TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Entwicklungsgenetik

Analysis of B Cell Antigen Receptor-Mediated NF-κB Activation

Uta Ferch

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. Siegfried Scherer
Prüfer der Dissertation:	1. Univ.-Prof. Dr. Wolfgang Wurst
	2. Priv.-Doz. Dr. Jürgen Ruland
	3. Priv.-Doz. Dr. Ulrich Keller

Die Dissertation wurde am 11.02.2008 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 17.07.2008 angenommen.

INDEX

INDEX 3

ZUSAMMENFASSUNG 6

ABSTRACT 8

1. INTRODUCTION 9

1.1 Adaptive Immunity 9
1.1.1 B Cells: Mediators of the Humoral Response 11
1.2 Signal Transduction in B Cells 12
1.2.1 Detection of Exogenous Signals in B Cells 13
1.2.1.1 Crosslinking of the B Cell Antigen Receptor 13
1.2.2 Spatial Regulation of B Cell Signalling: Lipid Rafts 15
1.2.3 BCR-Mediated NF-κB Activation 17
1.2.3.1 NF-κB 18
1.2.3.2 Ligand-Induced NF-κB Activation 19
1.2.3.3 Tonic Signalling to NF-κB 21
1.2.4 Regulation of B Cell Functions by NF-κB 22
1.2.5 NF-κB in Lymphomagenesis 23
1.3 Transgenic Mouse Models 24
1.3.1 *Bcl10* Knockout Mouse 25
1.3.2 *Malt1* Knockout Mouse 25
1.3.3 *BCL2* Transgenic Mouse 26
1.4 Specific Aims of this Project 27

2. MATERIALS AND METHODS 28

2.1 Materials 28
2.1.1 Reagents 28
2.1.2 Antibodies 28

2.1.3 Mouse strains and Genotyping Primers 28
2.2 Methods 29
2.2.1 Isolation of Primary B Cells 29
2.2.2 Culture and Stimulation of Primary B Cells 29
2.2.3 Survival and Proliferation Assays 29
2.2.4 Real Time Quantitative PCR 30
2.2.5 Western Blot 30
2.2.6 Co-Immunoprecipitation 31
2.2.7 Lipid Raft Preparation 31
2.2.8 Gel Mobility Shift Assay 31
2.2.9 Immunofluorescence 32
2.2.10 Statistics 32

3. RESULTS 33

3.1 Bcl10 and Malt1 Have Both Common and Individual Functions in BCR Signalling 33
3.2 Bcl10 Controls Malt1-Independent B Cell Proliferation 37
3.3 Bcl10 Controls IKK Signalosome Formation and Activation Independently of Malt1 38
3.4 Malt1 Couples the BCR Signal to c-Rel Activation 41
3.5 Malt1 Signalling Controls the Release of c-Rel from IκB 45

4. DISCUSSION 47

4.1 Bcl10 and Malt1 Mediate Tonic Signalling to NF-κB 47
4.2 Ligand Induced BCR-Signalling: the Canonical NF-κB Pathway is Bifurcated 48
4.2.1 Bcl10 is Essential for Recruitment and Activation of IKK in Lipid Rafts 48
4.2.2 Bcl10 is Essential for the Activation of RelA and c-Rel 49
4.2.3 Malt1 Directs Canonical NF-κB Signalling Selectively to c-Rel 50
4.2.4 Molecular Model of BCR-Mediated NF-κB Activation 51
4.3 Implications for the Presented Model in Other Cellular Contexts 53

ABBREVIATIONS **55**

CITATION INDEX **58**

PUBLICATIONS **66**

CURRICULUM VITAE **67**

DANKSAGUNG **68**

ZUSAMMENFASSUNG

Die Erkennung einer nahezu unbeschränkten Vielzahl an Pathogenen wird durch Antigenrezeptoren des adaptiven Immunsystems ermöglicht. Diese spezialisierten Rezeptoren auf der Oberfläche von Lymphozyten vermitteln durch die Induktion von Transkriptionsfaktoren eine streng regulierte Aktivierung bestimmter Immunzellfunktionen. Eine maßgebliche Rolle kommt dabei dem Transkriptionsfaktor “nuclear factor-kappaB“ (NF-κB) zu. In B Zellen führt die Stimulation des B Zell Antigenrezeptors (BZR) über den kanonischen NF-κB-Signalweg zur Aktivierung eines Multiprotein-Komplexes, dessen Stabilisierung spezialisierte Membran-Mikrodomänen, die “Lipid Rafts“ erfordert. Dieser aktivierte Multiprotein-Komplex reguliert die spezifische Aktivierung einer Vielzahl von Zielgenen anhand der selektiven Induktion von NF-κB-Dimeren, die die Untereinheiten RelA oder c-Rel enthalten. Die Adapterproteine Bcl10 und Malt1 stellen als direkte Interaktionspartner essentielle Komponenten der kanonischen NF-κB-Signalkaskade dar. Das vor dieser Arbeit bestehende Modell ging dabei von einer untrennbaren Funktion von Bcl10 und Malt1 in einem linearen Signalweg vom BZR nach NF-κB aus. Die molekularen Funktionen von Bcl10 und Malt1 sowie der Mechanismus der selektiven Aktivierung der NF-κB-Untereinheiten RelA oder c-Rel nach BZR-Stimulation waren jedoch unverstanden.

Im Rahmen der vorliegenden Arbeit wurde anhand der Analyse primärer B Zellen aus “Knock-out“-Maus-Modellen gezeigt, dass Bcl10 und Malt1 sowohl gemeinsame als auch unterschiedliche Funktionen in der BZR-vermittelten NF-κB Aktivierung ausüben. Bcl10 war für den Aufbau des “Lipid Raft“-assoziierten Signalosoms und die Aktivierung von RelA und c-Rel unentbehrlich. Funktionell konnte gezeigt werden, dass dieser Bcl10-abhängige Signalfluss die Inhibierung von Apoptose und Induktion von Zellteilung nach BZR Stimulation kontrolliert. Demgegenüber wirkten Bcl10 und Malt1 zwar in der Vermittlung von Überlebenssignalen zusammen, jedoch war Malt1 weitgehend verzichtbar für BZR-vermittelte Proliferation. Dies zeigte sich molekular in einem von Malt1-unabhängigen Aufbau des “Lipid Raft“-assoziierten Signalosoms und einer weitgehend unbeeinträchtigten Induktion von RelA. Demzufolge besteht die Funktion von Malt1 in der Ausrichtung des kanonischen NF-κB Signalflusses auf die selektive Aktivierung der Untereinheit c-Rel, wodurch die Kontrolle eines

bestimmten B Zell Subprogramms erfolgt. Die vorliegende Arbeit gibt mechanistischen Einblick in die Regulation BZR-vermittelter Überlebens- und Proliferationssignale. Darüber hinaus wurde eine bisher unbeschriebene Bifurkation des kanonischen NF-κB Signalwegs identifiziert, in der Bcl10 und Malt1 zu einer selektiven Aktivierung der NF-κB-Untereinheiten RelA und c-Rel führen.

ABSTRACT

NF-κB transcription factors differentially regulate distinct immune cell functions. In B cells, the B cell antigen receptor (BCR)-mediated canonical NF-κB pathway leads to the activation of RelA and c-Rel containing dimers. The scaffold proteins Bcl10 and Malt1 have been reported to cooperate for a linear pathway leading to "Inhibitor of κB kinase" (IKK) signalosome assembly and subsequent canonical NF-κB activation. However, the molecular roles of Bcl10 and Malt1 in BCR-induced IKK activation are unclear and how the functionally distinct RelA and c-Rel subunits are selectively controlled is unknown.

Using primary cells derived from genetic mouse models the present study revealed both common and distinct functions for Bcl10 and Malt1 in BCR signalling. In this context, a previously unrecognized bifurcation in the BCR-induced canonical signalling pathway at the level of Bcl10 and Malt1 has been identified. Bcl10 was required to recruit IKK into BCR associated lipid raft signalosomes for the activation of RelA and c-Rel containing NF-κB complexes. These Bcl10 dependent pathways controlled proliferation in activated B cells as well as cell survival of activated and of resting B cells. Malt1 participated in survival signalling. However, Malt1 was not required for IKK lipid raft recruitment or activation and largely dispensable for RelA activation or productive B cell proliferation. In contrast, Malt1 directed the canonical BCR signal from Bcl10 selectively to c-Rel to control a distinct B cell subprogram. The presented results provide mechanistic insights into BCR-induced survival and proliferation signals and demonstrate the selective control of c-Rel in the canonical NF-κB pathway.

1. INTRODUCTION

Humans are exposed to a vast array of potential pathogens daily, through contact, ingestion, and inhalation. The protection against these invading pathogens is mediated by a multitude of mechanisms referred to as the immune system. This comprises the cooperative action of a plethora of specialized cell types in two parallel but interrelated systems, termed innate and adaptive immunity. The innate immune response constitutes the first line of defence at direct sites of infection. Activation of innate immunity is triggered by pathogen-associated microbial-specific patterns that are recognized by a limited number of germline-encoded receptors. In addition to fighting infection directly, these responses enhance the activation of adaptive immunity in peripheral lymphoid organs, inducing a second wave of defence. Unlike innate immune responses, adaptive responses provide highly specific and long-lasting protection against reinfection with the same pathogen. A prerequisite for these functions is the high diversity of adaptive immune cell receptors that is generated by somatic recombination of gene segments encoding antigen-specific receptors. Both adaptive and innate immune receptors serve to recognize foreign microbial structures and transduce the external signal into activation of various transcription factors. They induce the expression of a broad spectrum of target genes that allow the mounting of a specific immune response. Among the large group of transcription factors, nuclear factor κB (NF-κB) plays a pivotal role in the direction of immune cell function. Receptor-mediated NF-κB activation controls the transcription of a wide variety of target genes, including cytokines, chemokines, adhesion receptors, antimicrobial peptides, cell cycle regulators, as well as cellular survival factors. These induced gene products are critically involved in the regulation of the immune response, as they control migration, maturation, proliferation and survival of immune cells (Ghosh, May et al. 1998), thus contributing to the clearance of infections.

1.1 Adaptive Immunity

Although innate immunity can effectively fight many infections, microbes have evolved to resist innate immunity consistently emerging new variants. To enable the immune system to respond to an almost unlimited diversity of antigens

specialized receptors are used. They are expressed on the surface of the key mediators of adaptive immunity, the lymphocytes. These lymphocyte receptors are collectively referred to as antigen receptors (AgR). AgRs are encoded by genes that are assembled from a series of gene segments by a unique form of genetic recombination that occurs early in lymphocyte development, independently from antigen encountering. This assembly process generates an enormous diversity of receptors. However, each lymphocyte expresses a unique AgR of only one single specifity that enables it to bind to a particular antigen. As this generation of AgRs occurs randomly, the recognition of self is unavoidable (Janeway and Bottomly 1994). Lymphocytes that would react against self molecules are either induced to go into apoptosis (`negative selection´), to re-edit their receptors or to be inactivated (`anergic´). The remaining collection of millions of single lymphocyte clones is called the lymphocyte repertoire and provides the basis for the diversity of adaptive immunity.

