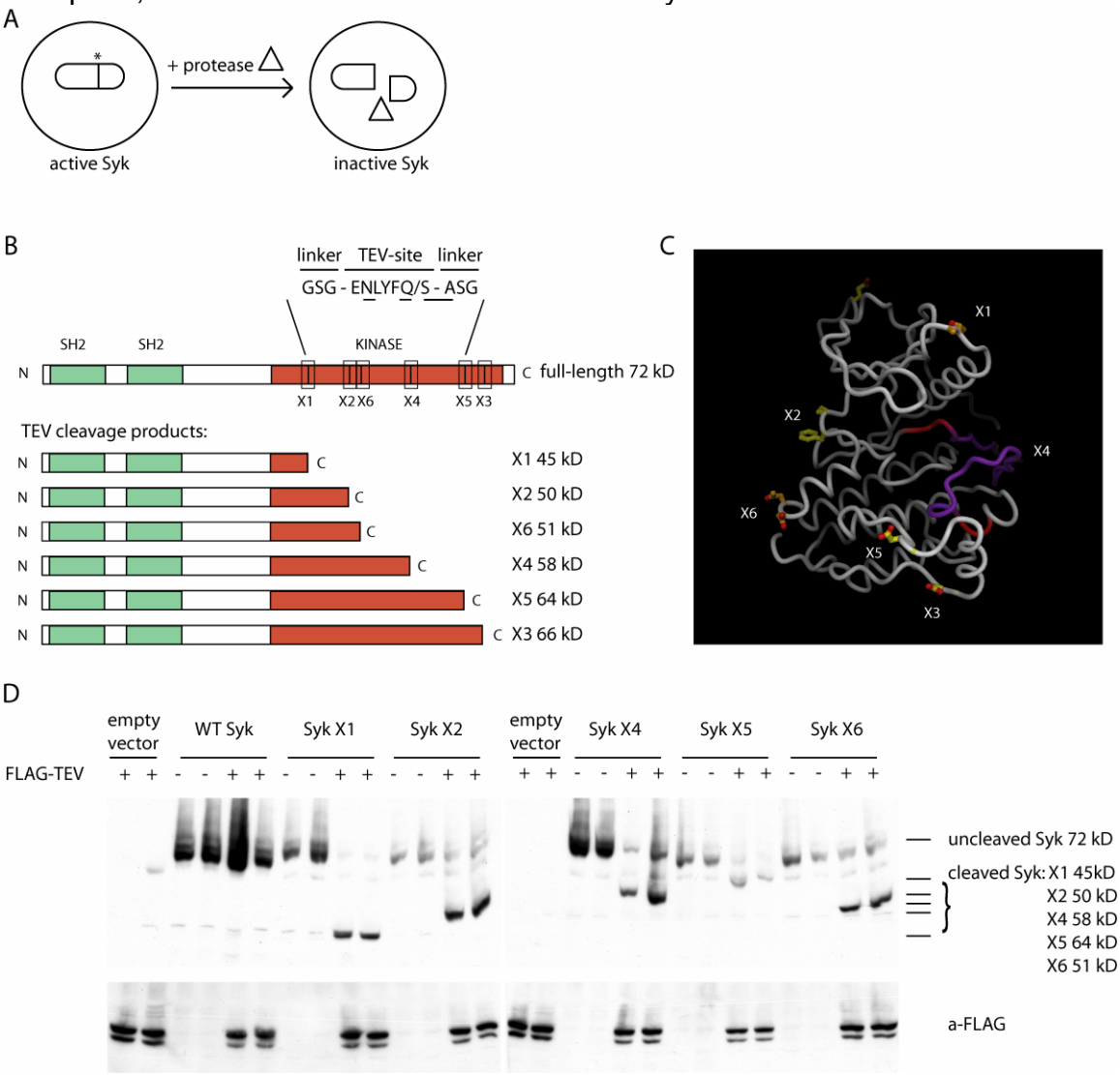


Figure 21: Development of an experimental system for rapid inducible ablation of Syk in mature B cells. A. Strategy for rapid inducible Syk inactivation through its proteolytic cleavage inside the cell. See text for details. B. The Syk domain structure indicating sites X1 through X6 and the inserted sequence including the TEV target site. Amino acid residues essential for TEV proteolytic activity are underlined and the slash indicates the site of cleavage between the conserved glutamine and serine. N-terminal Syk products resulting from TEV-mediated cleavage at sites X1 through X6 are depicted together with their expected size. C. Model of the Syk kinase domain shown in coil representation, generated using Modeller (Fiser and Sali, 2003) and the atomic coordinates of the EGFR kinase domain (PDB accession code 1M14, Stamos et. al., 2002). The activation loop and its flanking boundaries are colored in purple and red, respectively. Residues corresponding to the cleavage sites X1 through X6 are highlighted in yellow ball-and-stick representation. D. TEV-mediated cleavage of Syk X1 through X6 *in vivo*. HEK 293 cells were transiently transfected with the indicated

Syk-constructs in the absence or presence of FLAG-TEV. Cell lysates were subjected to immunoblot analysis using an N-terminal anti-Syk antiserum (top panel) and anti-FLAG antibody (lower panel). The sizes of full-length Syk and its N-terminal cleavage products are indicated.

under which TEV-activity is irreversibly inhibited (Ehrmann, et al., 1997). This result implies that TEV is indeed proteolytically active within live cells. TEV-protease appears as a double band in Western blotting with the lower band representing a catalytically inactive auto-cleavage product (Parks, et al., 1995).

The next aim was to determine, whether the modified Syk-versions were still capable of their normal functions to the same extend as the wild-type protein. This is a considerable concern given that the insertion of thirteen amino acids into the catalytically sensitive region of an enzyme may well not be tolerated. To assess whether insertion of the TEV target site was compatible with normal Syk-function, I made use of a Syk-deficient variant of the chicken B cell line DT40 (Takata, et al., 1994). DT40 cells are a popular model for studying BCR-induced signaling events. This is partially due to their high susceptibility to gene targeting, enabling the relatively easy deletion of individual signaling proteins (Buerstedde and Takeda, 1991). Furthermore, DT40 cells respond to BCR-ligation by inducing global tyrosine phosphorylation and Ca^{2+}-flux. Importantly, both of these responses are abrogated in Syk-null DT40 cells and can thus serve as a readout of Syk functionality upon reconstitution of Syk-deficient cells with the mutant Syk-versions in question. Wild-type and mutant Syk constructs were introduced into Syk-deficient DT40 cells by infection with a pantropic retro-virus in which the transgene precedes an IRES-GFP. Details regarding virus production and infection are given in the methods section. Infected cells were identified based on GFP-expression and positive clones were isolated by single cell sorting. Syk-expression was confirmed by Western blot in selected single clones which were used in subsequent experiments. To examine BCR-induced global tyrosine phosphorylation, cells were stimulated for two and five minutes with the anti-chicken IgM antibody M-4 and cell lysates were analyzed by Western blot using anti-phosphotyrosine antibody (figure 22A). BCR-ligation induces an increase in total tyrosine phosphorylation in Syk-sufficient but not -deficient cells and reconstitution of the latter with wild-type Syk restores the response as expected. Importantly, of the Syk-variants containing the TEV

target site, both X1 and X6 are capable of transducing the BCR-mediated

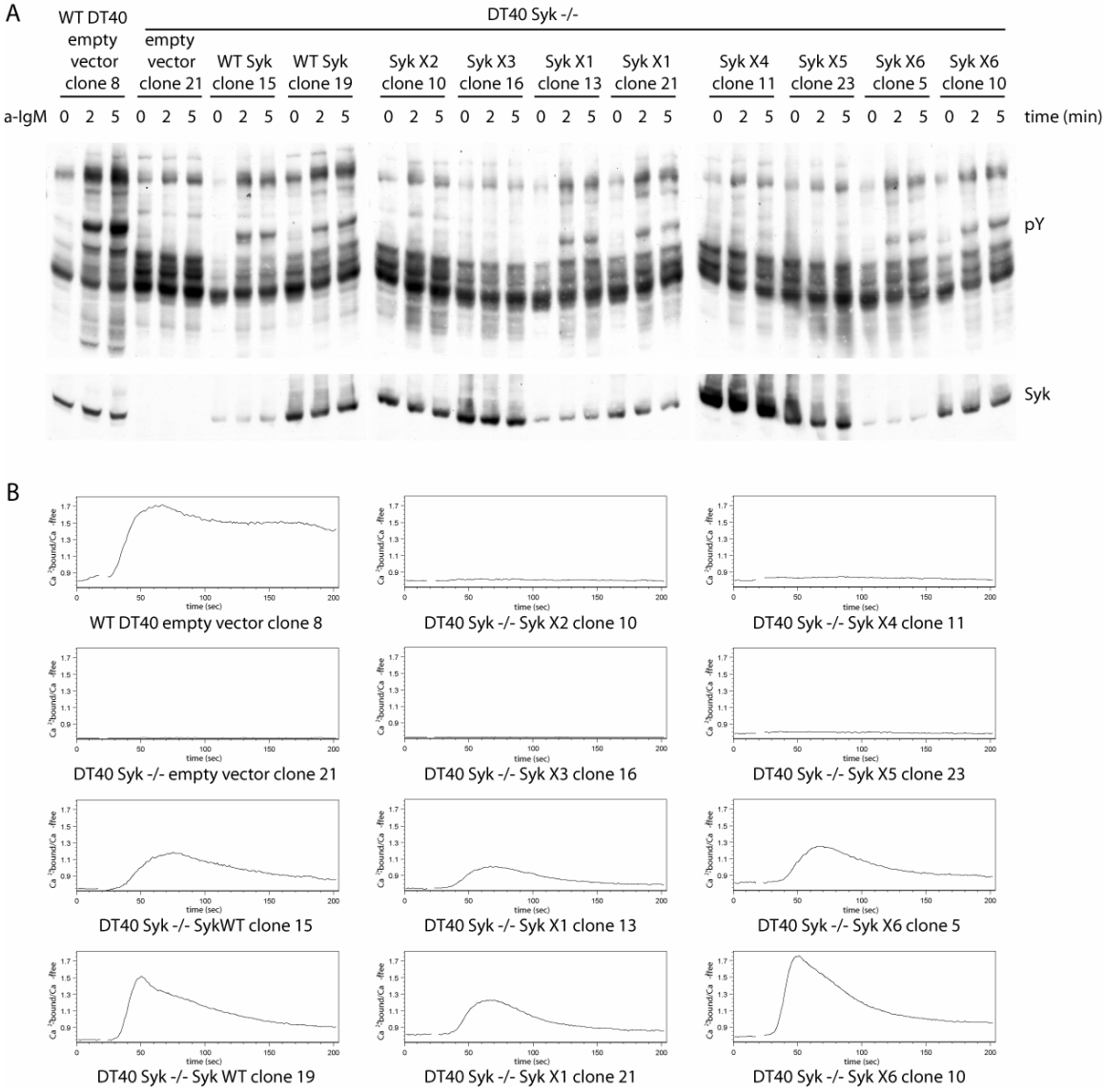


Figure 22: Functionality of the Syk mutants X1 through X6. DT40 cell clones expressing the indicated Syk constructs or empty vector were generated as described in the text. A. The indicated DT40 clones were stimulated with anti-chicken IgM antibody M-4 for 2 and 5 minutes. Cell lysates were subjected to immunoblot analysis using anti-phosphotyrosine (pY) antibody (top panel) and anti-Syk serum (bottom panel). B. The indicated DT40 clones were loaded with Indo-I, stimulated with anti-chicken IgM antibody M-4 and the ratio of Ca^{2+}-bound to Ca^{2+}-free dye was recorded for 200 seconds in a BD LSR flow-cytometer equipped with a UV laser.

signal while the mutants X2 through X5 remain insensitive to IgM-stimulation. Essentially very similar results were obtained when cells, loaded with a Calcium-sensitive fluorescent dye, were stimulated with anti-IgM and the ratio of Calcium-bound to Calcium-free dye was followed by flow cytometry (figure

22B). BCR cross-linking induces Calcium flux in wild-type cells as well as in reconstituted clones expressing either wild-type Syk or the variants X1 or X6, while Syk null cells and those expressing Syk X2 through X5 fail to respond to the stimulus. Identical responses were observed for several clones from each group expressing the same construct; at least two clones from the unresponsive and five clones from the responsive groups (figure 22B and data not shown). In summary, the Syk variants X1 and X6 are functional in transducing BCR-mediated responses to a degree roughly similar to the wild-type protein despite their carrying the TEV-target site.