Lymphocytes develop in a central lymphoid organ, giving rise to two major classes of lymphocytes: the B lymphocytes or B cells that are generated in the bone marrow, (BM; “B”, **b**one marrow) and the T lymphocytes or T cells that evolve in the thymus (“T”, **t**hymus). According to this, their antigen receptors are termed B cell receptor (BCR) or T cell receptor (TCR), respectively. Both B and T cells circulate continuously between the blood and lymph and accumulate in lymphoid organs. When a lymphocyte encounters its specific antigen in a peripheral lymphoid organ, the binding of the antigen to the receptor activates the lymphocyte, causing it both to proliferate and to differentiate into an effector cell. This process is referred to as `clonal selection´. The clonally selected B and T cells give rise to the two branches of adaptive immunity, the humoral immunity and the cell-mediated immunity. Humoral immunity is mediated by effector B cells, secreting antibodies, which can act over long distances to help eliminate extracellular pathogens and their toxins. Effector T cells, by contrast, act locally at sites of infection to either kill infected host cells or help other cells to eliminate pathogens, what is described as cell-mediated immunity. As part of the adaptive immune response, some lymphocytes differentiate into memory cells, which are able to respond faster and more efficiently the next time the same pathogen invades.

1.1.1 B Cells: Mediators of the Humoral Response

B lymphocytes are generated via a series of sequential differentiation steps (Rolink, Ghia et al. 1995). Generation of immature B cells in the BM is termed as central B cell development. B-lineage precursors in the BM proliferate and progress through a highly regulated maturation process that culminates in the production of immature B cells expressing a BCR on the cell surface. These immature B lymphocytes then migrate into the spleen, where they differentiate into mature, naïve B cells that have not encountered their antigen yet. This process is referred to as peripheral B cell development. Naïve, mature B cells recirculate through the bloodstream and enter peripheral lymph nodes (LN), gut-associated lymphoid tissues (GALT), mucosa-associated lymphoid tissues (MALT), peritoneal or pleural cavities, or the spleen where they respond to a specific antigenic challenge.

Distinct developmental clues give rise to three subsets of mature B cells: marginal zone (MZ) B cells, peritoneal (B-1) B cells, and follicular B cells or B-2 B cells (Martin and Kearney 2000). While MZ B cells are located in the MZ, a highly organized region of the spleen, B-1 B cells reside in the peritoneal and pleural cavities. As they differ from follicular B cells in expressing BCRs with a limited range of antigen specificities and confer to the rapid response against pathogens, they are often grouped as `innate-like´ B cells (Martin, Oliver et al. 2001) Conventional follicular B cells that are primarily present in the follicles of the spleen, by contrast, respond delayed and highly specific to antigen stimulation. Activated follicular B cells internalize the BCR-bound antigen and present processed peptide-fragments to a specialized T cell subset. T cell costimulation induces a sophisticated maturation process, the germinal centre (GC) reaction. The GC reaction is characterized by proliferation of the clone displaying the required receptor specifity (`clonal expansion´) and differentiation into effector cells including plasma cells (PC) and memory B cells.

PCs are the mediators of humoral immunity by producing antibodies that are identical to the specific BCR expressed on the cell surface except for the membrane binding region. Thus, the generated antibodies harbour the same unique antigen-binding site as the B cell clone selected by antigen. These antibodies are secreted into the circulation and mucosal fluids and protect the host from infection in three main ways. They neutralize the infectivity of pathogens or

microbial toxins by binding to them. By coating the pathogens, they activate accessory cells to ingest and kill the pathogen, a process called opsonization. Moreover, antibodies trigger activation of the complement system, a group of plasmaproteins directed against extracellular pathogens. Complement proteins strongly enhance opsonization, and directly lyse bacterial cells.

The characteristic biological response following antigen binding is mediated by five distinct classes of antibodies. A typical antibody molecule is composed of two identical heavy chains and two identical light chains. Both light and heavy chains are made up of repeating segments that form compact functional units called immunoglobulin (Ig) domains. Parts of both the heavy and light chains combine to form the antigen-binding sites. The five classes of antibodies (IgM, IgD, IgG, IgA and IgE), termed isotypes, are distinguished by a particular heavy chain that forms the tail (Fc region) of the antibody. The Fc region determines which other proteins will bind to the antibody and therefore what biological properties the antibody has.

1.2 Signal Transduction in B Cells

A fundamental feature of each living cell is to adapt to environmental changes. Therefore, exogenous stimuli have to be communicated to intracellular changes. This process is referred to as `signal transduction´. The multiplicity of extracellular signals is detected by disparate receptors expressed on the cell surface. Extracellular binding of a specific ligand is converted into intracellular biochemical events that are sustained and amplified in signalling cascades. These signalling cascades direct the formation of multiprotein complexes, often designated as `signalosomes´ that account for both spatial and temporal precision of signal transduction.

Thus, interaction modules consisting of adaptor proteins and enzymes act to localize proteins to specific subcellular sites and control enzyme activities. This is achieved by reversible covalent modifications and subsequent conformational changes generating a signal that is propagated downstream from protein to protein. On the one hand reversible covalent modifications confer directly to altered affinity for interaction partners or activation of enzymes. On the other hand, this signal is transmitted via adapted subcellular concentrations. This implicates

changed concentrations of the protein itself, as induced by the formation of oligomers, or of small molecules, called second messengers.

Important reversible covalent modifications are phosphorylation of tyrosine or serine/threonine residues, acylation to determine membrane localization and ubiquitination. Conjugation of lysine residues with ubiquitin has an important role in signal transduction and is catalyzed by three enzymatic reactions comprising an ubiquitin-activating, an ubiquitin-conjugating and an ubiquitin-protein ligase reaction. Ubiquitin itself contains further lysine residues that can be selectively attached to other ubiquitins to form a polyubiquitin chain. The chosen lysine residue of ubiquitin determines the fate of the marked protein. Typically, a protein is targeted for degradation (Chau, Tobias et al. 1989). Alternatively, `regulatory´ polyubiquitination provides new interaction sites to recruit and activate downstream proteins (Chen, Parent et al. 1996).

These biochemical mechanisms to propagate and amplify an intracellular signal culminate to a large degree in the activation of transcription factors. Activated transcription factors bind to regulatory sites in their target DNA to mediate the induction or repression of distinct target genes. Thus, via the process of signal transduction, the exogenous signal is converted into gene expression changes thereby regulating immediate and long-lived cellular responses.

1.2.1 Detection of Exogenous Signals in B Cells

A plethora of extracellular stimuli inform B cells to the presence of infectious conditions or acute stress. These stimuli are recognized by disparate receptors expressed on the B cell surface, such as proinflammatory cytokine receptors or Toll-like receptors (TLRs) and immunomodulatory receptors like CD40 or BAFF-R3 (B cell activating factor of the TNF family receptor 3) (Ruland and Mak 2003). Among the large group of receptors the antigen specific B cell receptor (BCR) exerts a key function in mediating B cell responses. The BCR signal can be modulated by signals simultaneously received from various coreceptors.

1.2.1.1 Crosslinking of the B Cell Antigen Receptor

Membrane-bound immunoglobulin expressed on the cell surface serves as B cell antigen receptor (BCR) (Figure 1). B cell activation occurs upon complementary

binding of a specific antigen to the BCR (`BCR crosslinking´). This leads to oligomerization of BCR molecules and signal induction. The structure recognized by the BCR is termed antigenic determinant and allows for a broad classification of antigens. Most antigenic macromolecules have many different antigenic determinants and are termed as multivalent (i.e. viral coat proteins)*;* if two or more of them are identical, the antigen is referred to as polyvalent (i.e. polysaccharide capsules of bacteria). Given that BCRs of naïve, mature B cells in principal comprise IgM isotypes, antigen-crosslinking can be mimicked by stimulation with an antibody binding to surface IgM.

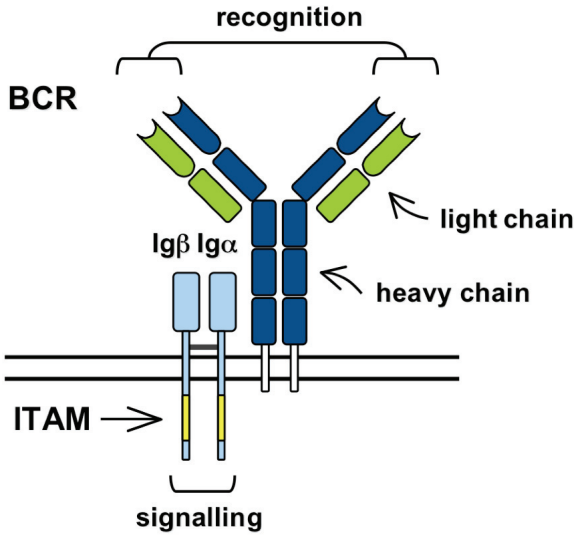

Figure 1. The B cell Receptor. The B cell Receptor is made up of cell surface immunoglobulin (heavy and light chains) and an invariant Igα/Igβ heterodimer. Each Igα and Igβ have a single immunoreceptor tyrosine-based activation motif (ITAM) in their cytosolic tails that enables them to signal when the B cell receptor is ligated with antigen.

The antigen-binding BCR is not directly involved in the transduction of biochemical signals. Signalling is mediated by a non-covalently associated Igα-Igβ heterodimer. Each Igα and Igβ contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Upon engagement of the BCR two tyrosine residues within the ITAMs are phosphorylated by the Src-family kinase Lyn (Figure 3). The BCR itself has no intrinsic kinase activity. The phosphorylated ITAMs provide a docking site for the SH2-domain kinase Syk, triggering downstream signalling cascades. Finally this leads to the transcriptional induction of a variety of genes associated with B cell activation, survival,

proliferation and differentiation. This role in the initiation of signalling represents one of the two important interrelated BCR functions. The second function of the BCR is the uptake and targeting of the antigen to the major histocompatibility complex (MHC) class II antigen processing and presentation pathway.

Based on mechanistic similarities in the initiation of signalling, the BCR is classified to the family of multichain immune recognition receptors (MIRR). This family further includes the T cell receptor for antigen (TCR) and the high-affinity receptor for IgE (FcεR1) expressed by mast cells and basophils. A common feature of this family of receptors is that signal initiation requires the presence of specialized membrane microdomains, termed `lipid rafts´ (Langlet, Bernard et al. 2000).

1.2.2 Spatial Regulation of B Cell Signalling: Lipid Rafts

The plasmamembrane is composed primarily of phospholipids, sphingolipids and cholesterol. The phospholipid bilayer is packed very loosely, allowing lateral movement of membrane-associated proteins. This is due to the unsaturated acyl chains of phospholipids that tend to be kinked.

Within this liquid-disordered phase float lateral heterogeneities in the outer leaflet of the plasmamembrane, which are referred to as `lipid rafts´ (Figure 2). They are enriched in sphingolipids and cholesterol. Sphingolipids are widely used as markers for lipid rafts; a common example is the glycosphingolipid GM1. Characteristically, sphingolipids consist of long, largely saturated acyl chains that allow them to pack tightly in a bilayer. The spaces between the acyl chains are occupied by cholesterol, which further condenses the packing of the sphingolipids. This promotes the formation of a liquid-ordered phase that allows only little lateral diffusion. The inner leaflet of lipid rafts is less well characterized but is probably composed of saturated phospholipids. The different packing of phospholipids and sphingolipids leads to their phase separation in membrane bilayers, forming a mosaic of raft and non-raft assemblages. This separation provides a mechanism for the compartmentalisation of signalling components within the membrane, concentrating certain components in lipid rafts and excluding others. Thus, lipid rafts exert a key function in resting cells in that they impede signal initiation in the absence of specific stimuli.

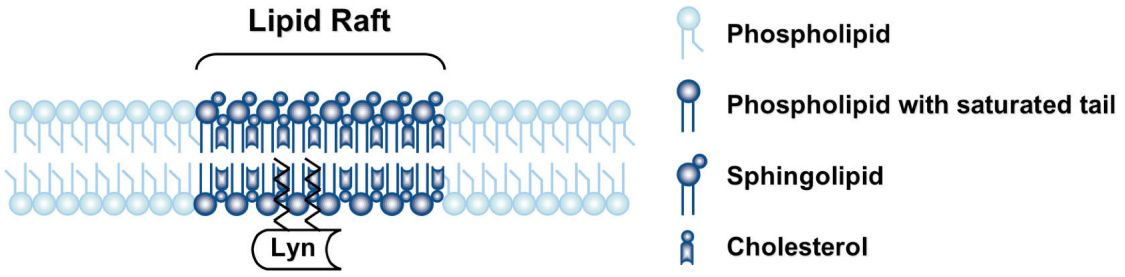

Figure 2. Compartmentalisation of the plasmamembrane: lipid rafts. Tight package of sphingolipids and cholesterol in the outer leaflet of the plasmamembrane leads to the formation of liquid-ordered phases referred to as lipid rafts. Due to their high condension they are separated from the loosely assembled phospholipid bilayer (liquid-disordered phase). Lipid rafts concentrate the Src tyrosine kinase Lyn.

While the vast majority of transmembrane proteins are excluded from lipid rafts, a small number of integral membrane proteins constitutively partition into these membrane microdomains, which are usually dually acylated by saturated fatty acids. A third, very small group of proteins can be distinguished that reside constitutively outside rafts but become raft associated upon activation. The MIRRs are important examples of this group. The monomeric BCR in resting mature B cells is predominantly excluded from lipid rafts (Pierce 2002) (Figure 3). In contrast, lipid rafts have been shown to concentrate the Src family kinase Lyn (Casey 1995). Binding of antigen to the BCR leads to receptor oligomerization and induces a conformational change that increases the affinity for rafts. This is followed by a transient translocation into these membrane microdomains. Within the lipid rafts the BCR is brought into association with Lyn. Lyn phosphorylates the ITAMs of the Igα/Igβ heterodimer and initiates the signalling cascade, resulting in the recruitment of additional signalling components. Lipid rafts coalesce to clusters, which serve as platforms for signal transduction. Thus, in stimulated cells, the second important function of lipid rafts is to facilitate signalosome assembly and to stabilize the formed complex. Stabilization is achieved by preventing the dissociation of complex components and by selective exclusion of negative regulators. This exemplifies the role of lipid rafts in spatial regulation of signal transduction mediated by selective exclusion and inclusion of signalling components.

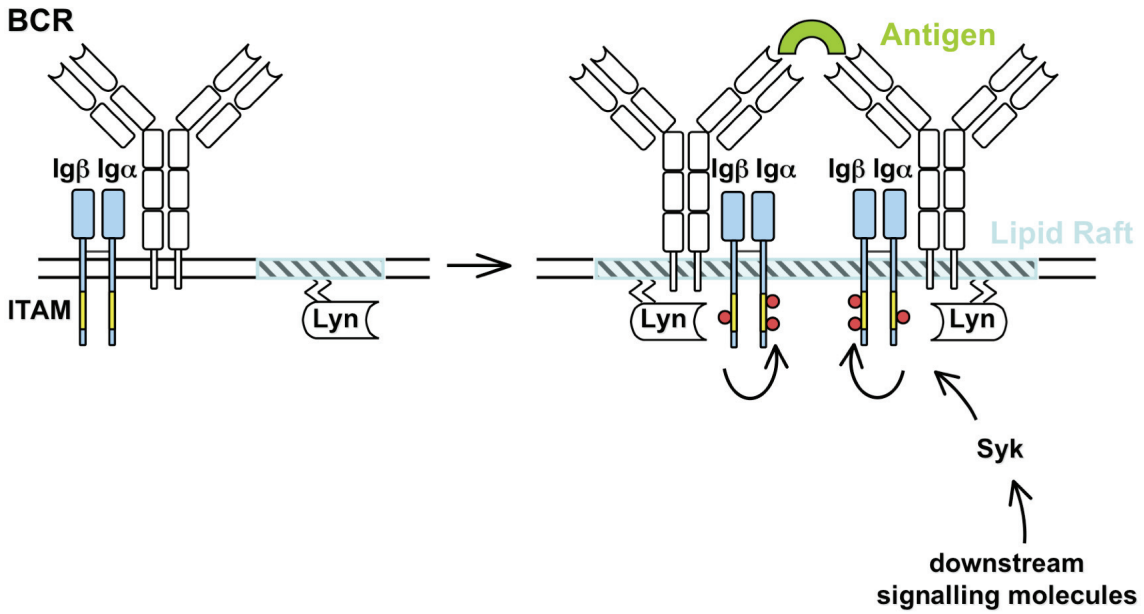

Figure 3. Initiation of BCR signalling depends on lipid rafts. The monomeric BCR is predominantly excluded from lipid rafts, which concentrate the Src tyrosine kinase Lyn. Antigen binding and receptor oligomerization induce translocation into these membrane microdomains, where Lyn phosphorylates the ITAMs of the Igα/Igβ heterodimer. This leads to the recruitment of the tyrosine kinase Syk and further downstream signalling molecules. ITAM, immunoreceptor tyrosine-based activation motif.

1.2.3 BCR-Mediated NF-κB Activation

Signalling pathways triggered by BCR crosslinking lead to the activation of several key transcription factors, including nuclear factor κB (NF-κB) and activator protein 1 (AP-1) families.

Induction of AP-1 transcription factors is initiated by guanine-nucleotide exchange factors (GEFs) that become associated with the receptor and activate small GTP-binding proteins. These in turn engage via a cascade of tyrosine/threonine phosphorylations mitogen-activated protein kinases (MAP kinases); three major groups can be distinguished in immune cells: the extracellular signal regulated protein kinases (ERK), the p38 MAP kinases and the c-Jun N-terminal kinases (Jnk). Dually phosphorylated MAP kinases translocate to the nucleus and phosphorylate transcription factors, finally culminating in AP-1 activation. AP-1 regulates the expression of many genes involved in cell growth.

Independently from the important function of AP-1, in a remarkable number of instances critical changes in gene expression upon BCR stimulation are mediated

by the NF-κB family of transcription factors that exert a crucial role in B cell function (Hayden and Ghosh 2004).

1.2.3.1 NF-κB

NF-κB represents a family of structurally related and evolutionary conserved proteins that comprises five members in mammals: RelA, c-Rel, RelB, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) (Ghosh, May et al. 1998) (Figure 4a). These NF-κB proteins are present as homo- and heterodimers and are hence often referred to as NF-κB subunits. Characteristic is the conserved N-terminal Rel-homology domain (RHD) that mediates dimerization, association with IκB inhibitory proteins and binding to a variety of related target DNA sequences called κB sites (Ghosh, May et al. 1998).

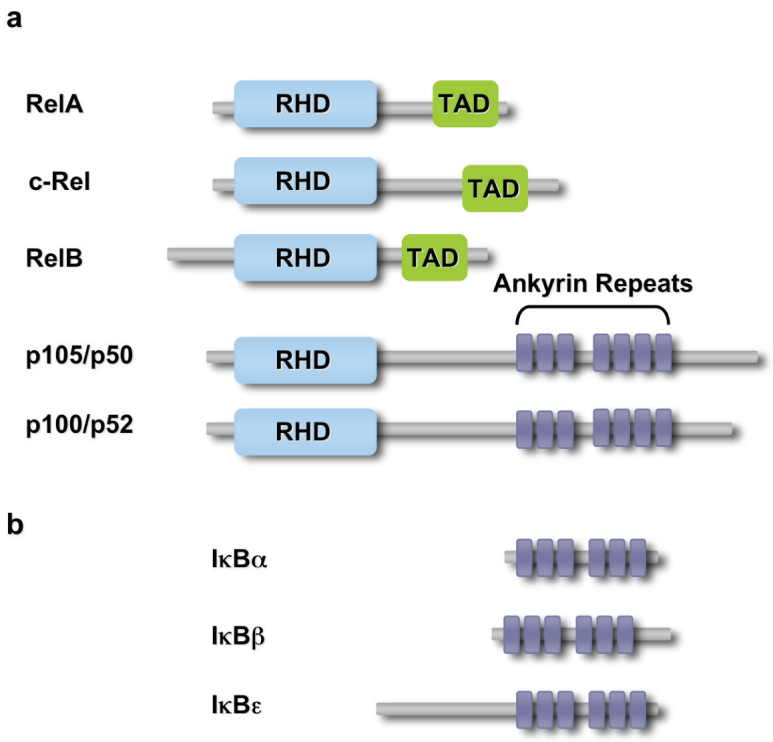

Figure 4. NF-κB subunits and inhibitory κB proteins. (a) The NF-κB family comprises five members: RelA, c-Rel, RelB, p105/p50 (NF-κB1) and p100/p52 (NF-κB2). Characteristic is the structurally conserved amino-terminal Rel-homology domain (RHD), which contains the dimerization, nuclear-localization and DNA-binding domains. RelA, c-Rel and RelB also possess a carboxy-terminal transactivation domain (TAD). **(b)** Prototypic members of the inhibitor of NF-κB (IκB) family are IκBα, IκBβ and IκBε. Ankyrin repeats of IκBs and NF-κB precursors p100 and p105 serve to prevent nuclear translocation.

Based on their ability to activate transcription, NF-κB subunits can be classified into two groups. Only RelA, c-Rel and RelB contain a C-terminal transcription activation domain (TAD), which promotes transcription by facilitating the recruitment of coactivators and the displacement of repressors. p50 and p52, in contrast, lack TADs and hence are not able to drive gene expression, rather can serve as transcriptional repressors (Zhong, May et al. 2002).

In resting cells, the NF-κB dimers are maintained in an inactive state by binding to inhibitory κB (IκB) molecules that retain NF-κB dimers in the cytoplasm (Figure 4b). In addition to the conventional IκB molecules IκBα, IκBβ and IκBε, the precursor proteins p100 and p105 serve to prevent NF-κB nuclear translocation. This function is mediated by the presence of ankyrin repeats that interact with NF-κB via the RHD (Ghosh, May et al. 1998).