Having generated Syk variants which are functional in BCR-mediated signal transduction as well as susceptible to cleavage by TEV-protease, the next aim was to test for TEV-mediated Syk-inactivation. In order to achieve acute Syk-cleavage in cultured cells, several methods were considered of which the administration of an active cell-permeable TEV-protein was chosen for the following reasons: direct application of the active enzyme to the target represents the most straight forward and least invasive way of directly regulating Syk activity *in vitro* in a dose-dependent manner. It avoids extensive cell manipulation which would be required for transgenic TEV-expression by viral infection. A potential alternative in the form of a mouse model harboring an inducible TEV-transgene would create an additional set of challenges. First, one would have to find a reliable system for inducible gene expression in B cells in mice which is by no means a trivial task. Second, for the purpose of acute TEV-mediated Syk cleavage, inducible TEV gene expression harbors the disadvantage that accumulation of the active enzyme increases over time from the onset of TEV gene transcription whereas transduction of cell-permeable TEV-protease would allow for the delivery of a controlled of amount of active enzyme at a specific time point. Due to the common use of TEV for biochemical applications, established protocols for the purification of high amounts of the recombinant active protease exist (Lucast, et al., 2001: ; Parks, et al., 1995). Importantly, a variety of peptide-sequences have recently been identified whose attachment to a cargo enables the transduction of the entire fusion protein into live cells (figure 23A). Such peptides, collectively termed protein transduction domains (PTDs), have been derived from the TAT-protein of HIV (Nagahara, et al., 1998), the Kaposi fibroblast growth factor (Rojas, et al., 1998) and the *Drosophila melanogaster* transcription factor antennapedia (Derossi, et al., 1998).

Based on these considerations, I chose to prepare an array of recombinant fusion proteins composed of TEV and a PTD, with the expectation that such

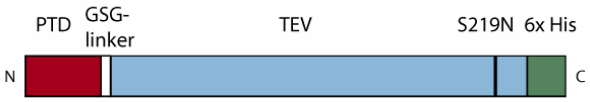
proteins should mediate intracellular Syk-cleavage upon their transduction

A

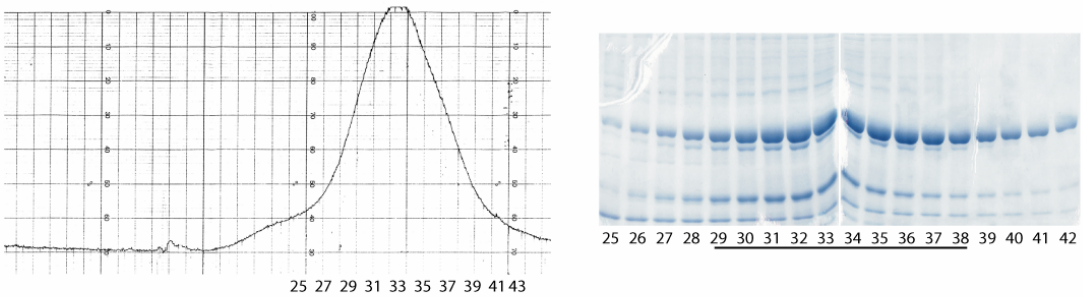
PTD	Sequence	Origin	Reference
TAT	YGRKKRRQRRR	TAT protein (HIV)	Fawell et al.; Nagahara et al.
Penetratin (Pen)	RQIKIWFQNRRMKWKK	Antennapedia (D. melanogaster)	Derossi et al.; Perez et al.
MTS	AAVLLPVLLAAP	modified from Kaposi fibroblast growth factor	Rojas et al.; Lin et al.

B

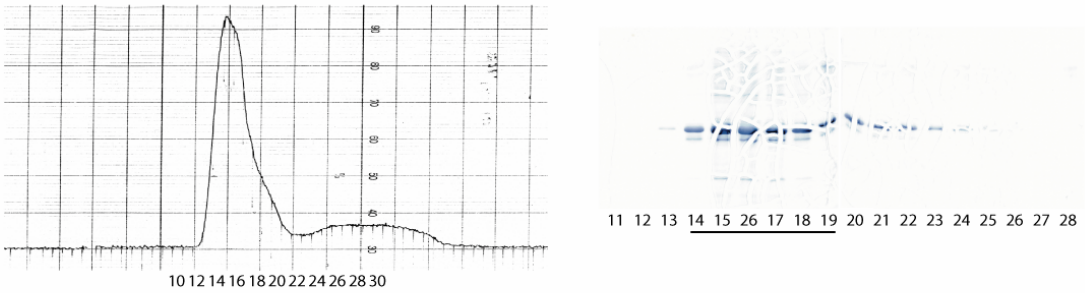
WT	pET15b-TEV-HIS
TAT	pET15b-TAT-TEV-HIS
TAT-NES	pET15b-TAT-NES-TEV-HIS
Pen	pET15b-Pen-TEV-HIS
MTS	pET15b-MTS-TEV-HIS



C



D



E

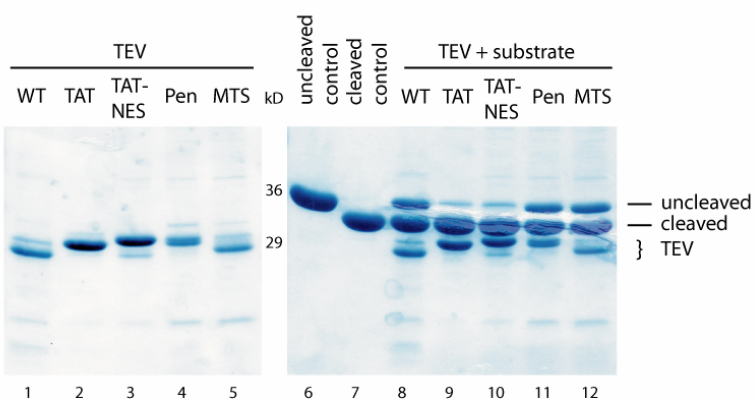


Figure 23: Generation of cell-permeable TEV-protease. A. Protein transduction sequences (PTDs) used in this study are listed. See text for details. B. Schematic representation of the recombinant proteins. Constructs and their abbreviations are listed on the left. C. Representative elution profile (left) of one PTD-TEV following affinity chromatography on a HisTrap Ni^{2+}-Sepharose column. Numbers at the bottom

correspond to the fractions whose protein content was further analyzed by SDS-PAGE and Coomassie-staining (right). The underlined fractions were pooled and purified further. D. Representative elution profile (left) of one PTD-TEV following purification over a HiTrap size exclusion column. Numbers at the bottom correspond to the fractions whose protein content was further analyzed by SDS-PAGE and Coomassie-staining (right). The underlined fractions were pooled and represent purified PTD-TEV. E. Purity and *in vitro* activity of all five recombinant TEV-proteins. Right: a recombinant substrate protein containing a TEV cleavage site (lane 6: 36 kD before cleavage, lane 7: 33 kD after cleavage) was incubated with the indicated TEV-proteins *in vitro*. Reactions were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. The size of the full-length and the cleaved substrate is indicated together with the size of the different TEV-proteins. Recombinant TEV-proteins without substrate are shown on the left.

into cells. Bacterial expression constructs were designed and cloned using standard molecular biology techniques. The fusion proteins consist of an N-terminal PTD, followed by the TEV coding region and a C-terminal hexa-histidine-tag to facilitate affinity purification (figure 23B). The PTDs used correspond to the above mentioned (figure 23A) with one exception. Because TAT is believed to mediate intranuclear localization as opposed to the predominantly cytoplasmic localization of Syk, a nuclear export signal (NES) derived from mitogen-activated protein kinase kinase (MAPKK, (Fukuda, et al., 1996) was included in one construct with the intention to facilitate co-localization of the enzyme and its target. In all constructs, the TEV cDNA contains a serine to asparagine mutation at position 219 which renders it resistant to inactivation by auto-cleavage (Lucast, et al., 2001). The PTD-TEV fusion proteins were expressed in *E. coli* BL21 (DE3) cells and purified as outlined in the methods section. Briefly, recombinant protein contained in bacterial inclusion bodies was solubilized using denaturing reagents and affinity-purified using the hexa-histidine-tag over a Ni^{2+}-charged sepharose column (figure 23C). Protein-containing fractions were pooled and dialyzed against a salt buffer containing 50% glycerol for protein re-folding. Concentrated, refolded protein was further purified on a size exclusion column (figure 23D). Final protein containing fractions were concentrated to a maximum of approximately 2 mg/ml and stored at -80ºC. This purification procedure resulted in the preparations of recombinant fusion proteins with little degree of contamination (figure 23E left). The protease activity of all TEV-preparations was tested in an *in vitro* assay through digestion of a substrate protein containing a TEV-cleavage site. In this assay, protease

activity is visualized by conversion of a 36 kD substrate to a 33 kD product. All TEV-preparations displayed high proteolytic activity on the substrate *in vitro*, demonstrating that the presence of the PTD does not interfere with TEV catalytic activity (figure 23E right).