The activation of NF-κB occurs via two main signalling pathways, the canonical and the alternative pathway (Bonizzi and Karin 2004). The alternative pathway that is engaged by a restricted set of tumor necrosis factor family members involves IKKα-induced processing of p100 to 52 to induce RelB containing comlexes (Hayden and Ghosh 2004). As most NF-κB inducing stimuli, triggering of the antigen receptor engages the canonical pathway, which is characterized by phosphorylation of conventional IκB proteins by the IκB kinase complex. This activates predominantly heterodimers consiting of p50, RelA and c-Rel subunits (Hayden and Ghosh 2004). Most critical is the translocation of p50:RelA and p50:c-Rel dimers (Ghosh and Karin 2002). As distinct NF-κB dimers essentially confer to target gene specifity (Baldwin 1996; Leung, Hoffmann et al. 2004), it is a major task to elucidate the mechanisms of selective induction of RelA and c-Rel containing complexes. To date, in most systems it is unresolved how their selective release from cytoplasmic inhibition is controlled.

1.2.3.2 Ligand-Induced NF-κB Activation

Signal initiation upon BCR crosslinking occurs via successive activation of receptorproximal tyrosine kinases including Lyn and Syk, leading to the reorganization of adaptor and scaffold proteins at the activated BCR complex (Figure 5). This induces the activation of various enzymes, in particular phospholipase C-gamma 2 (PLCγ2) that mediates the synthesis of lipid second

messengers inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) followed by enhanced calcium mobilization. DAG and calcium activate protein kinase C-beta (PKC-β), which is essential for BCR-induced NF-κB activation (Su, Guo et al. 2002; Moreno-Garcia, Sommer et al. 2006). Activated PKC-β phosphorylates the adaptor protein Caspase recruitment domain (CARD) membrane associated guanylate kinase (Carma1), thus destabilizing inhibitory intramolecular interactions (Matsumoto, Wang et al. 2005; Sommer, Guo et al. 2005). This conformational change renders Carma1 amenable to self-oligomerization and to interactions with downstream molecules. While a small fraction of Carma1 constitutively resides in lipid rafts, self-oligomerization or interaction with a presently unknown protein lead to substantial recruitment of additional Carma1 molecules into these membrane microdomains. This promotes an `oligomerization cascade´ (Sun, Deng et al. 2004) resulting in the formation of a lipid raft-associated signalosome. Initially, Carma1 assembles B-cell lymphoma 10 (Bcl10) and the paracaspase mucosa associated lymphoid tissue lymphoma translocation gene 1 (Malt1) (Gaide, Favier et al. 2002; Egawa, Albrecht et al. 2003; Rawlings, Sommer et al. 2006) into the complex. As downstream effectors of Malt1 the ubiquitin ligases Traf2 and especially Traf6 (TRAF, TNF-receptor associated factor) have been implicated (Sun, Deng et al. 2004). Activated Traf6 is autoubiquitinated and the resulting regulatory polyubiquitinated Traf6 might directly recruit the inhibitory kappaB kinase (IKK) complex via the ubiquitin binding protein IKKγ (Wu, Conze et al. 2006). Subsequent association of the IKK holocomplex consisting of the regulatory subunit IKKγ and the catalytic subunits IKKα and IKKβ leads to the phosphorylation of regulatory serines in the kinase subunits and to the induction of IκB kinase activity (Rothwarf, Zandi et al. 1998; Yamaoka, Courtois et al. 1998; Ghosh and Karin 2002). The TGF-β-activated kinase-1 (Tak1) has been suggested to catalyze the phosphorylation of IKKα and IKKβ (Wang, Deng et al. 2001). As the activated IKK, their substrates, the IκB proteins, are recruited into the lipid rafts (Su, Guo et al. 2002). Two prototypic isoforms of IκB, IκBα and IκBβ, are serine phosphorylated and therefore targeted for polyubiquitin mediated proteasomal degradation. NF-κB dimers are released to translocate to the nucleus and bind to specific DNA motifs, thus regulating the expression of target genes.

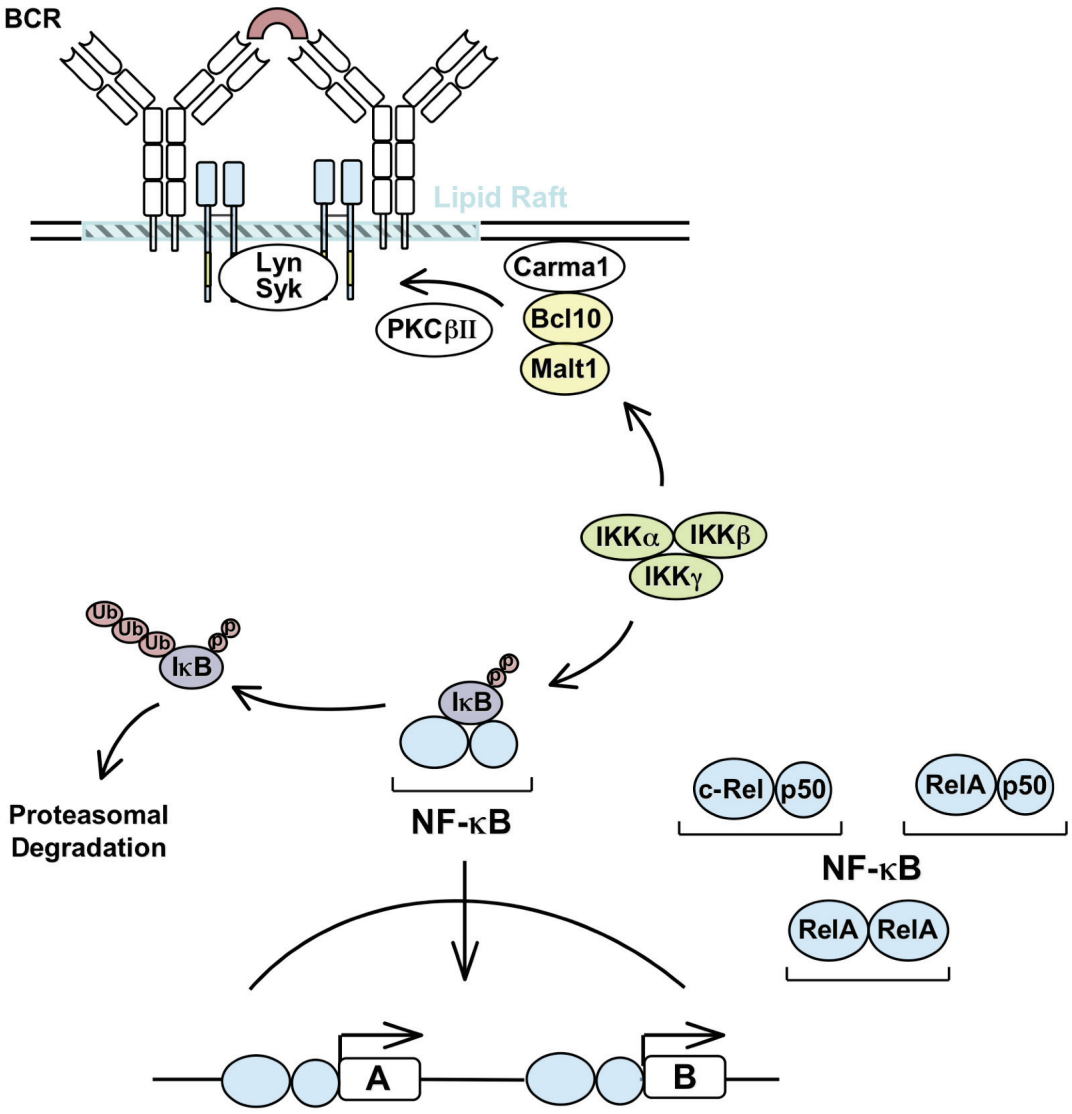

Figure 5. Simplified scheme of BCR-signalling to NF-κB. BCR-induced receptorproximal phosphorylation events involving the kinases Lyn and Syk culminate in the activation of PKC-β. This induces an oligomerization cascade: Carma1 interacts with Bcl10 and Malt1, accompanied by a preferential recruitment into lipid rafts. Subsequent recruitment and activation of IKK leads to phosphorylation and ubiquitin-mediated proteasomal degradation of IκB molecules. NF-κB dimers are released to translocate to the nucleus, where they bind to their common sequence motif, the κB-site and regulate the expression of target genes.

1.2.3.3 Tonic Signalling to NF-κB

To understand the regulation of survival and differentiation by BCR mediated NF-κB activation it is important to distinguish between ligand-independent or tonic signalling and conventional antigen mediated signal transduction. Hence, even in the absence of a specific ligand, signals from the pre-BCR and the BCR are

transmitted. Due to the accessibility of negative regulators and the absence of ligand-induced processes for stabilization and amplification, these tonic signals are transient, asynchronous and occur randomly (Monroe 2006). By contrast, antigen-triggered aggregation leads to oligomerization of the BCR and compartimentalisation into lipid rafts, both of which could modulate the effect of negative regulators, resulting in positive feedback regulation. To date, it is not possible to distinguish between tonic- and ligand-induced signalling to NF-κB in precisely quantitative or qualitative terms. In spite of this, tonic signalling via the pre-BCR and the BCR has an important role in directing appropriate fate decisions of differentiating and mature B cells.

1.2.4 Regulation of B Cell Functions by NF-κB

In mature B cells, antigen receptor mediated NF-κB activation governs differentiation, survival and proliferation (Kurosaki 2002) (Ruland and Mak 2003; Thome 2004; Siebenlist, Brown et al. 2005). The discrimination between these functional outcomes is difficult, due to mutual influence.

The regulation of survival and proliferation has been correlated to NF-κB-mediated induction of essential target genes. Important pro-survival genes of the B cell lymphoma 2 (*Bcl-2*) family, Bcl-2-like 1 (*Bcl-x$_L$*) and Bcl2-related protein A1 (*A1*), have been shown to be activated by BCR-triggered NF-κB activation (Chen, Edelstein et al. 2000; Vigorito, Gambardella et al. 2005) as well as cellular FLICE inhibitory protein (*c-Flip*) (Schram and Rothstein 2003). While Bcl-x$_L$ and A1 maintain mitochondrial integrity and thus interfere with the intrinsic apoptosis pathway, c-Flip inhibits the death receptor induced apoptosis pathway. Besides the regulation of anti-apoptotic molecules further NF-κB gene products have major impact on mature follicular B cell proliferation, as has been demonstrated for cyclin D2, cyclin D3 and cyclin E (Solvason, Wu et al. 2000) (Joyce, Albanese et al. 2001) that mediate cell cycle progression.