The next aim was to determine whether the PTD-TEVs were capable of mediating intracellular Syk-cleavage. To this end, DT40 clones expressing TEV-sensitive Syk X1 or X6 were incubated with increasing amounts of recombinant protein for one hour. Cells were analyzed two hours after treatment with the protease by SDS-PAGE. Under these conditions, very little to no cleavage of Syk could be detected using any of the recombinant PTD-TEVs (figure 24A and data not shown). TEV-mediated Syk-cleavage was not improved by variations in the experimental protocol concerning the concentration of recombinant TEV (between 1 µM and 16.5 µM), the incubation time (between 1 and 36 hours), the time between incubation and cell lysis or the cell type (data not shown). PTD-TEV-mediated Syk cleavage was also not enhanced by addition of a peptide which is believed to facilitate

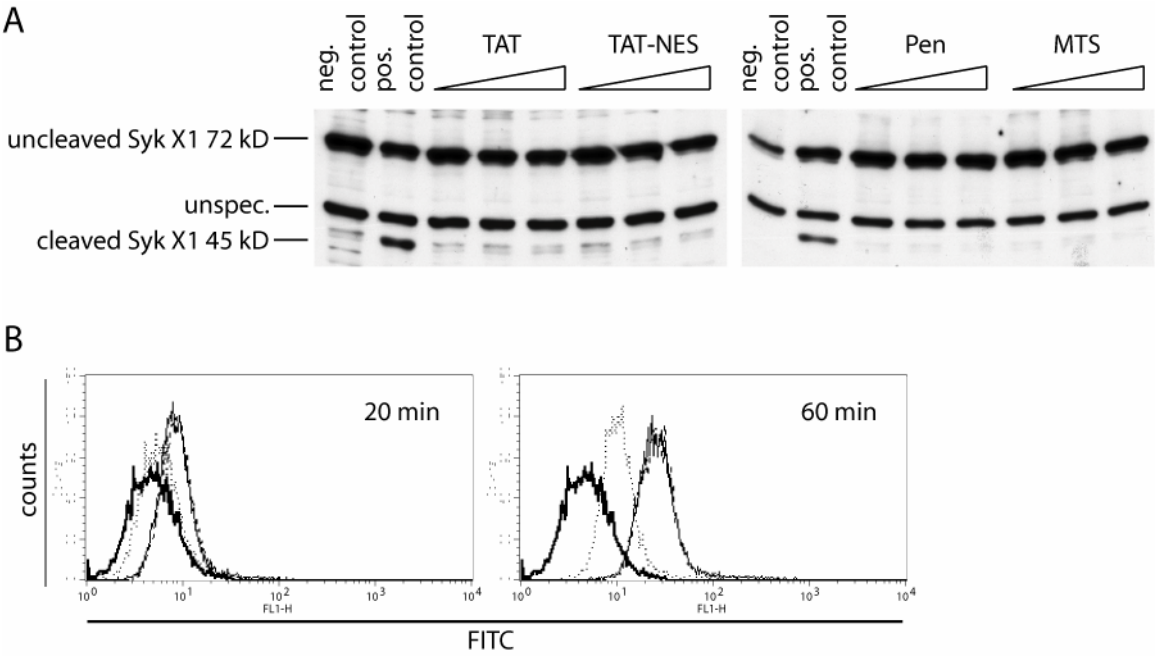


Figure 24: PTD-TEV activity *in vivo*. A. DT40 Syk X1 cells were incubated with buffer or with 3.3 µM and 16.5 µM of the indicated PTD-TEVs for 1 hr. Cell lysates were analyzed by immunoblot using an N-terminal anti-Syk antibody. The sizes of uncleaved and cleaved Syk X1 and a non-specific band are indicated. B. Jurkat cells were incubated for 20 and 60 min with medium (thick black line) or 100 nM FITC-BSA (thin dotted line), FITC-WT-TEV (thin dashed line) or FITC-TAT-TEV (thin black line) and analyzed by flow-cytometry under pH 6.5 buffer conditions.

release of a PTD-protein following its translocation into the cell (Wadia, et al., 2004) and data not shown).

In order to assess, whether the lack of PTD-TEV-mediated Syk-cleavage was due to a failure of PTD-TEV to translocate into cells or to a problem with PTD-TEV activity inside the cells, the recombinant protease was labeled with the fluorescent dye FITC and the cellular uptake of fluorescently-labeled TEV was measured by flow-cytometry. Jurkat cells were incubated with FITC-labeled TAT-TEV, wild-type TEV not containing a PTD or BSA for 20 or 60 minutes, washed extensively and resuspended in a pH 6.5 buffer prior to flow-cytometric analysis. The low pH was used as FITC-fluorescence is quenched with increasing acidity, such that any fluorescence detected should stem exclusively from the intracellular FITC-labeled protein pool and not from any labeled protein potentially attached to the cell surface (Veldhoen, et al., 2005). Cells incubated with FITC-TAT-TEV showed strong FITC-staining in a time-dependent manner (figure 24B), suggesting intracellular uptake of the protein. However, cell treatment with a control protein composed of TEV only without any PTD yielded a virtually identical result. Furthermore, weaker but significant fluorescent staining was also observed with mere FITC-conjugated BSA. The significance of this result in judging the capacity of the recombinant PTD-TEV to translocate into cells therefore remains questionable. Similar observations were obtained by microscopic inspection of cells incubated with FITC-labeled TEV, PTD-TEV or BSA (data not shown). In summary, Syk-cleavage through direct application of cell-permeable PTD-TEV turned out not to be a feasible approach to achieve instant Syk-inactivation.

4 Discussion and future perspective

Within the B cell compartment, the life-span of individual cells varies dramatically depending on the developmental stage of the cell and the extracellular environment. During early B lymphopoiesis in the bone marrow, life versus death decisions are made at every transitional step of developmental progression (Rolink, et al., 2001). It is estimated that, in the mouse, every day 20 million cells manage to survive this rigorous selection process and are released from the bone marrow into the periphery (Osmond, 1993). The size of the peripheral B cell pool is constant at approximately 100 million cells and the vast majority of these have a life-expectancy of several weeks to month (Forster and Rajewsky, 1990). This is in stark contrast to the newly emigrated B cells from the bone marrow, 95% of which are believed to die within a few days. This massive cell death is a consequence of the necessity to compete with the mature long-lived B cell pool for extracellular survival cues whose availability is limited in the peripheral lymphoid organs. This mechanism of B cell homeostasis serves at once to (1) sustain the survival of a mature naive B cell pool with a constant size of approximately 100 million cells, (2) maintain a diverse repertoire of antigen receptor specificities corresponding to the number of B cells, (3) prevent inappropriate expansion of the peripheral B cell pool with potentially adverse side effects to the organism, (4) mediate a low level of dynamic exchange to the composition of the long-lived peripheral B cell pool and (5) enable very efficient repopulation of a lymphopenic peripheral environment in a very short time frame. Within the scope of this study, the focus has been on the mechanisms which enable the longevity of the mature naive B cell pool. It is worth mentioning, however, that a different set of regulatory mechanisms come into play in the event of a B cell's encounter with antigen. The immunogenic differentiation program of activated B cells creates both extremely short and long-lived cell types. For example, cell death is a frequent outcome of affinity maturation during the course of the germinal center reaction, but this contrasts with the extreme longevity of memory B cells and a subset of plasma cells which is the basis for an organisms lasting immunity to previously encountered pathogens (Janeway, et al., 2001).

The aim of this study was to illuminate the survival of mature naive B cells in the periphery which is achieved through two distinct mechanisms: an internal signal originating from the BCR and the external pro-survival cytokine BAFF. During the course of this work, significant advances were made in

characterizing the effect of BAFF on B cell physiology as well as towards deciphering its signaling mechanisms. Furthermore, the protein tyrosine kinase Syk was identified as an integral component of the tonic BCR survival signal.

4.1 BAFF-mediated effects on B cell physiology

The viability of many cell types is closely associated with their trophic state (Hammerman, et al., 2004: ; Khaled and Durum, 2002). This concept is well established for the effect of nerve growth factor on neurons, for IL-3, IL-5 and granulocyte-macrophage colony stimulating factor (GM-CSF) on various hematopoietic precursors and for IL-7 and IL-4 on primary T and B lymphocytes (Deckwerth and Johnson, 1993: ; Rathmell, et al., 2001: ; Vander Heiden, et al., 2001). While cell survival is one important function of growth factor signaling, their impact on cellular physiology is more complex and extends to the regulation of energy homeostasis, protein synthesis, growth and cell cycle progression. Despite the striking effect of BAFF on B lymphocyte viability, its potential to act as a growth factor on B cells in analogy to the previous examples had not been investigated. In an attempt to close this gap in knowledge, I found that, indeed, the BAFF-imposed effects on B cells exceeded mere survival. Rather, it caused significant changes to B cell physiology which impacted energy homeostasis, protein synthesis, cell growth and cell cycle progression.

One of the most striking observations was that BAFF literally acts as a growth factor in increasing B cell size. Cell growth is tightly linked to nutrient availability, protein synthesis and survival through an ancient mechanism which is essentially conserved in all eukaryotes and centers around mTOR (Wullschleger, et al., 2006). The principle function and regulation of mTOR have been detailed in a previous section. Several lines of evidence suggest regulation of mTOR activity by BAFF in B cells. First, the BAFF-induced increases in cell size and protein synthesis are key cellular outcomes of mTOR-signaling. On a molecular level, enhanced protein synthesis was reflected by BAFF-mediated phosphorylation of TSC2, 4E-BP1 and ribosomal S6 protein. In light of the recent discovery of Akt-mediated mTOR regulation via PRAS40, it would also be of interest to determine whether BAFF enhances PRAS40-phosphorylation and weakens its interaction with mTOR. Of note, although 4E-BP1 can be phosphorylated by mTOR, the same function can be performed by the oncogenic kinase Pim-2. BAFF had a

profound effect on Pim-2 expression at the RNA and protein level (data presented herein and (Lesley, et al., 2004: ; Xu, et al., 2002). It is therefore conceivable, that BAFF's impact on protein synthesis is mediated by two independent mechanisms, namely mTOR and Pim-2. One possibility of experimentally distinguishing these pathways and assessing the individual importance of either one for protein synthesis and growth could make use of the immunosuppressant rapamycin which selectively inhibits mTOR.

The potential of BAFF to influence cellular energy homeostasis was evidenced both by an elevation of the inner mitochondrial membrane potential as well as by the transcriptional upregulation of glycolytic enzymes. This observation is not necessarily contradictory, but rather suggests that BAFF enhances the cellular uptake of glucose which is used for glycolytic energy derivation. This metabolic pathway can feed back into mitochondrial respiration by providing essential intermediates of the electron transport chain and the citric acid cycle. Glycolytic energy derivation and that via mitochondrial respiration are intimately linked by the subcellular location of hexokinase. This important enzyme is required for glucose-phosphorylation at the 6-position, a rate-limiting event for glycolysis which requires ATP. Hexokinase localization at the mitochondrial membrane enhances the efficiency of this process, by facilitating ATP-supply and thus altering the enzyme's kinetics in favor of glucose-phosphorylation (Robey and Hay, 2006). Of note, the subcellular localization of hexokinase is influenced by Akt-activity (Gottlob, et al., 2001: ; Majewski, et al., 2004). In view of BAFF's effect on glucose metabolism and Akt-activity (see below), a similar function in enhancing mitochondrial hexokinase association could be anticipated.

In contrast to the BAFF-dependent regulation of cellular growth and energy homeostasis, its potential to promote cell cycle progression came somewhat as a surprise. Although cell cycle entry is a common consequence of growth factor stimulation, no such effect had been observed in BAFF-treated B cells. Quite the contrary, ever since its first characterization, BAFF has been described to exclusively promote B cell viability without inducing cell division (Schneider, et al., 1999). While it does enhance B cell proliferation in response to different mitogens, this phenomenon is due to heightened cell survival. Yet, compelling evidence presented herein suggests that BAFF alters the profile of cell cycle regulating molecules in a manner which is indicative of cell division. In a simplified model of the cell cycle, entry into the G1-phase is initiated by activation of Cdk4 by D-type cyclins which results in phosphorylation of Rb, alleviating the latter's inhibitory effect on the

transcription factor E2F. Expression of E2F-controlled genes including cyclin E activates Cdk2 which further inhibits Rb and results in entry into S-phase (Sherr and Roberts, 2004). The second cell cycle checkpoint at the transition from G2- into M-phase is controlled by activation of the phosphatase Cdc25C which results in activation of the Cdk1/cyclin B complex. Cdc25C activity is controlled by multiple input signals including the Polo-related kinase (Plk) and Cdk1 itself (Myer, et al., 2005).

Based on BAFF-induced upregulation of Cdk4, cyclins D and E as well as Rb-phosphorylation, one would expect B cell entry into S-phase. This assumption is further supported by enhanced expression of Mcm2 and 3 as well as Ki67, as these proteins function in DNA-replication and Ki67 is in fact commonly used as a proliferation marker (Gerdes, 1990). Yet, in agreement with previous reports, I found that BAFF-treatment alone elicited neither DNA-replication nor cell division. A possible explanation to reconcile these contradictory observations centers on the regulation of Cdk-activity by Cdk-inhibitors whose degradation is an integral part of productive cell cycle progression (Sherr and Roberts, 1999). p16 and p18 are members of the INK4-family of proteins which inhibit Cdk4/cyclin D complexes, while p27 and p21 are well characterized negative regulators of the Cdk2/cyclin E complex. It is conceivable, that in contrast to mitogens such as anti-IgM or LPS, BAFF fails to inactivate such Cdk-inhibitors which would prevent Cdk-activity despite the presence of Cdk4/cyclin D and Cdk2/cyclin E complexes. BAFF-mediated phosphorylation of Rb and expression of cyclin E indicate enzymatic activity of the Cdk4/cyclin D complex and thus a potential for BAFF to negate the action of INK4-inhibitors. In contrast, there is no functional evidence for activity of the Cdk2/cyclin E complex, suggesting that BAFF may be unable to inactivate p27 or p21. This model could be tested by directly measuring the enzymatic activities of the respective Cdk-complexes isolated from BAFF-treated B cells. Resting and anti-IgM stimulated B cells could serve as negative and positive controls, respectively. Using a different approach, an independent study found that BAFF-treatment induced S-phase entry in a significant fraction of B cells which contained elevated levels of cyclin E and lacked p27 (Huang, et al., 2004). Together, the results argue in favor of a model in which BAFF elicits B cell exit from the resting state G0 and into the G1-phase including activation of Cdk4/cyclin D complexes, Rb-phosphorylation, transcription of E2F-target genes and expression of proteins involved in DNA-replication. However, BAFF-mediated cell cycle progression just stops short of passing the final G1- to S-phase checkpoint, possibly due to a failure of inducing Cdk2/cyclin E

activity. In a physiological context, this molecular signature translates to a situation in which BAFF would keep B cells constantly on the verge of cell division, but still in need of an additional final trigger. Considering the primary function of B cells in the detection of and fight against foreign antigens, this effect of BAFF appears very well suited to enable the most effective immunogenic response, both by positioning cells on the verge of S-phase entry and by establishing a cellular pool of proteins which will be essential at later stages of cell cycle progression. This pool includes the proteins required for DNA-replication mentioned above, but apparently also a multitude of proteins required for mitosis. From the latter group of proteins, we only confirmed the expression of survivin on the protein level, but the results from the global gene expression analysis indicate BAFF-mediated upregulation of numerous factors involved in chromosome condensation, kinetochore formation, cohesion and assembly of the spindle apparatus. A BAFF-mediated preparation for cell cycle progression upon B cell triggering was supported by the observation that cellular pre-treatment with BAFF prior to mitogenic stimulation shortened the response time between cell triggering and DNA-replication. The suggested hypothesis could also be of use in explaining the synergistic effect of BAFF on B cell expansion in response to mitogens as well as help explain the strikingly short doubling time of some B cell subsets. For example, cycling B cells at the height of the germinal center response replicate within seven hours (MacLennan, 1994).

4.2 A novel BAFF-induced signaling mechanism

The physiological changes that were observed in B cells in response to BAFF, including cell survival, growth, regulation of energy homeostasis, protein synthesis and cell cycle progression, are all processes which are known to be regulated by the PI3K-Akt pathway in other cellular contexts (Massague, 2004), prompting an examination of the potential sensitivity of this pathway to BAFF in B cells. The experimental evidence presented herein, including BAFF-mediated activation of PI3K as well as PI3K-dependent activation of Akt and several of its target proteins, supports this hypothesis. Furthermore, inactivation of TSC2, FoxO1 and GSK-3β in response to BAFF provides a mechanistic link to the before mentioned cellular outcomes.

BAFF-stimulation enhanced Akt-phosphorylation at both of its catalytically relevant residues in a biphasic manner with a rapid initial peak at approximately 10 minutes and a delayed maximum at circa 24 hours. This

pattern of Akt-activity is not unusual and has been observed in other contexts, although the time frame may vary depending on the individual circumstances. Biphasic PI3K- or Akt-activation at early and late time points has been observed in platelets stimulated through the thrombin receptor (Banfic, et al., 1998), in TNFα-primed neutrophils treated with N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Condliffe, et al., 2005: ; Guillou, et al., 2007), in estradiol-treated hepatoma cells (Marino, et al., 2003), in fibroblasts in response to zinc (Kim, et al., 2000), in mesangial cells in response to mechanical strain (Krepinsky, et al., 2005), in Kaposi sarcoma cells in response to TAT (Deregibus, et al., 2002) and in various cell types upon viral infection (Ehrhardt, et al., 2006: ; Flaherty, et al., 2004). In the experimental setup used in the present study, mock treatment of cells with control medium rather than BAFF caused a modest but discernable increase in early Akt-phosphorylation, indicating that a fraction of the phospho-Akt signal in response to short-term BAFF-treatment is of unspecific nature. For future studies, an effort should be made to optimize the experimental conditions towards minimal background Akt-phosphorylation.

With regard to the mechanism of BAFF-mediated Akt-activation, both its dependency on PI3K-activity and its phosphorylation of the catalytical threonine 308 by PDK1 are parts of a generic mechanism which are essentially invariant in all cases studied to date. The main question to resolve concerned the mechanism by which Akt's C-terminal serine 473 was phosphorylated following BAFF-treatment, as there appears to be significant heterogeneity to this process. Depending on the cellular context and stimulus, the panel of implicated S473-kinases include PDK1, ILK1, MAPKAP-K2, DNA-PK, ATM, mTORC2 and PKCβ (reviewed in (Fayard, et al., 2005). The results presented in this study and elsewhere suggest that PKCβ phosphorylates Akt at S473 in B cells in response to BAFF. This conclusion is based on the ability of PKCβ to phosphorylate Akt at S473 *in vitro* (Kawakami, et al., 2004) and the specific lack of this modification in BAFF-treated PKCβ-deficient B cells while Akt T308 phosphorylation remains intact. Residual Akt-phosphorylation which was observed in the absence of PKCβ could represent the non-specific component of the signal mentioned earlier or indicate a certain amount of flexibility in the mechanism of BAFF-induced Akt S473-phosphorylation. The latter scenario does not seem unlikely given the large number of previously identified Akt S473 kinases, some of which may be able to partially compensate for a lack of PKCβ. PKCβ-independent Akt-activation following BCR-ligation further suggests a context-specific function of PKCβ in BAFF-

mediated induction of the PI3K-Akt pathway. In support of this model, BAFF appeared to activate PKCβ, as judged by its translocation to the plasma membrane, and enhanced its physical association with Akt. However, other BAFF-dependent signaling mechanisms besides Akt-activation appeared to be unperturbed in the absence of PKCβ, arguing against a universal role of this kinase in BAFF-mediated signaling.

Previous experimental evidence had suggested the existence of BAFF-mediated survival signaling mechanisms in addition to NF-κB-activation. A comparison between the signaling capacities of the three BAFF receptor proteins' intracellular domains had shown that BAFF-R, TACI and BCMA are all capable of conferring a survival effect on B cell lines, while at the same time differing in the molecular responses which they elicit (Craxton, et al., 2005). In this experimental system, BAFF-R had an impact on NF-κB activation and Bim-phosphorylation, whereas TACI only affected Bim and BCMA did not elicit any of these responses. With the expanded knowledge of BAFF-induced signaling events since the publication of this report, it would be of great interest to assess the sensitivity of PKCδ and Akt to the individual receptor proteins in this system. In any case, this interesting study implies that the three BAFF receptors are at least partially unique in their signaling capacity. The fact that all three proteins positively affected cell survival despite the inability of some to regulate NF-κB and Bim suggests the existence of additional BAFF-induced survival mechanisms. A similar conclusion was reached by Bender and colleagues based on the response of c-Myb deficient B cells to BAFF (Thomas, et al., 2005). In this study, the authors documented normal BAFF-dependent NF-κB activation, but impaired regulation of PKCδ subcellular localization which correlates with a reduction in BAFF-R expression and partial refractiveness of c-Myb-deficient B cells to BAFF-mediated survival. The discovery of BAFF-mediated activation of the PI3K-Akt pathway in the present study suggests that this could be part of the previously proposed but as yet undefined mechanism which contributes to BAFF-mediated B cell survival in addition to the regulation of NF-κB, PKCδ and Bim.

The dependency of BAFF-induced Akt-activation on PKCβ combined with the apparent lack of PKCβ-involvement in other BAFF-induced signaling pathways provided an opportunity to test this hypothesis. While a lack of PKCβ affected B cell viability *in vitro*, this was not restricted to BAFF-mediated B cell survival. This result was not entirely unexpected given the previously described function of PKCβ in BCR-mediated NF-κB activation (Saijo, et al.,

2002: ; Su, et al., 2002) and suggests that PKCβ may have a dual role in the regulation of B cell survival via tonic BCR-signaling and BAFF. *In vivo*, reduced B cell numbers and alteration of the splenic B cell compartment towards a more immature phenotype were consistent with a defect in BAFF-mediated survival signaling. The relative mildness of the phenotype compared with that of BAFF-deficient mice argued that PKCβ-dependent Akt-activation contributes to BAFF-mediated B cell survival without being its sole regulator.

Judging the physiological importance of BAFF-mediated Akt-activation on the responses of PKCβ-deficient B cells may be misleading to some extend since residual Akt-S473 phosphorylation in the absence of PKCβ could indicate a certain amount of flexibility and thus compensation for this function. One way to further confirm the conclusions made in this study would be to characterize BAFF-dependent responses in B cells which lack the individual Akt-isoforms 1, 2 and 3 or a combination of those. This type of analysis could be hampered by potential redundancy of the different Akt proteins, but could also point out isoform-specific functions, particularly in light of Akt-regulation by PHLPPs (see below). Alternatively, B cells deficient for the regulatory or catalytic subunit of PI3K could be tested for their response to BAFF. However, lack of p85α or p110δ compromises B lymphopoiesis (Okkenhaug and Vanhaesebroeck, 2003) and it might be problematic to obtain sufficient amounts of cells which match the maturation stage of wild-type follicular B cells. If such technical difficulties can be overcome, the characterization of BAFF-mediated responses in B cells devoid of components of the PI3K-Akt pathway could help clarify the importance of this signaling chain for B cell survival, growth or cell cycle entry.

As mentioned previously, one central conclusion of the present study is that BAFF-mediated effects on B cells are not restricted to mere survival but include the regulation of energy homeostasis, protein synthesis, cell growth and cell cycle progression. This diversity of BAFF-mediated cellular responses raises the possibility that certain BAFF-dependent molecular changes could act as general regulators of BAFF-signaling and affect the various cellular outcomes equally or, alternatively, that individual B cell physiological changes might be dependent on a specific signaling pathway. Our analysis of the responses of PKCβ-deficient B cells to BAFF *in vitro* suggest that Akt-activation plays a prominent role in B cell growth in response to BAFF and this hypothesis could be investigated further by analyzing BAFF-mediated regulation of translational initiation in the absence of PKCβ.