The role of NF-κB in BCR-mediated differentiation is barely understood and experimental findings are often controversial. BCR-mediated stimulation of distinct developmental subsets results in opposite responses. In immature B cells BCR signals triggered by self-antigens can either lead to deletion (apoptosis), inactivation (anergy), or receptor editing, while BCR ligation in mature B cells

confers to B cell activation. The underlying regulatory mechanisms still have to be elucidated. Concerning the development of mature B cell subsets, the generation of marginal zone and peritoneal B cells in contrast to follicular B cells is known to require ligand induced positive selection through the BCR (Berland and Wortis 2002; Martin and Kearney 2002). Given the different anatomical locations of marginal zone and peritoneal B cells compared with follicular B cells, unique signals might affect the development of these subsets (Niiro and Clark 2002). Due to its crucial function in mediating survival, the role of NF-κB in mature follicular B cell development is difficult to determine. Disruption of B cell receptorproximal signalling cascades and of IKK activation as point of convergence for various pathways to NF-κB activation leads to a block in follicular B cell development (Niiro and Clark 2002). Experimental evidence exists that NF-κB mediated induction of pro-survival molecules fails to rescue B cell maturation in Syk- and Btk-deficient mice (Turner, Gulbranson-Judge et al. 1997; Solvason, Wu et al. 1998). This allows the conclusion that survival and differentiation signals generated by the BCR can be dissociated (Niiro and Clark 2002; Su, Guo et al. 2004). Thus, the function of NF-κB is to help developing lymphocytes to survive (Siebenlist, Brown et al. 2005).

Regardless of the particular meaning of BCR signalling in mediating survival, NF-κB is activated furthermore upon stimulation of distinct receptors expressed on the B cell surface. In this context, the role of the pro-survival factor BAFF has been demonstrated in the induction of Bcl-2-family proteins (Schiemann, Gommerman et al. 2001; Hsu, Harless et al. 2002). Hence, crosstalk to other receptor signalling pathways may influence B cell differentiation and survival of distinct developmental stages.

1.2.5 NF-κB in Lymphomagenesis

With respect to the crucial function of NF-κB in inducing anti-apoptotic genes and genes that positively regulate cell-proliferation, an aberrant activation of NF-κB is a hallmark of tumorigenesis. Deregulation of the canonical signalling cascade may be involved in human lymphomagenesis (Alizadeh, Eisen et al. 2000; Shipp, Ross et al. 2002; Ngo, Davis et al. 2006; Jost and Ruland 2007). *BCL10* and *MALT1*, in particular, are recurrent targets of oncogenic chromosomal translocations in MALT

lymphoma (Isaacson and Du 2004). These translocations lead to either overexpression or `gain of function´ mutations of BCL10 or MALT1. Growing evidence indicates that this gives rise to sustained oligomerization and subsequent constitutive activation of the canonical NF-κB pathway. Overexpression of BCL10 and MALT1 emerges from two prevalent genetic abnormalities in MALT lymphoma that juxtapose either the *BCL10* gene (t (1; 14) (q22; q32)) or the *MALT1* gene (t (14; 18) (q32; q21)) to the Ig-heavy-chain gene enhancers (Willis, Jadayel et al. 1999; Zhang, Siebert et al. 1999; Sanchez-Izquierdo, Buchonnet et al. 2003; Streubel, Lamprecht et al. 2003). Gain of function has been proposed in MALT lymphomas with t (11; 18) (q21; q21); the resulting API2-MALT1 (API2, apoptosis inhibitor 2) fusion product is believed to self-oligomerize through the baculovirus IAP repeat (BIR) domain of the IAP2 molecule (Akagi, Motegi et al. 1999; Dierlamm, Baens et al. 1999; Uren, O'Rourke et al. 2000; Lucas, Yonezumi et al. 2001) (Ruland, Duncan et al. 2003).

Besides this constitutive activation of NF-κB via deregulation of signal transduction components, genetic alterations that directly affect the activity and expression of cellular NF-κB/REL proteins have also been linked to lymphoma and leukaemia. Initial evidence that affiliated NF-κB to lymphoid malignancies came from the *v-Rel* oncogene, which was shown to cause aggressive lymphoma and leukaemia in chickens (Gilmore 1999). A unique oncogenic activity has been attributed to c-Rel (Gilmore, Kalaitzidis et al. 2004). Chromosomal amplifications of *REL* (the gene encoding c-Rel) are frequently detected in various lymphoma subtypes (Jost and Ruland 2007), although their pathogenic function has to be further evaluated. In contrast, amplifications or chromosomal rearrangements that affect the *RelA* locus are rare (Karin, Cao et al. 2002).

1.3 Transgenic Mouse Models

Of the genetically well explored organisms, mice are much the closest to humans in evolutionary terms. Because of the comparatively high level of sequence conservation between human and mouse coding sequences, orthologous gene mutations are more likely to produce similar phenotypes in humans and mice. The ability to construct mice with pre-determined genetic modifications to the germline by gene-targeting in embryonic stem cells has been a powerful tool for addressing

questions related to the functional importance of a gene, for identifying pathways and underlying mechanisms (Mak, Penninger et al. 2001).

1.3.1 *Bcl10* Knockout Mouse

Bcl10 cannot be assigned to any known protein families, but is characterized by an N-terminal caspase recruitment domain (CARD). The C-terminus is rich in serine and threonine residues. Bcl10 can bind to several NF-κB signalling molecules. Best explored are the interactions with Malt1 (Uren, O'Rourke et al. 2000; Lucas, Yonezumi et al. 2001) and Carma1 (Gaide, Martinon et al. 2001; Wang, Guo et al. 2001). Bcl10 and Carma1 proteins interact via their CARD-domains, whereas the association of Bcl10 and Malt1 depends on aa 106-120 of Bcl10.

Targeted disruption of exon two and three within the *Bcl10* CARD-domain in mice led to embryonic lethality by exencephaly in one third of *Bcl10*$^{-/-}$ embryos studied (Ruland, Duncan et al. 2001). Surviving *Bcl10*$^{-/-}$ mice were severely immunodeficient. They had reduced immunoglobulin concentrations in the serum and defects in T and B cell mediated immunity. *Bcl10*$^{-/-}$ B and T lymphocytes were defective in antigen receptor- or PMA/ionomycin induced activation. While receptorproximal tyrosine phosphorylation, activation of ERK and AP-1, and Ca^{2+} mobilization in T cells were normal, antigen-induced canonical NF-κB signalling was absent in B and T cells. In addition, *Bcl10* knockout mice have reduced numbers of follicular mature B cells (Xue, Morris et al. 2003) and a substantial decrease in the amount of peritoneal and marginal zone B cells (Xue, Morris et al. 2003).

1.3.2 *Malt1* Knockout Mouse

MALT1 has been allocated to the protein family of paracaspases, which are characterized by the presence of a caspase-like domain (CLD) (Uren, O'Rourke et al. 2000). MALT1 contains a C-terminal CLD with the conserved cysteine-histidine dyad, but so far no proteolytic activity has been identified for MALT1 (Snipas, Wildfang et al. 2004). MALT1 contains an N-terminal death-domain (DD) and two Ig-like domains that mediate protein-protein interactions. The two Ig-domains have been shown to be essential for the binding of BCL10 (Uren, O'Rourke et al. 2000; Lucas, Yonezumi et al. 2001). Upon Bcl10-mediated oligomerization, the

C-terminus of Malt1 has been implicated in regulatory ubiquitination of IKKγ and activation of the canonical IKK complex. Whether Malt1 itself possesses intrinsic ubiquitin ligase activity or whether it recruits, oligomerizes and activates the ubiquitin ligase Traf6 to catalyze IKKγ ubiquitination is still controversial (Sun, Deng et al. 2004; Zhou, Wertz et al. 2004).

Mice with a targeted disruption of exon 8, which encodes the N-terminal region of the *Malt1* CLD, were viable, but displayed a severe immunodeficiency (Ruland, Duncan et al. 2003). *Malt1*$^{-/-}$ mice demonstrated a reduction in serum immunoglobulin levels and defects in T cell activation. As in *Bcl10*$^{-/-}$ T cells, receptorproximal tyrosine phosphorylation and activation of ERK and AP-1 were normal, but TCR stimulation failed to induce proliferation and canonical NF-κB activation. In contrast to Bcl10 disruption, however, targeting of *Malt1* had only mild effects on B cell activation.

While the shown defects in T cell immunity are in line with an independently generated *Malt1* knockout mouse, in which exon 11 and 12 of the CLD have been deleted, this study gained divergent results concerning the requirement for Malt1 in B cell activation (Ruefli-Brasse, French et al. 2003).

1.3.3 *BCL2* Transgenic Mouse

BCL2 is the most prominent member of a family of pro-survival molecules that interfere with the intrinsic or stress-mediated apoptosis pathway (Cory and Adams 2002). BCL2 protects the integrity of the mitochondrial membrane to prevent the release of cytochrome *c* and subsequently inhibits the activation of caspase-9.

Transgenic overexpression of the human *BCL2* gene in mice by utilizing the promoter of the *vav*-gene, which is expressed throughout the hematopoetic compartment, leads to a marked elevation in T and B lymphocytes and an enlargement of the spleen due to a 4-6 fold increase in total cellularity (Ogilvy, Metcalf et al. 1999).

1.4 Specific Aims of this Project

Bcl10 and Malt1 have been reported to cooperate for a linear pathway leading to canonical NF-κB activation in various contexts (Thome 2004; Rawlings, Sommer et al. 2006). They are essential components of a multiprotein complex that is formed upon AgR stimulation in lymphocytes. But how they regulate BCR induced NF-κB activation in detail is still very poorly understood. Moreover, recent findings also indicated differential functions for Bcl10 and Malt1 in signal transduction. First insights into separate protein functions were gained by the analysis of primary B cells derived from *Bcl10*- and *Malt1*-knockout mice (Ruland, Duncan et al. 2001; Ruland, Duncan et al. 2003). On the one hand, *Bcl10*$^{-/-}$ B cells failed to activate NF-κB upon AgR crosslinking. However, targeted disruption of *Malt1* in B cells did not block antigen receptor mediated NF-κB signalling (Ruland, Duncan et al. 2003). Thus, the two major aims of this study were to deliver insights into the molecular mechanisms of signalosome assembly and to identify potentially isolated functions of Bcl10 and Malt1.

To this end, in primary splenic B cells from *Bcl10*- and *Malt1*- knockout mice, firstly the constitutive and BCR mediated initiation of functional outcomes like survival and proliferation should be characterized in detail.

The second part of the project was to reveal the molecular differences that led to a block in BCR-mediated activation of *Bcl10*$^{-/-}$ cells, but only to a mild impairment of *Malt1*$^{-/-}$ B cells.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

If not otherwise stated, all chemicals were purchased from Sigma.
Primers were synthesized by MWG Biosciences.

2.1.2 Antibodies

Antibodies were used against Bcl-x_L, c-Flip, p-Erk, Erk, p-Lyn, p-IKKα;β, IκBα (NEB), cyclin D2, cyclin D3; cyclin E, A1, p-Tyrosine, Bcl10, Lyn, IκBβ, c-Rel, RelA, RelB, NF-κB p50, Lamin B (Santa Cruz Biotechnology), Malt1 (kind gift from V. Dixit), IKKα, IKKβ, IKKγ, NF-κB p52 (Upstate), β-actin or α-tubulin (Sigma Aldrich). Secondary HRP-conjugated antibodies from NEB or Santa Cruz Biotechnology were utilized. Fluorescently labeled antibodies against B220, CD21 and CD24 were purchased from Becton Dickinson or ebioscience.