Within the scope of this study, I found evidence for an involvement of PI3K and PKCβ in BAFF-mediated activation of Akt. Yet, several aspects of this pathway continue to be unclear. Most notably, the proximal signaling events which lead from BAFF-treatment to the activation of PI3K and PKCβ remain to be investigated. The first question in this respect concerns the binding of BAFF to its receptor protein on the B cell surface. As mentioned earlier, three proteins have been identified to date, which are capable of BAFF-binding, BAFF-R, TACI and BCMA. Judging by the consequences which result from genetic inactivation of the individual receptors, the survival effect of BAFF on B cells appears to occur primarily through BAFF-R, making it the prime candidate for mediating BAFF-induced activation of the PI3K-Akt pathway. By contrast, the previously mentioned study comparing the individual receptor proteins' survival and signaling capacity supports the notion that all three proteins can in principle enhance cell viability (Craxton, et al., 2005). Differential expression of BAFF receptor proteins on various B cell subsets, the second potential ligand APRIL and potential interaction with proteoglycans could all be factors which would have to be considered in a complete picture of B cell survival through this protein family.

In addition to establishing which BAFF receptor protein accounts for Akt-activation, it would be of great interest to identify the missing components of the molecular signaling chain which link the receptor to PI3K and PKCβ. Very little is currently known about the molecules associated with the intracellular portions of the BAFF receptor proteins and which would be expected to be the primary acceptor and transducer of the activating signal. The specific interaction of BAFF-R with TRAF3 could serve as a starting point for further analysis (Xu and Shu, 2002), although, to date, TRAF3 has not been implicated in the regulation of the PI3K-Akt pathway. It is therefore equally conceivable that as yet unidentified receptor-associated proteins might be critical mediators in BAFF-mediated activation of this pathway. A promising strategy in the search for such factors could focus on binding partners of the very C-terminal eight residues of BAFF-R which are altered in the A/WySnJ mutant, as B cell lymphopenia in these mice has been attributed to a dramatically impaired BAFF-R signaling capacity (Thompson, et al., 2001: ; Yan, et al., 2001). The significance of the identification of BAFF-responsive proximal signaling factors is heightened by the fact that they could provide insights not only into the activation of the PI3K-Akt pathway, but also the remainder of the BAFF-induced molecular responses. To date, the mechanism by which the BAFF-signal is relayed to NF-κB2 p100-processing,

PKCδ regulation and Erk-mediated phosphorylation of Bim is largely elusive. The search for novel BAFF receptor associated signaling factors could thus be potentially daunting but very rewarding.

An alternative approach in illuminating the BAFF-mediated activation of the PI3K-Akt-pathway could focus on those factors which have been identified as key regulators of this pathway in other cellular contexts. As this is a tremendously complex field, not all potential candidates can be discussed here, but several recent novel insights definitely deserve closer inspection. One very interesting family of Akt-regulators are the recently discovered PH domain leucine-rich repeat phosphatases (PHLPPs) 1 and 2 (Brognard, et al., 2007: ; Gao, et al., 2005). Like PTEN, PHLPP 1 and 2 are phosphatases which negatively regulate Akt-activity, but where PTEN functions in the breakdown of the essential Akt co-activator PIP_3, the PHLPPs act directly on Akt by dephosphorylating the C-terminal hydrophobic motif residue serine 473. The importance of S473-phosphorylation to Akt's catalytic activity is related to its stabilizing effect on the active protein confirmation and this has long been believed to be an integral aspect of the enzyme's activity. Only recently has it emerged that S473-phosphorylation may in fact be dispensable in the regulation of a subset of Akt targets under certain conditions (Guertin, et al., 2006: ; Jacinto, et al., 2006: ; Shiota, et al., 2006: ; Yang, et al., 2006). Nevertheless, PHLPP-mediated Akt S473-dephosphorylation plays a role in the regulation of cell survival and cell cycle progression (Mendoza and Blenis, 2007). Since both of these cellular responses are subject to control by BAFF in B cells, it could well be worthwhile to explore a potential regulation of PHLPPs by BAFF. Another very interesting aspect of the PHLPPs is that they appear to at least partially distinguish between the different Akt-isoforms 1, 2 and 3 as substrates for S473-dephosphorylation. While PHLPP1 preferentially acts on Akt 2 and 3, PHLPP2 favors Akt 1 and 3. This differential pattern of substrates coincides with a partially unique profile of activity of the Akt-targets. While TSC2, FoxO1 and Gsk3β are apparently sensitive to both PHLPPs and all three Akts, the CDK-inhibitor p27 seems to be exclusively regulated by Akt 3 and PHLPP2. This latter finding is of pivotal interest in light of the fact that a lack of p27-inactivation could account for BAFF's failure to surpass the G1 to S phase checkpoint of the cell cycle. While it would have to be confirmed whether this exact mechanism of p27-regulation by Akt and PHLPP is conserved in B cells, the differential regulation of certain Akt-targets by specific isoforms of Akt and/or PHLPP represents an intriguing mechanism by which the generic outcomes of the PI3K-Akt pathway could be modulated to

reflect the particular effects of BAFF on B cells. Within the scope of the present study, I did not distinguish between the different Akt isoforms, but the very recent findings in connection with the discovery of the PHLPPs suggest this to be an undertaking well worthwhile.

Another very interesting group of Akt regulatory proteins is the proto-oncogene family TCL1 (T cell leukemia), which comprises the proteins TCL1, MTCP1 and TCL1b (reviewed in (Noguchi, et al., 2007). The TCL1 proteins interact with the PH-domain of Akt and function as a co-activator of its catalytic activity. Curiously, although TCL1 expression in mice is almost exclusively restricted to embryogenesis, it is expressed in some lymphoid lineages including mature B cells, suggesting that it may play an important role in the regulation of peripheral B cell survival. In support of this hypothesis, overexpression of TCL1 is a common feature of many B and T cell malignancies and transgenic TCL1 family member expression in mice results in the development of lymphatic neoplasias (Gritti, et al., 1998: ; Hoyer, et al., 2002: ; Virgilio, et al., 1998). Conversely, loss of TCL1 in mice reduces the cellularity of the B cell compartment in both spleen and bone marrow (Kang, et al., 2005). The lack of manifestation of a more dramatic phenotype is most likely owing to a compensatory effect of other members of the TCL1 family. Given the expression pattern and the *in vivo* evidence for involvement of TCL1-family members in regulation of the B cell compartment, it would be of great interest to investigate, whether BAFF has the capacity to alter the protein content of individual TCL1-proteins in B cells or modify their association with Akt upon BAFF-treatment.

Of note, an independent study has investigated the potential of BAFF to act as a growth factor on B cells (Woodland, et al., 2006). In line with the results presented here, the investigators observe BAFF-mediated B cell growth, activation of the pro-survival kinases Pim-2 and Akt as well as phosphorylation of effector proteins including FoxO transcription factors, mTOR and 4E-BP1. In addition, the authors report increased glucose metabolism and nutrient uptake in response to BAFF which represents further proof for the growth factor function of BAFF.

A meticulous understanding of the signaling mechanisms which mediate BAFF-dependent survival of mature B cells constitutes not only an intellectual exercise, but has the potential to offer new alternatives for the treatment of B cell-related pathologies. As mentioned in a previous section, excess amounts of BAFF promote the persistence of self-reactive B cells and systemic

autoimmune disease is a common consequence of misregulation of factors whose activity is BAFF-controlled. In this sense, BAFF and the survival signaling mechanisms induced by it are an attractive therapeutic target and clinical efforts are underway to reduce BAFF-titers in patients through administration of soluble BAFF receptor proteins (Martin and Chan, 2006). Components of the PI3K-Akt pathway have long been the subjects of pharmacological interest owing to their association with neoplastic disease (Hennessy, et al., 2005). The results obtained as part of the present study could expand the application of potential drugs targeting the PI3K-Akt pathway for the treatment of autoimmune diseases such as SLE, rheumatoid arthritis or Sjogren's syndrome. In addition to the striking association of BAFF to autoimmunity, there is increasing evidence that BAFF and its relative APRIL may play a role in several B cell malignancies including mature B cell-derived non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (B-CLL) as well as plasma cell-derived multiple myeloma (Tangye, et al., 2006). This is based on the observation that BAFF and APRIL promote the survival and proliferation of neoplastic cells as well as on higher serum titers of BAFF and APRIL in NHL and B-CLL patients. Furthermore, the malignant B cells themselves were found to synthesize BAFF and APRIL, suggesting the existence of an autocrine loop which promotes the expansion of tumor cells. Given the extensive connection of the PI3K-Akt signaling pathway to tumorigenesis, it is interesting to speculate that BAFF-induced activation of this pathway may at least in part account for its effect on malignant B cells. In support of this hypothesis, Akt-activation has been observed in BAFF- and APRIL-treated multiple myeloma cell lines (Moreaux, et al., 2004).

4.3 The role of Syk in mature B cell survival

In the present study, the role of Syk in the survival of mature resting B cells was investigated using a mouse model in which Syk-expression was inducibly ablated by Cre-recombinase. Surprisingly, Syk-deficient mice turned out to be viable in rare cases despite the profound defect in vascular development which caused severe hemorrhaging in such animals (Abtahian, et al., 2003). Of note, Syk-null mice only resulted from crosses of parents with a mixed genetic background and ceased to appear on a pure C57Bl/6 background. It is possible that the mixed background confers a somewhat greater robustness such that the severe vascular defect caused by lack of Syk penetrates to a lesser extend and can in some instances be compatible with an animal's

survival. Apart from this surprising finding, the phenotypic properties of the B cell compartment in the absence of Syk overlapped very well with those derived from fetal liver chimeras (Cheng, et al., 1995: ; Turner, et al., 1995) and can essentially be summarized as a complete absence of B lymphocytes with the exception of bone marrow resident pro B cells.

Regulated ablation of the Syk allele according to the expression pattern of the B cell-specific marker CD19, showed that the arrest in B cell development is caused by a defect which is an intrinsic property of the affected cell type. This excludes the possibility that aberrant B lymphopoiesis could be related to a Syk-dependent function in bone marrow resident stromal cells or other cell types which create the environment necessary for B cell development. The onset of CD19-Cre mediated Syk-deletion at the late pro B stage also showed that the inability to progress through B cell development is not a consequence of Syk-deficiency in stem cells, but rather, that Syk is specifically required at the pro to pre B transition following the onset of CD19-expression. In addition to these conclusions, the use of this experimental system exposed the potential problems which arise from inefficient Cre-mediated deletion in conditional knock-outs and which can dramatically affect the phenotypic appearance at first glance. When using Cre-mediated gene inactivation, it is therefore always essential to control for the efficiency of this process and, if required, an effort has to be made to distinguish mutant from wild-type cells to allow for correct interpretation of the phenotype. Such considerations are of particular importance when ablation of the target gene is detrimental to the viability of the cell type of interest, as this could evoke a substantial counter-selection pressure. For these reasons, a genetic system was used in the subsequent experiment in which the event of successful Cre-mediated recombination is marked by the acquisition of a fluorescent signal. This enabled the flow-cytometric identification of Syk-deleted cells upon induction of Cre-expression in mice transgenic for the Mx-Cre gene.

This system revealed significant differences in the efficiency of Cre-mediated recombination between different lymphoid organs. While hematopoietic cells in the bone marrow overwhelmingly lost Syk and showed characteristic alterations indicative of Syk-deficiency, Cre-mediated recombination occurred to a much lesser degree in splenocytes. This was not primarily due to a potential disadvantage of Syk-null cells, as deletion efficiency did not reach beyond 20% percent even when the cells retained a functional, Cre-insensitive Syk-allele. While it could be possible to improve peripheral deletion efficiency to some extend by repeated induction of Cre-expression, this option

would have interfered with the primary purpose of the experiment, namely to monitor the fate of mature naïve B cells immediately following Syk-removal. Despite the comparatively modest extend of Cre-mediated recombination in splenocytes, a significant difference in the size of the recombination-positive B cell compartment was apparent between mice who retained a wild-type Syk allele and those which were rendered Syk-deficient. While the former constituted approximately a quarter of splenic B cells following induction of Cre-expression, the latter were virtually absent. No such difference occurred in splenic non-B cells whose viability is expected to be unaffected by lack of Syk.

Together, these observations lead to the conclusion that the constitutive presence of Syk is indispensable for the maintenance of the mature resting B cell compartment and Syk-loss is incompatible with B cell persistence in the periphery and triggers B cell demise. This result strongly argues that Syk is a critical mediator of the tonic BCR-signal which ensures the survival of the mature B cell repertoire. In view of the homeostatic equilibrium model proposed to explain tonic BCR signaling (Monroe, 2006), the results presented herein support the notion that Syk mediates transient phosphorylation of ITAMs in the BCR complex or that its transient activation transduces a low level of signaling to downstream targets such as PLCγ or BLNK. The mechanistic details of the role of Syk in tonic BCR signaling remain an important topic for future studies.

4.4 Development of a novel protein knockout system for Syk inactivation in B cells

While the present work established a function of Syk in tonic BCR survival signaling, the precise action of Syk during this process remained unknown. My aim was to create an experimental system by which it would be possible to study how tonic BCR-induced Syk-activity is translated into precise cellular events responsible for B cell viability. This task was not brought to a satisfying conclusion within the scope of this study. While the development of a functional Syk-kinase which is susceptible to proteolytic cleavage was successful, the main technical problem that remained concerned the efficient delivery of the protease to its target inside the cell. While considerable effort was invested in the generation of an active cell-permeable protease, the complete range of possibilities which could potentially improve the method

has not been fully exploited.

One possible option could be the use of a different peptide sequence to mediate protein transduction. The three different peptides which were used during the course of this work had previously been described as highly efficient and were selected on that basis. Judging from the published literature, the most commonly used peptide for protein transduction appeared to be TAT. The capacity of full-length HIV TAT protein to translocate into cells has been known for some time (Frankel and Pabo, 1988: ; Green and Loewenstein, 1988). Chemical cross linking of a 36 residue TAT-domain to cargo proteins as well as the preparation of fusion proteins composed of an 11 amino acid TAT-peptide and a cargo protein have been successfully used to achieve transport of a diverse collection of proteins across the plasma membrane (Fawell, et al., 1994: ; Vocero-Akbani, et al., 2000). The fusion proteins range in size from 15 to 115 kD and include β-galactosidase, Gst, Cre, ovalbumin, Cdk inhibitors p16 and p27, HPV-E7, GFP, cdc42 and cyclins D3 and E (Ezhevsky, et al., 1997: ; Hsia, et al., 2002: ; Huang, et al., 2004: ; Kim, et al., 1997: ; Lissy, et al., 1998: ; Nagahara, et al., 1998: ; Peitz, et al., 2002: ; Schwarze, et al., 1999: ; Su, et al., 2005: ; Tyagi, et al., 2001: ; Vocero-Akbani, et al., 2000). In virtually all cell and tissue types tested, protein transduction proved to be very efficient. This includes various murine and human cell lines such as NIH3T3, HEK 293, HeLa and CHO *in vitro* as well as whole mouse organs including liver, kidney, heart and brain *in vivo*. Of particular importance for my purpose, TAT-mediated protein transduction seems to work very efficiently in the lymphoid compartment as judged by results on Jurkat T and BJAB B cell lines**,** human and murine peripheral blood lymphocytes, splenocytes as well as purified T and B cells (Hsia, et al., 2002: ; Huang, et al., 2004: ; Krautwald, et al., 2004: ; Peitz, et al., 2002: ; Schwarze, et al., 1999: ; Su, et al., 2005: ; Veldhoen, et al., 2005: ; Vocero-Akbani, et al., 2000). While functional read-outs in all the studies mentioned above indicate correct protein function upon transduction, direct enzymatic activity has been observed for catalytically active proteins such as β-galactosidase, cdc42 and Cre, justifying the expectation that a PTD-TEV-protease should retain its proteolytic activity upon intracellular translocation.

Although ample evidence exists on the efficiency of the TAT-peptide in protein transduction, the repertoire of PTDs used within the scope of this study was expanded to include additional peptides to which the capacity for protein transduction has been ascribed. One such sequence is derived from the *Drosophila melanogaster* homeodomain protein antennapedia and has been

termed Penetratin. It has mainly been used for the intracellular delivery of peptide cargos (Derossi, et al., 1998: ; Horng, et al., 2001). The MTS-peptide derived from Kaposi fibroblast growth factor has been shown to mediate transmembrane transport of both oligo- and polypeptides including Gst and Cre, although a later report has questioned the effectiveness of this sequence in protein transduction (Derossi, et al., 1996: ; Jo, et al., 2001: ; Peitz, et al., 2002: ; Rojas, et al., 1998). Another sequence to which protein transduction capacity has been ascribed is derived from the Herpes simplex virus-type 1 tegument protein VP22 (Elliott and O'Hare, 1997). Beyond mere translocation from the extracellular environment into the cellular interior, VP22 has actually been shown to mediate transcellular protein migration. Later studies have questioned the protein transduction potential of VP22, although there are conflicting reports which strengthen the original claim (Bennett, et al., 2002: ; Lundberg and Johansson, 2001). Due to the ongoing controversy, this particular PTD was not included in the present study, but it may still be worthwhile testing this sequence for the purpose of intracellular protease delivery. Very recently, a novel cell-permeable peptide has been derived from the human transcription factor Hph-1 (Choi, et al., 2006) which shows an impressive capacity to mediate protein translocation into Jurkat T cells and various other cell lines as well as mouse organs *in vivo*, making it another candidate for PTD-TEV translocation.

The mechanism by which PTD-linked proteins are able to cross the cell membrane from the extracellular environment to the cells' interior has not been conclusively settled. Originally, it had been suggested that PTD-proteins could directly cross the lipid bilayer in an unfolded state and refold once inside the cell (Schwarze, et al., 2000). However, such a scenario is somewhat difficult to picture mechanistically and, at present, a different hypothesis is considered more likely. With the exception of the MTS-sequence whose functionality has been questioned independently, one feature that all the PTDs mentioned here have in common is their high content of positively-charged amino acids. In fact, a repeat of nine to eleven arginines confers translocation capacity to attached cargo proteins with at least equal efficiency to TAT (Futaki, et al., 2001: ; Matsui, et al., 2003: ; Wender, et al., 2000). The polybasic PTD-peptide is believed to mediate cargo-protein attachment to negatively charged heparan sulfate proteoglycans which coat the extracellular cell surface, such that proteins can enter cells through an endocytotic mechanism (Belting, 2003: ; Tyagi, et al., 2001). Although the further intracellular processing of the transduced proteins and their potential release

from endosomes are unknown, several observations support this model. Most importantly, cells genetically incapable of synthesizing fully sulfated heparan sulfate proteoglycans are refractive to the translocation of TAT fusion proteins (Tyagi, et al., 2001). This model could also help explain the phenomenon of cell membrane translocation of histones considering their high basic charge (Hariton-Gazal, et al., 2003). Based on this model, it has been suggested that a peptide derived from the haemagglutinin (HA) protein of influenza virus could enhance protein transduction efficiency by facilitating the exit of transduced proteins trapped inside endosome vesicles (Wadia, et al., 2004). I tested the effect of this HA-peptide on protein transduction efficiency of TAT-TEV, but was unable to detect any improvement in Syk-cleavage (data not shown). While this observation does by no means prove or disprove the current model, it would certainly be desirable to gain a better understanding of the exact mechanism of protein transduction, and, with this in mind, make strategic amendments to improve the efficiency of the procedure.

Based on the wealth of published information on the successful intracellular transduction and activity of PTD-fusion proteins, the expectation that recombinant PTD-TEV would translocate into cells and be capable of Syk-cleavage appeared reasonable. Unfortunately, this was not the case under the conditions used in the present work. However, given the widespread use of this technology especially in lymphocytes, the present results may still be improved by using slightly modified strategies. As evident from the extensive description of PTDs above, the full repertoire of possible peptide sequences has not been exploited within the scope of this study. Promising PTD-candidates could be VP22, Hph-1 or poly-arginine which could be fused to TEV-protease in a similar manner to the ones described in this study. Alternatively, TEV-protease, despite its high catalytic activity and lack of cytotoxicity may not be the ideal protease of choice for this purpose. One problem encountered with this enzyme during the course of this study was that the recombinant protein could only be concentrated to a maximum of 2 mg/ml and that its buffer composition proved to be toxic to cells. From this ensues a limitation on the maximum concentration of PTD-TEV which can be used for cellular incubation. If it were possible to prepare a more concentrated protease stock solution, the amount of enzyme that could be applied to cells would proportionally increase, which may positively effect the protein transduction efficiency. In addition, proteases other than TEV may be soluble in a buffer whose composition is less toxic to cells. One candidate for a potentially superior enzyme could be PreScission protease (Cordingley, et al.,

1990). Similar to TEV, this protease is derived from a virus (human rhinovirus), has a very high specificity for its target sequence and is commonly used for biochemical purposes. To my knowledge, potential cytotoxicity of the enzyme has not been tested in eukaryotic cells, but it seems rather unlikely given its high degree of target sequence specificity. For these reasons, it may be worthwhile testing the capacity of PreScission protease for the purpose of inducible protein inactivation.

4.5 Other potential methods for rapid inducible Syk inactivation

Lastly, the approach used herein for inducible Syk inactivation may be internally flawed to some extend as it may be difficult to achieve one hundred percent of target cleavage depending on the abundance of Syk, the maximal amount of protease transduction and the ability of the protease to find its target inside the cell. Yet, complete protein inactivation is a priority when addressing the function of Syk in tonic BCR survival signaling as, most likely, even residual amounts of Syk would be capable of mediating this presumably weak signal. In this sense, other ways of inducible protein inactivation may be better suited for this particular purpose.