2.1.3 Mouse strains and Genotyping Primers

Bcl10$^{-/-}$, *Malt1*$^{-/-}$ and vav-*BCL2* transgenic mice (Ogilvy, Metcalf et al. 1999; Ruland, Duncan et al. 2001; Ruland, Duncan et al. 2003) backcrossed onto a C57BL/6 background (N=6) were used at 6 to 12 weeks of age. Primers for genotyping are listed as follows:

Primer	**Sequence `5 - 3´**
Bcl10-wt	TTG GCT CTC TGC TCT CCT CAC T
Bcl10-com	CGC TCT GAG GAC TGT GGG ACT G
Malt1-wt	CTG CTG CTG ACA TGC TAC AAT ATG CTG
Malt1-com	CTG CTG CTG ACA TGC TAC AAT ATG CTG
NEO	GGG TGG GAT TAG ATA AAT GCC TGC TC
vav-*BCL2*-a	GCC GCA GAC ATG ATA AGA TAC ATT GAT G
vav-*BCL2*-c	AAA ACC TCC CAC ACC TCC CCC TGA A

2.2 Methods

2.2.1 Isolation of Primary B Cells

For survival and proliferation assays, mature splenic B cells were purified via fluorescence activated cell sorting using B220, CD21 and CD24 cell surface markers (Loder, Mutschler et al. 1999) and a high speed MoFlo cytometer (DAKO Cytomation) by Dr. Markus Schiemann. For biochemical analyses splenic B cells were purified by negative selection using magnetic beads (Dynabeads, Invitrogen). To this aim, myeloid cells were labeled with rat anti-CD11b antibody and depleted with sheep anti-rat beads. T cells were removed using anti-Thy1.2 coupled beads. Successful B cell isolation was confirmed by flow cytometry via B220 staining.

2.2.2 Culture and Stimulation of Primary B Cells

Primary B cells were cultured in complete RPMI 1640 medium (Invitrogen), supplemented with 0.1 % β-mercaptoethanol (Invitrogen) and either 0.2 % bovine serum albumine (for short term incubation up to 4 hours, Roth) or 5 % very low endotoxin fetal bovine serum (for culturing time up to 72 hours, Hyclone). BCR crosslinking was accomplished by incubation with 10 μg/ml anti-IgM $F(ab')_2$ fragment (Jackson Immuno Research). As a strong pharmacological surrogate for the antigen receptor signal phorbol-12 myristate 13-acetate ester and calcium ionophore (PMA + Ionomycin, 10 ng/ml each, Sigma) were used. TLR-4 was stimulated with 10 μg/ml LPS (Sigma). For the analysis of IκB-degradation upon BCR stimulation B cells were incubated in the presence of Cycloheximide (5 μg/ml) (Sigma) to avoid a resynthesis of IκB-proteins.

2.2.3 Survival and Proliferation Assays

Viability of mature B cells was analysed upon Propidium Iodide (PI, Sigma) staining or by forward/side scatter profiling. Cell proliferation was determined after 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE, Sigma) staining. After 24, 48 and 72 hours cells were subjected to flow cytometry (FACScalibur, Becton Dickinson). Data were analyzed using CellQuest (Becton Dickinson) and FlowJo software (Tree Star) according to standard protocols.

2.2.4 Real Time Quantitative PCR

RNA was isolated with TRIzol (Invitrogen) and reversely transcribed using SuperScript II (Invitrogen) according to manufacturers´ protocols. Specific primer pairs were designed that span exon-exon boundaries:

Primer	**Sequence `5 - 3´**
c-Flip$_L$-fwd	CCT CCG CAC ATC CGT GAA
c-Flip$_L$-rev	AGG TCT CTT GAA GAT ATT TTG TGT CGT T
A1-fwd	GGA ATG GAG GTT GGG AAG AT
A1-rev	AGT GTT ACT TGA GGA GAA AGA GCA TT
Tbp-fwd	CCA CCA GCA GTT CAG TAG CTA TGA
Tbp-rev	TGC TCT AAC TTT AGC ACC TGT TAA TAC AAC

PCR reactions were carried out utilizing qPCR Core Kit for SYBR Green I (Eurogentec). The amount of specifically amplified cDNA was monitored using ABI PRISM 7700 Sequence Detection System Instrument and SDS software (Applied Biosystems) according to standard protocols. Differences in the cDNA content of distinct samples were normalized to the expression of the housekeeping gene *Tbp* and for relative quantification the $\Delta\Delta$Ct method was employed.

2.2.5 Western Blot

Standard Western Blot extracts were generated using TritonX-100 (1%, 50 mM TRIS pH 8.0, 150 mM NaCl, 10 mM NaF, 1 mM $NaVO_4$ and protease inhibitors (Calbiochem)). To investigate subcellular fractions distinct lysis procedures were employed. For fractionation into cytoplasmic and nuclear extracts lymphocytes were first mildly lysed using Buffer A (0.2 % Nonidet P40, 10mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF and protease inhibitors). Protein extracts of pelleted nuclei as well as total cell extracts were generated using RIPA buffer (0.5 M Tris-HCl pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% Nonidet P40, 10mM EDTA, 10 mM NaF, 1 mM $NaVO_4$ and protease inhibitors).

Western blotting was carried out as described previously (Ruland, Duncan et al. 2001). Densiometry of immunoblots was performed using Scion Imaging Software

(Scion Corporation) and relative changes in the abundance of target proteins were determined upon normalization to β-actin expression.

2.2.6 Co-Immunoprecipitation

B cells were lysed in co-immunoprecipitation buffer (0.2 % Nonidet P40, 150 mM NaCl, 50 mM HEPES pH 7.5, 1 mM Glycerol, 10 mM NaF, 8 mM β-glycero-phosphate, 1 mM Na_3VO_4 and protease inhibitors) as described previously (Wegener, Oeckinghaus et al. 2006). Upon preclearing primary antibodies were incubated over night with gentle agitation. Depending on the species and clonality of the primary antibody precipitation was carried out with Sepharose A or Sepharose G beads (Amersham) for 30 minutes. Upon washing and detaching lysates were subjected to western blot analysis.

2.2.7 Lipid Raft Preparation

Sucrose gradient raft fractionation was carried out as described (Zhang, Trible et al. 1998). In brief, 5 x 10^7 splenocytes were stimulated with anti-IgM (10 μg/ml) at 37°C for 5 minutes and lysed using 1 ml ice-cold Brij-lysis buffer (1 % Brij97, 150 mM NaCl, 20 mM TRIS (pH 7.5), 2 mM EDTA, 10 mM NaF, 1 mM $NaVO_4$ and protease inhibitors). Postnuclear supernatant was mixed with an equal volume of 85 % sucrose (w/v) in MBS (25 mM MES pH 6.5, 150 mM NaCl, 5 mM EDTA), overlaid with 2 ml of 35 % and 1 ml of 5 % sucrose in MBS. After 3.5 hours of ultracentrifugation (200 000 g, 4°C) (Zhang, Trible et al. 1998) eleven 400 μl fractions were collected from the top of the gradient. Separation of lipid raft and cytoplasmatic fractions was confirmed by detection of the lipid raft marker GM1 with HRP-labelled Choleratoxin B subunit (Sigma). Raft-associated protein was concentrated via CHCl3/methanol precipitation and resuspended in 1 x Laemmli buffer. Fractions 2 to 5 (raft fractions) and 8 to 11 (cytoplasmatic fractions) were pooled and subjected to Western Blot analysis.

2.2.8 Gel Mobility Shift Assay

Nuclear extracts of 2 x 10^7 B cells were prepared as described previously (Ruland, Duncan et al. 2001). Extracts (4 μg protein) were incubated in 20 μl binding buffer

(5 mM HEPES (pH 7.8), 50 mM KCl, 0.5 mM dithiothreitol, 1 μg Poly (dI-dC) and 10 % glycerol) with ^{32}P-labeled, double-stranded oligonucleotide probes (5`-ATC AGG GAC TTT CCG CTG GGG ACT TTC CG-3´) in the presence or absence of superhift antibodies to NF-κB p50, NF-κB p65 or c-Rel (Santa Cruz Biotechnology) and electrophoretically separated on a 5% polyacrylamide gel.

2.2.9 Immunofluorescence

B cells were incubated in the pesence of Cycloheximide (5 μg/ml) with or without stimulus on object slides. Upon fixation with 4% formalin cells were permeabilized (0.25 % TritonX-100 in PBS) and stained with antibodies to RelA or c-Rel (Santa Cruz Biotechnology). Incubation of fluorochrome-conjugated secondary antibodies (Molecular Probes) was followed by staining of the nuclei with DAPI (Sigma Aldrich). Localization of RelA and c-Rel signals relative to DAPI positive nuclei was monitored using confocal microscopy. At least 100 cells per condition were counted to determine the frequencies of c-Rel- or RelA-positive nuclei.

2.2.10 Statistics

P-values were determined by applying Student's two-tailed t-test for independent samples, assuming equal variances on all experimental data sets using the Microsoft excel t-test calculator.

3. RESULTS

3.1 Bcl10 and Malt1 Have Both Common and Individual Functions in BCR Signalling

To identify individual roles of Bcl10 and Malt1 in BCR-mediated B cell activation, B cells lacking Bcl10 or Malt1 were investigated for their ability to induce survival and proliferation. To this end, splenic mature B cells (M cells) from Bcl10- and Malt1-deficient mice (Ruland, Duncan et al. 2001; Ruland, Duncan et al. 2003) were enriched using FACS as described previously (Loder, Mutschler et al. 1999). First, tonic and ligand-induced B cell survival was assessed. Culture of resting Bcl10 and Malt1-deficient M cells in the absence of any stimuli revealed an enhanced death compared to wild-type M cells (Figure 6a). Both Bcl10- and Malt1-deficient B cells died with similar kinetics, indicating that Bcl10 and Malt1 control a common signalling pathway mediating survival of mature, resting B cells.

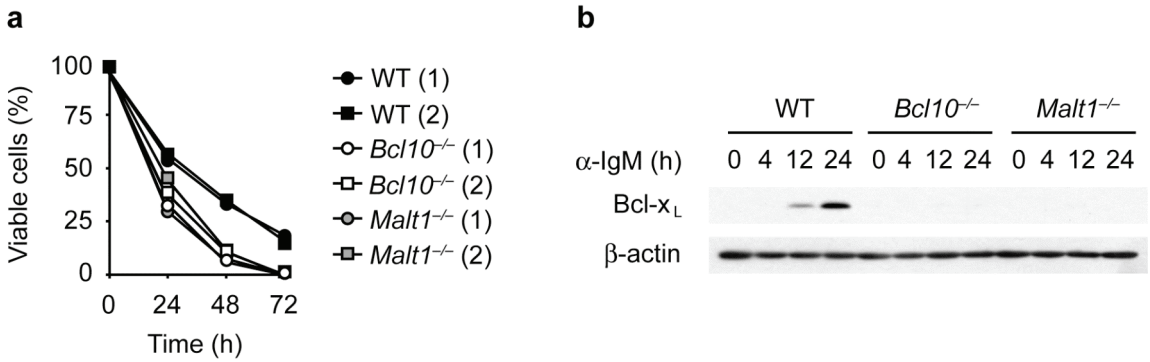

Figure 6. Common functions for Bcl10 and Malt1 in signalling for the survival of mature B cells. (**a**) Contribution of Bcl10 and Malt1 to the survival of resting B cells. Flow cytometry of the viability of mature B lymphocytes from individual wild-type (WT), Bcl10- or Malt1-deficient mice sorted by FACS and cultured without specific stimuli, assessed after propidium iodide staining. Numbers in parentheses indicate individual mice. (**b**) Immunoblot analysis of the BCR-induced upregulation of Bcl-x_L in purified wild- type, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ B cells stimulated with anti-IgM (α-IgM; 10 μg/ml). β-actin, loading control. Results are representative of three (**a**) or four (**b**) individual experiments.