One very attractive system called chromophore-activated light inactivation (CALI) exploits the property of some fluorochromes to generate reactive oxygen species (ROS) in response to high-intensity illumination. Attachment of a suitable fluorochrome to the target protein either via a dye-conjugated antibody or genetically makes the target protein susceptible to ROS-mediated inactivation upon illumination (Jay, 1988: ; Jay and Sakurai, 1999). This technique has been described as highly efficient and offers an ideal means of spatiotemporal regulation as target inactivation can be induced under any conventional fluorescent microscope. The use of genetically encoded fluorochromes is particularly intriguing as it avoids the need to deliver an exogenous protein into the cells' interior, the step which impeded the present approach. Genetically encoded dyes suited for CALI include GFP, although this is not particularly efficient (Rajfur, et al., 2002). In an alternative technique, a short tetracysteine-tag is genetically attached to the target protein which renders it sensitive to the binding of non-fluorescent membrane-permeable biarsenic compounds FlAsH or ReAsH (Marek and Davis, 2002: ; Tour, et al., 2003). FlAsH and ReAsH emit green or red light, respectively, upon binding to the tetracysteine moiety and both function in CALI with the

efficiency of ReAsH being somewhat greater. One drawback of this elegant system is that it may cause damage not only to the target, but also to other proteins in a close enough vicinity to be affected by the fluorochrome-generated ROS. Also, the biarsenic dyes may not only bind to the tetracysteine tag of the target protein, but show some affinity for endogenous cysteine-containing proteins, thus creating some background staining (Stroffekova, et al., 2001). Non-specific cytotoxicity may be reduced by further optimization of the tetracysteine-motif's dye-affinity (Tour, et al., 2003) or by using multi-photon over single-photon excitation which is associated with less phototoxicity (Tanabe, et al., 2005). In another modified and less cytotoxic approach, the target protein is fused to a FKBP12 homologue which displays high affinity to a synthetic, cell-permeable fluorescein-coupled ligand (Marks, et al., 2004). Very recently, a new GFP homologue termed "KillerRed" has also been shown to by highly effective for CALI (Bulina, et al., 2006). In principle, KillerRed could be an attractive CALI-fluorochrome since it is entirely genetically encoded and does not require any type of invasive cell manipulation. However, its application is currently restricted by its obligate dimerization. In summary, although certain details may have to be optimized, CALI in principle is likely to be a good approach for the purpose of rapidly inducible Syk-inactivation in cells *in vitro*.

Another elegant approach for targeted protein inactivation has been demonstrated for tyrosine and serine/threonine kinases. The method relies on the sensitization of the target enzyme, but not any other kinases, to a modified inhibitor. This is achieved by the modification of a single conserved amino acid termed the gatekeeper, thus expanding the kinase's ATP-binding site by an additional pocket. At the same time, a bulky group is added to a general protein kinase inhibitor, thus preventing its binding to any native kinases. However, due to its enlarged ATP-binding site, the target kinase is susceptible to inhibition by the modified inhibitor (Bishop, et al., 2000: ; Liu, et al., 1998). Strategies as this are termed chemical genetics and offer several advantages (Knight and Shokat, 2007). For one, the kinase inhibitors are readily cell-permeable, which ablates the main problem I encountered with the present strategy. Second, direct administration of the inhibitor to cultured cells without the need for any additional equipment would be expected to cause instantaneous kinase-inactivation in a dose-dependent manner. Importantly, in contrast to conventional pharmacological inhibitors, this strategy confers absolute specificity for the selected target kinase. For these reasons, chemical genetics represent a highly attractive method for targeted Syk

inactivation. While the strategy has to be optimized for every individual kinase, it has been successfully employed on Src-kinases as well as EGFR, the latter's kinase domain sharing a high degree of homology to Syk's (see above). It therefore seems plausible that the genetic sensitization of Syk to a modified inhibitor should be fairly straight forward. Very recently, the chemical genetics approach has been extended by modification of an additional residue in the ATP-binding pocket which serves as the anchor point for covalent binding of a modified inhibitor and thus irreversible kinase inactivation (Blair, et al., 2007). Furthermore, addition of a fluorochrome to the irreversible inhibitor creates a means to specifically label the portion among the total pool of target kinase molecules which are activated in response to a certain stimulus. Such a measurement would be of enormous interest regarding the role of Syk and other kinases in tonic versus immunogenic BCR signaling.

While the alternative tactics discussed above may offer some considerable advantages for the purpose of rapid inactivation of Syk in cultured B cells, the approach attempted herein may still be of significant interest for other applications. For one, the information on the structural flexibility of Syk's kinase domain may be exploited in a scenario where the addition of ectopic loops to the kinase domain core may be desired. This could for example be with regard to creating novel binding sites which would mediate synthetic interaction with other cellular proteins or exogenous pharmacological compounds. Quite remarkably, the insertion of at least thirteen residues at at least two different positions within the kinase domain appears to be compatible with kinase activity, suggesting that significant additions can be made to this catalytic module at certain sites. Such alterations might also be suited to regulate the degree of enzymatic activity. In addition, kinase inactivation through proteolytic cleavage could be of use under conditions which involve a cell-free context, ablating the problem of intracellular protease delivery.

4.6 B cell survival mechanisms which could represent targets of the Syk-mediated tonic BCR signal

Should it be possible to surmount the technical challenges involved in rapid inducible Syk-inactivation, this system could be used to investigate the sensitivity of molecular cell survival signatures to loss of the Syk-mediated tonic BCR signaling. While not all potential targets of this pathway can be thoroughly discussed here, some important mechanisms which deserve

mentioning include the Bcl-2 protein family, members of the transcription factor NF-κB as well as the B cells' capacity to respond to BAFF-induced survival signals.

One family of proteins whose members have been briefly mentioned throughout this work was originally defined by its founding member Bcl-2 (reviewed in (Chao and Korsmeyer, 1998). The family encompasses both anti- and pro-apoptotic members, the former being multi-domain proteins consisting of several Bcl-2 homology domains (BH1 to 4). Pro-apoptotic family members include the multi-domain proteins Bax (Bcl2-associated X protein) and Bak (BCL2-antagonist/killer) as well as the so-called BH3-only proteins comprised of Bad, Bid (BH3 interacting domain death agonist), Bim and others. In recent years, significant advances have been made in understanding the mechanism how this family of proteins regulates cell survival (Willis and Adams, 2005). Briefly, in viable cells, Bax and Bak are believed to exist as monomers in the cytoplasm or at the outer mitochondrial membrane. A pro-apoptotic signal induces homo-oligomerization of Bax and Bak within the outer mitochondrial membrane, causing its permeabilization and release of intermembrane-resident proteins including cytochrome C. BH3-only proteins are mediators whose expression and activity is controlled by diverse apoptosis-regulating signals and who in turn assist in the activation of Bax and Bak. Their pro-apoptotic potential is believed to be contained by the pro-survival Bcl-2 family members through their sequestration away from Bax and Bak. This general scheme is likely to be a simplification to some extend and, in addition, defined regulatory functions probably exist for the individual proteins.

In the B cell compartment, anti-apoptotic family members Bcl-2, Bcl-xL, A1 (BCL2-related protein A1) and Mcl-1 are expressed at different stages throughout development. In mature peripheral B cells, Bcl-2 and A1 are highly expressed, whereas Bcl-xL seems to play a more dominant role at earlier B cell stages (Grillot, et al., 1996: ; Merino, et al., 1994: ; Tomayko and Cancro, 1998). Mcl-1 expression appears to be very dynamic as judged by its high protein turnover, its induction by cytokine-treatment and its decline in cells undergoing atrophy and death (Lomo, et al., 1996). Lymphocyte development in the absence of Bcl-2 is initially normal, but lymphocyte populations dramatically collapse later on due to massive apoptosis in the lymphoid organs (Veis, et al., 1993). Lack of Bcl-xL impairs the survival of immature lymphocytes, but their mature descendents appear to be less affected, whereas ablation of Mcl-1 blocks B lymphopoiesis at a very early stage

(Motoyama, et al., 1995: ; Opferman, et al., 2003). Conversely, inactivation of Bim causes B cell hyperplasia and impairs the negative selection of auto-reactive B cells (Bouillet, et al., 1999: ; Enders, et al., 2003: ; Oliver, et al., 2006). Combined inactivation of Bak and Bax also causes lymphoid hyperplasia and resistance to death by neglect (Lindsten, et al., 2000).

It is attractive to speculate that the tonic BCR-signal influences the composition of Bcl-2 family proteins in resting B cells, ensuring sufficient expression of Bcl-2, A1 and Mcl-1 while keeping at bay the upregulation of Bak, Bax and BH3-only proteins. As mentioned before, the distinct outcomes of tonic versus immunogenic BCR-signaling are unknown. However, antigenic BCR-stimulation effects the expression of several Bcl-2 family proteins which tends to coincide with their developmental fate of survival versus death (Enders, et al., 2003). In this context, it is interesting to note that ectopic overexpression of Bcl-2 prolongs the presence of receptorless B cells in the periphery, but fails to prevent their eventual demise (Lam, et al., 1997). In this sense, it is conceivable that tonic BCR-mediated survival is partially, although not completely, accomplished by regulation of Bcl-2 family proteins.

One protein family which has simultaneously been implicated in the regulation of Bcl-2 proteins as well as lymphocyte survival is the transcription factor NF-κB. NF-κB comprises a group of five mammalian proteins and ablation of individual members as well as proteins which function in NF-κB activation affect B cell survival. Loss of individual NF-κB proteins negatively infuences B cell viability under various conditions (reviewed in (Siebenlist, et al., 2005). More severely, combined ablation of both NF-κB1 and NF-κB2 or of c-Rel and p65 diminishes the mature peripheral B cell compartment (Franzoso, et al., 1997: ; Grossmann, et al., 1999: ; Grossmann, et al., 2000). This coincides with decreased expression of A1 and Bcl-2 in B cells while exogenously expressed Bcl-2 largely restores peripheral B cell maturation and survival in c-Rel/p65 double mutant mice (Claudio, et al., 2002: ; Grossmann, et al., 2000). As mentioned previously, two modes of NF-κB activation exist which are activated by different signals. BCR-engagement triggers the canonical pathway of NF-κB activation dependent on PKCβ, the adaptor complex CARMA1/BCL-10/MALT1 as well as IKKβ and γ which results in the formation of transcriptionally active p50/p65 heterodimers. On the other hand, alternative NF-κB activation for example in response to BAFF, involves NIK and IKKα and leads to the generation of p52/RelB heterodimers (Siebenlist, et al., 2005). Disruptions of either the canonical or the alternative pathway of NF-κB-activation affect the B cell compartment. Thus, inactivation mutations of

either CARMA1/BCL-10/MALT1, IKKβ and IKKγ or NIK and IKKα reduce the frequency of mature peripheral B cells (Thome, 2004). Overall, there is ample evidence for a tight correlation between NF-κB activity and B cell survival. Anti-apoptotic NF-κB target genes include Bcl-2, Bcl-xL and A1 (Dutta, et al., 2006), suggesting that NF-κB-dependent activation of Bcl-2 family gene expression could constitute an outcome of the tonic BCR signal which mediates B cell survival.