Next, inducible survival signals were studied upon mimicking antigen-mediated BCR engagement with antibody to surface IgM (anti-IgM). As BCR-regulated expression of the antiapoptotic NF-κB target gene encoding Bcl-x_L is involved in B

cell survival (Solvason, Wu et al. 1998), next, the induction of Bcl-x_L in response to BCR ligation was assessed. Wild-type B cells robustly upregulated Bcl-x_L as both mRNA and protein (data not shown and Figure 6b). However, both *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ B cells failed to induce Bcl-x_L expression (Figure 6b). These findings indicate that in addition to cooperate for resting B cell survival, Bcl10 and Malt1 also have common functions in ligand-induced survival signalling after B cell activation.

To address, whether the deficiency for Bcl10 or Malt1 might interfere with BCR-induced proliferation, primary B cells were labelled with carboxyfluorescein diacetate N-succinimidyl ester (CFSE). As a control, B cells were triggered with the Toll-like receptor 4 agonist lipopolysaccharide (LPS), which has been shown to drive cell division independently from Bcl10 and Malt1 (Ruland, Duncan et al. 2001; Ruland, Duncan et al. 2003). In response to LPS B cells of either genotype proliferated vigorously without discernible differences as gauged by CFSE distribution (Figure 7a). Wild-type B cells also induced cell proliferation upon BCR engagement, and underwent several division cycles after 48 h and 72 h. Notably, although the percentage of viable *Malt1*$^{-/-}$ cells was lower than that of wild-type cells (Figure 7b) surviving *Malt1*$^{-/-}$ B cells also divided robustly after BCR stimulation, albeit with delayed kinetics (Figure 7a). To test for possible implications of known NF-κB target genes, which mediate proliferation, the induction of distinct cyclins was assessed (Joyce, Albanese et al. 2001; Sasaki, Derudder et al. 2006). Parallel to the observed delayed onset of proliferation, *Malt1*$^{-/-}$ B cells displayed a retarded, but full upregulation of cyclin D2 expression after 24 hours of stimulation (Figure 7c) as well as robust induction of cyclin D3 and cyclin E (Figure 7d). *Bcl10*$^{-/-}$ B cells acted very differently. In response to BCR ligation, *Bcl10*$^{-/-}$ cells underwent apoptosis beginning 16 to 24 hours of stimulation (Figure 7b and data not shown). Moreover, the induction of the NF-κB-dependent cell cycle regulators cyclin D2, cyclin D3 and cyclin E at early and later time points was severely impaired (Figure 7c,d) and Bcl10-deficient B cells did not divide at all (Figure 7a). Notably, after 72 hours of BCR ligation, almost all *Bcl10*$^{-/-}$ B lymphocytes were dead (Figure 7a,b). Thus, these results indicate the existence of BCR-mediated pathways that signal via Bcl10, but largely independent of Malt1. These Malt1-independent pathways are essential to block apoptosis and are prerequisites for B cell division after BCR ligation.

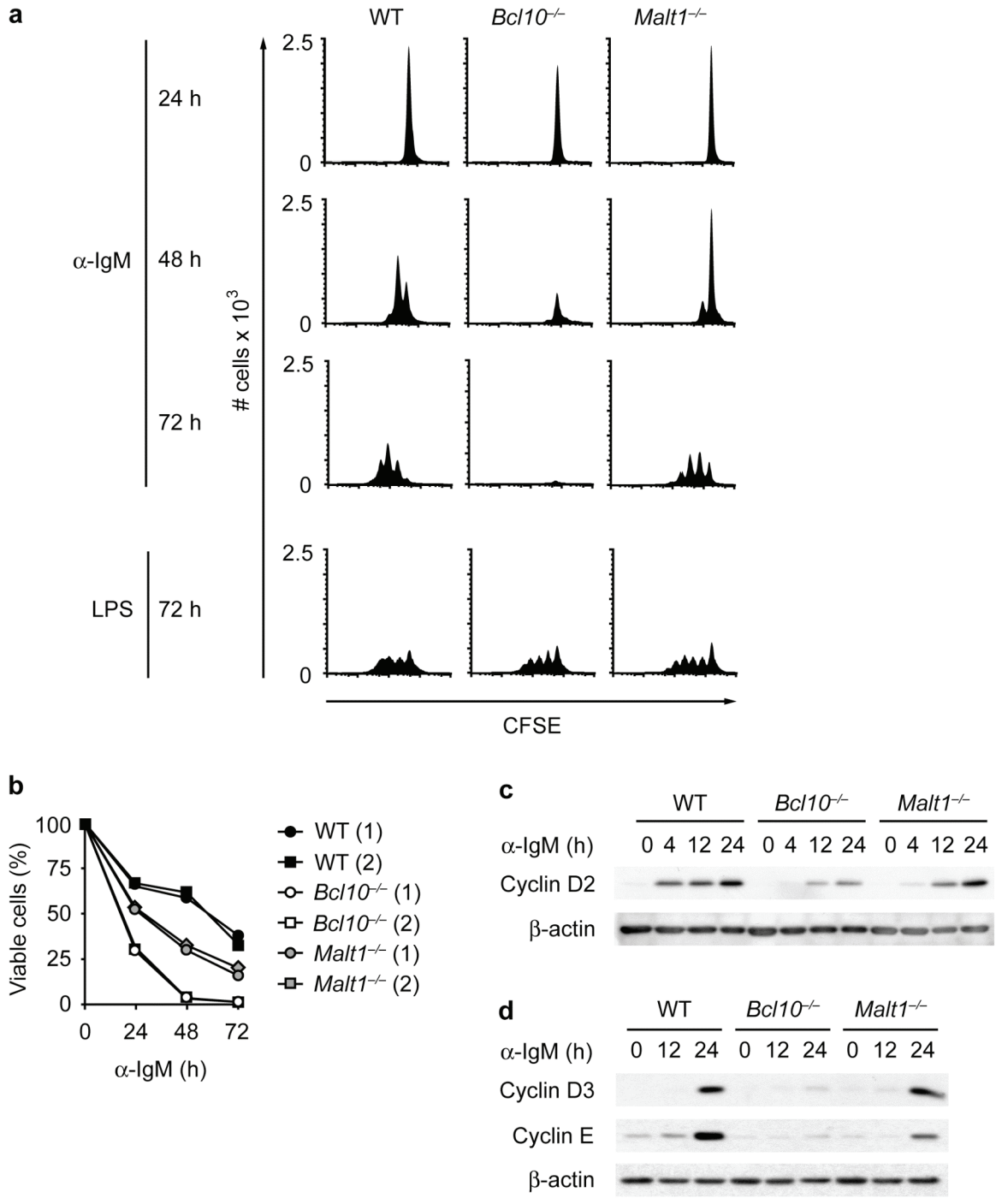

Figure 7. Distinct functions for Bcl10 and Malt1 after B cell activation. Proliferation of mature wild-type (WT), *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ (2 x 10^5) B cells labelled with CFSE and stimulated with anti-IgM (10 μg/ml) or LPS (10 μg/ml); Division was tracked by flow cytometry by analysis of CFSE fluorescence in 3 x 10^4 viable cells or, for *Bcl10*$^{-/-}$ cells stimulated with anti-IgM, in all remaining viable cells. (**b**) Viability after BCR ligation of B cells cultured in the presence of anti-IgM (10 μg/ml), assessed by flow cytometry. Numbers in parentheses indicate individual mice. (**c,d**) Immunoblot analysis of the BCR-mediated induction of cyclin D2 **(c)** and upregulation of cyclin D3 and cyclin E **(d)** in B cells stimulated with anti-IgM (10 μg/ml). β-actin, loading control. Data are representative results of three (**a**), six (**b**) or four (**c,d**) independent experiments.

To characterize these Bcl10-dependent pathways in more detail, the induction of additional antiapoptotic NF-κB target genes was monitored. The *BCL2* family member *A1* and the inhibitor of death receptor signalling *c-Flip* have previously been shown to be regulated by BCR-mediated NF-κB activation (Schram and Rothstein 2003; Vigorito, Gambardella et al. 2005). B cells of the three genotypes were BCR-stimulated for 4 hours and the induction of mRNAs encoding A1 and c-Flip was determined (Figure 8a).

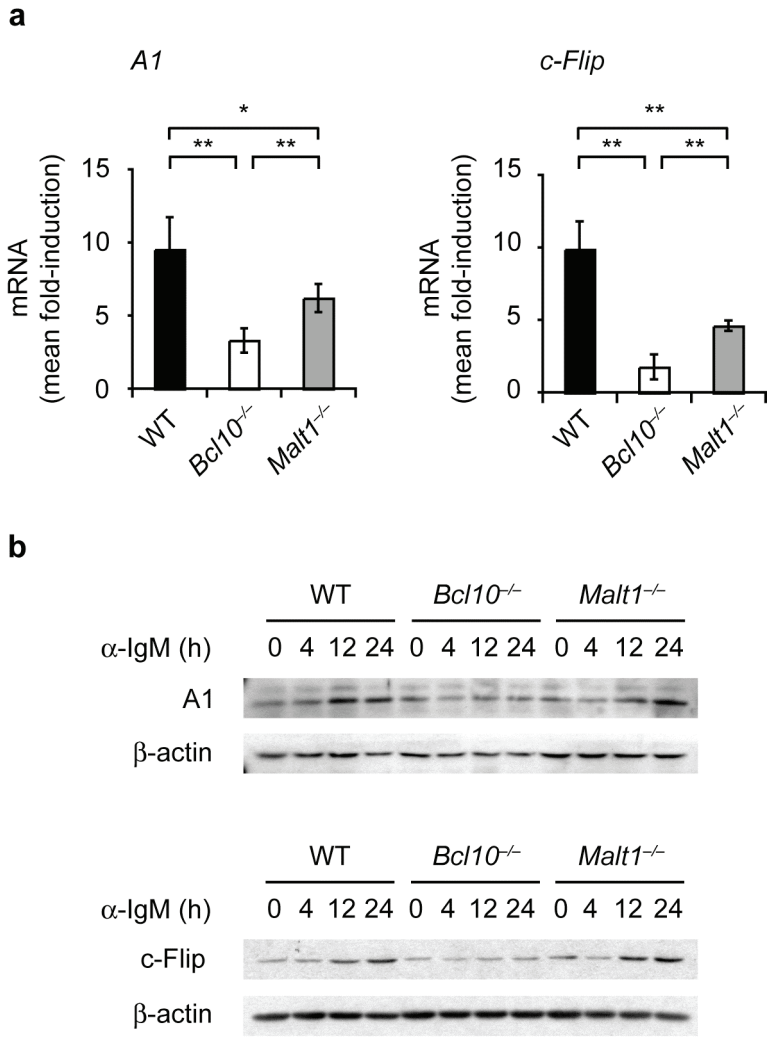

Figure 8. Bcl10 and Malt1-dependent control of survival programs in activated B cells. (**a**) Real-time PCR analysis of BCR-induced expression of A1 and c-Flip mRNA in B cells stimulated with anti-IgM (10 μg/ml). The abundance of these transcripts among total mRNA is normalized to that of TATA box binding protein (`housekeeping gene´) expression; `fold induction´ (mean ± s.d.) is relative to the abundance in unstimulated cells. *, $P < 0.05$; **, $P < 0.01$. Data are from three independent experiments. (**b**) Immunoblot analysis of BCR-induced A1, c-Flip and β-actin protein expression in B cells stimulated with anti-IgM (10 μg/ml). Data are one of three independent experiments with similar results.