The discovery of BAFF as a second essential factor for mature resting B cell survival has expanded the possible mechanisms by which the tonic BCR signal could mediate B cell viability. It is an attractive hypothesis that at least part of the tonic BCR signal is somehow involved in the regulation of BAFF-mediated survival signaling. In its simplest form, tonic BCR-signaling could affect the expression of BAFF receptors, thus sensitizing B cells to the BAFF-mediated survival function. No such direct correlation has yet been reported. However, BAFF-R expression is affected by the small G-proteins Rac1/2 and the transcription factor c-Myb, both of which could themselves be targets of BCR-signaling (Thomas, et al., 2005: ; Walmsley, et al., 2003). It would also be very interesting to follow BAFF-R expression in B cells devoid of Syk or surface BCR prior to their complete demise, but such data have yet to be reported. Alternatively, it would be conceivable that tonic BCR-signaling impacts downstream factors such that they become susceptible to the BAFF-mediated signal or that the two pathways operate synergistically. For example, both NF-κB and Akt are responsive to activating signals from both BAFF and the BCR. The proximal signaling events appear to be at least partially distinct based on the canonical versus alternative mechanism of NF-κB activation and the differential requirement for PKCβ in Akt-activation. Yet, it is noteworthy, that both pathways appear to intersect at the level of the generic survival hallmarks NF-κB and Akt and it is conceivable that there might be a synergistic rather than mere additive effect to this mode of regulation. Similarly, PKCβ plays a role downstream of both BCR- and BAFF-mediated signaling in the activation of NF-κB and Akt, respectively. One more interesting aspect which has not been investigated to date is the potential regulation of PKCδ by tonic BCR signals.

5 Summary

The persistence of a large pool of mature naive B cells with a broad B cell receptor (BCR) repertoire is the basis for an appropriate immunological response to antigenic challenge in mice and humans. The longevity of mature peripheral B cells in the order of several weeks or month in a resting state is dependent on at least two survival cues, a tonic signal originating from the BCR and the cytokine B cell activating factor of the TNF family (BAFF).

The protein tyrosine kinase Syk has been implicated in signaling from the BCR during early B cell developmental stages and upon cross-linking of the BCR-complex by antigen. Using a mouse model in which Syk was inducibly ablated in the B cell lineage or in response to an exogenous trigger, an indispensable function for Syk in the survival of mature peripheral B cells was shown. The results of this study strongly suggest that Syk is a critical mediator of the tonic BCR survival signal.

Investigation of the effect of BAFF on B cells had so far been restricted to its survival function. The present study has uncovered a novel role for BAFF in ensuring the functional efficiency of mature peripheral B cells. BAFF was found to influence B cell growth, metabolism, energy homeostasis and entry into the cell cycle. These cellular changes are mediated by at least two BAFF-induced mechanisms: upregulation of the oncogenic kinase Pim-2 and activation of a signaling transduction pathway which centers around phosphoinositide-3-kinase (PI3K) and Akt. The latter controls the activity of key effector proteins, including transcription factors of the forkhead box O family and the master regulator of cellular metabolism mammalian target of rapamycin, whose activation state is sensitive to B cell stimulation with BAFF. Protein kinase C (PKC) β, a member of the classic family of PKCs, was found to mediate the activation of Akt in response to BAFF-treatment and PKCβ-deficient mice show signs of partial refractiveness of the B cell compartment to BAFF. Misregulated BAFF-signaling has been implicated in a variety of B cell pathologies, ranging from lymphopenia to hyperplasia and auto-immunity in mice and humans. Involvement of the PI3K-Akt pathway in BAFF-mediated B cell signaling may propose novel ways of interfering with pathological BAFF signaling in disease states.

6 Zusammenfassung

Eine große Anzahl reifer naiver B Lymphozyten mit einem ausgedehnten B Zellrezeptor Repertoire ist die Grundlage für eine angemessene Immunantwort im Falle einer Begegnung mit einem fremden Antigen in Mäusen und Menschen. Die Langlebigkeit reifer periphärer B Zellen in einer Größenordung von mehreren Wochen oder Monaten ist abhängig von mindestens zwei Überlebenssignalen, einem tonischen Signal, welches vom B Zellrezeptor ausgeht, und dem Zytokin B Zell aktivierender Faktor der TNF-Familie (BAFF).

Die Proteintyrosinkinase Syk steht im Zusammenhang mit Signaltransduktion vom B Zellrezeptor während früher Stadien der B Zellentwicklung sowie im Falle der B Zellrezeptorstimulation mit fremdem Antigen. Im Zuge dieser Arbeit wurde ein Mausmodell verwendet, in welchem Syk entweder spezifisch in der Familie der B Lymphozyten oder auf induzierbare Art und Weise durch einen äußeren Auslöser inaktiviert wurde. Die von dieser Untersuchung gewonnenen Ergebnisse lassen darauf schliessen, dass Syk unverzichtbar für die Vermittlung des tonischen B Zellrezeptorsignals ist, welches das Überleben reifer B Zellen ermöglicht.

Die Untersuchung des Effekts von BAFF auf B Lymphozyten hatte sich bis dato auf dessen Überlebensfunktion beschränkt. Im Zuge der vorliegenden Arbeit wurde eine neue Rolle dieses Zytokins in der Erhaltung der Funktionstüchtigkeit von reifen periphären B Zellen entdeckt. BAFF hat Einfluss auf das Wachstum, den Metabolismus, den Energiehaushalt und die Regulierung des Zellzyklus in B Zellen. Diese physiologischen Veränderungen werden von mindestens zwei BAFF-induzierten Mechanismen vermittelt: einer erhöhten Expression der onkogenen Kinase Pim-2 zum einen, sowie der Aktivierung eines Signaltranduktionsweges, welcher sich um Phosphatidylinositol-3-Kinase (PI3K) und Akt dreht, zum anderen. Letzterer kontrolliert entscheidende Effektorproteine, einschliesslich Transkriptionsfaktoren der forkhead box O (FoxO) Familie sowie dem Hauptregulator des zellulären Metabolismus, mammalian target of rapamycin, deren Aktivierungszustand auf B Zellstimulation mit BAFF reagiert. Protein Kinase C β, ein Mitglied der klassischen Familie von PKCs, vermittelt die Aktivierung von Akt in Folge der B Zellstimulation mit BAFF und B Zellen aus PKCβ-defizienten Mäusen zeigen Anzeichen von Unsensitivität gegenüber BAFF-Stimulation. Misregulierte BAFF-abhängige Signaltransduktion ist mit verschiedenen B Zellpathologien in Mäusen und Menschen in Verbindung

gebracht worden, die sowohl Lymphopenie als auch Hyperplasie und Autoimmunität umfassen. Die Mitwirkung des PI3K-Akt Signaltransduktionsweges an den physiologischen Auswirkungen von BAFF auf B Lymphozyten, kann möglicherweise neue Wege eröffnen, durch die gezielt in pathologische BAFF-Effekte eingegriffen werden kann.

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9 Erklärung

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt und die verwendeten Quellen und Hilfsmittel vollständig angegeben habe, daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat und daß sie, abgesehen von den unten angegebenen Teilpublikationen, noch nicht veröffentlicht worden ist. Die Bestimmungen dieser Promotionsordnung sind mir bekannt.

Teilpublikationen:

Saijo K, Schmedt C, Su IH, Karasuyama H, Lowell CA, Reth M, Adachi T, **Patke A**, Santana A, Tarakhovsky A. Essential role of Src-family protein tyrosine kinases in NF-kappaB activation during B cell development. Nat Immunol. 2003 Mar;4(3):274-9.

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Patke A, Mecklenbrauker I, Erdjument-Bromage H, Tempst P, Tarakhovsky A. BAFF controls B cell metabolic fitness through a PKC beta- and Akt-dependent mechanism. J Exp Med. 2006 Oct 30;203(11):2551-62.

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10 Publications

Diploma thesis: „Untersuchung des Einfluß von Proteinkinase C auf die Antigenrezeptor und Phorbolester induzierte Aktivierung von NF-κB und JNK in B-lymphoiden Zellen“. Freie Universität Berlin, 2001

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11 Appendix

Table 5: Gene IDs corresponding to the GO categories in figure 9B (Cluster 1) and C (Cluster 2)

GO category	Gene ID
Cluster 1	
cellular carbohydrate catabolism	Aldoc, Tpi1, Pkm2, Pfkl, Pfkp, Pgam1
monosaccharide metabolism	Aldoc, Galk1, Tpi1, Fabp5, Pkm2, Pfkl, Pfkp, Pgam1
monosaccharide catabolism	Aldoc, Tpi1, Pkm2, Pfkp, Pfkl, Pgam1
alcohol catabolism	Aldoc, Tpi1, Pkm2, Pfkl, Pfkp, Pgam1
hexose catabolism	Aldoc, Tpi1, Pkm2, Pfkl, Pfkp, Pgam1
hexose metabolism	Galk1, Aldoc, Tpi1, Fabp5, Pkm2, Pfkl, Pfkp, Pgam1
glucose metabolism	Aldoc, Tpi1, Fabp5, Pkm2, Pfkl, Pfkp, Pgam1
glucose catabolism	Aldoc, Tpi1, Pkm2, Pfkl, Pfkp, Pgam1
glycolysis	Aldoc, Tpi1, Pkm2, Pfkl, Pfkp, Pgam1
Cluster 2	
cell cycle	Kntc1, 2810406C15Rik, Cdkn3, Cdk4, Bub1b, Esco2, Rad51, Dmwd, Espl1, Ccnb1, Mybl2, Aurka, Csks2, E2f7, Mki67, Gmnn, Tfdp1, Wwox, Chek1, Ect2, Nek2, Kif11, Cdc20, Cenph, Sycp3,Cdca5, Stmn1, Ccnd2, Uhrf2, Sfn, Uhrf1, Sspn, Brrn1, Ccnf, Tacc3, Mcm3, E2f1, Myc, Incenp
regulation of cell cycle	Cdkn3, Cdk4, Ccnd2, Sfn, Sspn, Ccnb1, Mybl2, Ccnf, Gmnn, Tacc3, Tfdp1, E2f1, Chek1, Wwox, Myc, Ect2
mitotic cell cycle	Cdc20, Kntc1, Cenph, 2810406C15Rik, Sycp3, Cdc5a, Bub1b, Stmn1, Brrn1, Ccnb1, Aurka, Ccnf, Tfdp1, Chek1, Nek2, Incenp
meiotic cell cycle	Sycp3, Rad51, Cks2, Mki67, Dmwd, Nek2, Espl1
M phase	Cdc20, Kntc1, Cenph, 2810406C15Rik, Sycp3, Cdc5a, Bub1b, Stmn1, Rad51, Dmwd, Espl1, Brrn1, Ccnb1, Cks2, Ccnf, Mki67, Nek2, Incenp

chromosome segregation	Cenph, 2810406C15Rik, Brrn1, Top2a, Espl1, Nek2
regulation of progression through cell cycle	Cdkn3, Cdk4, Ccnd2, Sfn, Sspn, Ccnb1, Mybl2, Ccnf, Gmnn, Tacc3, Tfdp1, Chek1, Wwox, E2f1, Myc, Ect2
M phase of mitotic cell cycle	Cdc20, Kntc1, Cenph, 2810406C15Rik, Sycp3, Cdc5a, Bub1b, Stmn1, Brrn1, Ccnb1, Ccnf, Nek2, Incenp
M phase of meiotic cell cycle	Sycp3, Cks2, Rad51, Mki67, Dmwd, Nek2
mitosis	Cdc20, Kntc1, Cenph, 2810406C15Rik, Sycp3, Cdc5a, Bub1b, Stmn1, Brrn1, Ccnb1, Ccnf, Nek2, Incenp
meiosis	Sycp3, Rad51, Cks2, Mki67, Dmwd, Nek2, Espl1
chromosome condensation	2810406C15Rik, Brrn1, Top2a
mitotic sister chromatid segregation	2810406C15Rik, Brrn1, Nek2