A1 and *c-Flip* mRNAs were upregulated in wild-type B cells and to a reduced extent in Malt1-deficient cells. In contrast, no induction could be observed in *Bcl10*$^{-/-}$ B cells. Studying the BCR-induced expression of A1 and c-Flip protein over a 24 hour time interval, a successive increase in A1 and c-Flip protein concentrations in wild-type and in *Malt1*$^{-/-}$ cells was detected (Figure 8b). After 24 hours of stimulation the A1 and c-Flip protein abundance was finally similar in wild-type and *Malt1*$^{-/-}$ cells. However, no increase in either A1 or c-Flip expression was found in Bcl10-deficient B cells. Thus, it can be concluded that Bcl10 signalling induces the expression of a core set of BCR-controlled factors in a way that is at least partly independent from Malt1. The failure of *Bcl10*$^{-/-}$ cells to upregulate a series of essential survival molecules above a critical threshold presumably causes their death after BCR ligation.

3.2 Bcl10 Controls Malt1-Independent B Cell Proliferation

Due to the rapid death of Bcl10-deficient cells in response to BCR stimulation, the specific function of Bcl10 signalling for BCR mediated cell division could not be gauged as far. To explicitly investigate this function without taking onto consideration the effects of Bcl10 on survival, we introduced a *BCL2*-transgene (Ogilvy, Metcalf et al. 1999) into the *Bcl10*$^{-/-}$ background. Transgenic *BCL2* expression blocked the apoptosis of resting wild-type and *Bcl10*$^{-/-}$ B lymphocytes (Figure 9). Notably, enforced BCL2 expression also overcame the survival defects of BCR-stimulated *Bcl10*$^{-/-}$ cells (Figure 9).

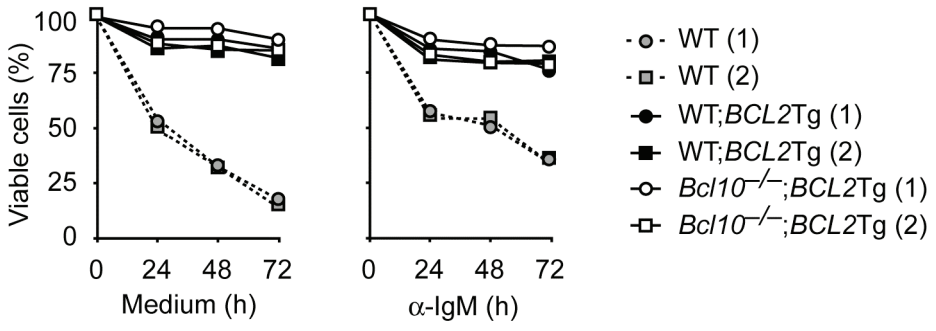

Figure 9. Enforced BCL2 expression blocks apoptosis of BCR stimulated *Bcl10*$^{-/-}$ B cells. Viability of B cells isolated from wild-type control mice (WT), *BCL2* transgenic control mice (WT;*BCL2*Tg) and *BCL2* transgenic *Bcl10*$^{-/-}$ mice (*Bcl10*$^{-/-}$;*BCL2*Tg) and left unstimulated (Medium) or stimulated with anti-IgM (10 μg/ml), assessed by flow cytometry. Numbers in parentheses indicate individual mice. Experiments were repeated three times with similar results.

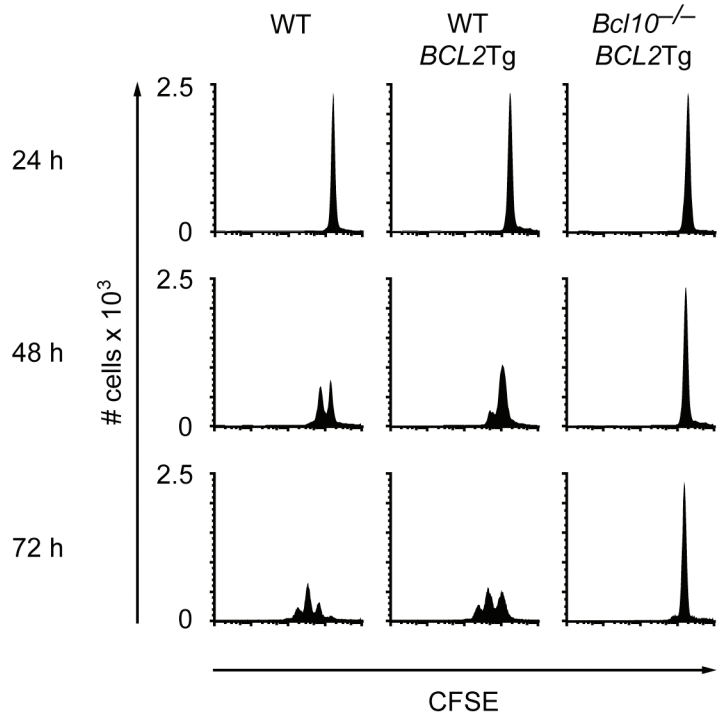

Figure 10. Enforced BCL2 expression does not rescue the proliferation of BCR stimulated *Bcl10*$^{-/-}$ B cells. Proliferation of wild-type control, *BCL2* transgenic control or *BCL2* transgenic *Bcl10*$^{-/-}$ B cells stained with CFSE and stimulated with anti-IgM (10 μg/ml) assessed by measurement of CFSE fluorescence in 3 x 10^4 cells by flow cytometry. Experiments were repeated four times with similar results.

However, whereas control *BCL2*-transgenic B cells proliferated vigorously in response to BCR ligation, *BCL2*-transgenic *Bcl10*$^{-/-}$ cells showed no signs of division even after a prolonged stimulation (Figure 10). According to this, in addition to protecting activated B cells from apoptosis, Bcl10 signalling is also required to directly drive a Malt1-independent B cell division program.

3.3 Bcl10 Controls IKK Signalosome Formation and Activation Independently of Malt1

So far, our genetic experiments demonstrated both common and distinct functions for Bcl10 and Malt1 in B cell signalling. To obtain mechanistic insights into these functions, signal transduction from the BCR to NF-κB was gradually investigated.

Firstly, BCR proximal events were addressed (Figure 11a). BCR crosslinking results in the rapid phosphorylation of multiple receptor proximal signalling proteins (Kurosaki 2002). Immunoblot analysis of wild-type, *Bcl10*$^{-/-}$ and

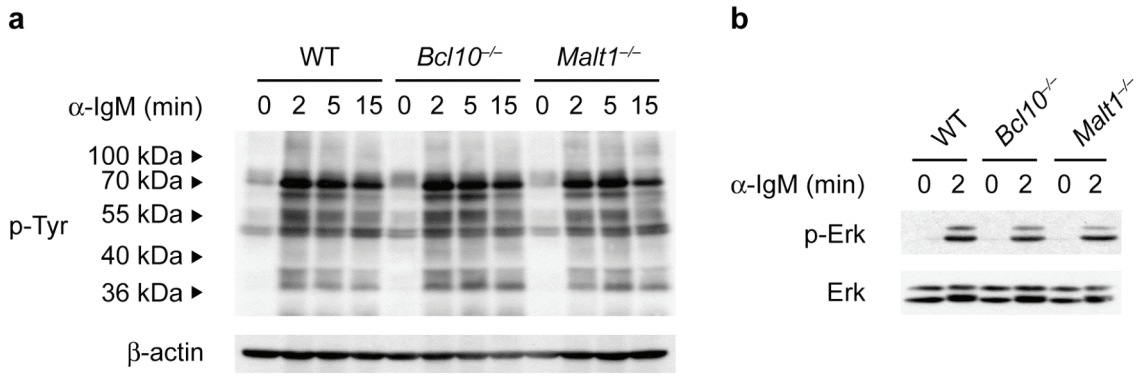

Figure 11. Lack of Bcl10 or Malt1 does not affect receptorproximal events. (**a**,**b**) BCR proximal signalling and Erk activation in B cells left untreated or stimulated with anti-IgM (10 μg/ml); total tyrosine phosphorylation (p-Tyr; **a**) and Erk phosphorylation (p-Erk; **b**) were assessed by immunoblot. Bottom, membranes reprobed with anti-β-actin (**a**) or antibody to total Erk (**b**) to confirm equal loading. Left margin (**a**), molecular sizes in kilodaltons. Data are one experiment representative of three.

Malt1$^{-/-}$ B cells stimulated with anti-IgM showed similar tyrosine phosphorylation patterns among the three genotypes (Figure 11a), indicating that BCR proximal tyrosine activation does not require Bcl10 or Malt1. Consistent with those findings, a normal activation of the mitogen-activated kinase Erk in the absence of Bcl10 or Malt1 could be noted (Figure 11b).

In the following the specific control of the NF-κB pathway was focused. Due to the fact that BCR signalling to NF-κB requires the recruitment of IKK into lipid rafts (Su, Guo et al. 2002), next the formation of BCR induced lipid raft signalosomes was studied (Figure 12). The receptor proximal tyrosine kinase Lyn resides constitutively in lipid rafts (Cheng, Dykstra et al. 1999). In line with Bcl10- and Malt1-independent proximal signalling, a rapid BCR induced phosphorylation of raft-associated Lyn for all three genotypes could be detected. This indicates that initial raft integrity likewise does not depend on Bcl10 or Malt1. BCR ligation further induced a recruitment of Bcl10 into the raft fractions of wild-type and *Malt1*$^{-/-}$ B cells. However, Malt1 was recruited only into the rafts of wild-type B cells, not those lacking Bcl10, suggesting an essential function for Bcl10 `upstream´ of Malt1. Moreover, wild-type and *Malt1*$^{-/-}$ cells displayed an enhanced BCR-induced recruitment of IKKα, IKKβ and IKKγ into the raft domains. As opposed to that, BCR engagement in *Bcl10*$^{-/-}$ cells failed to concentrate the IKK subunits within lipid rafts, indicating a specific requirement for Bcl10 in

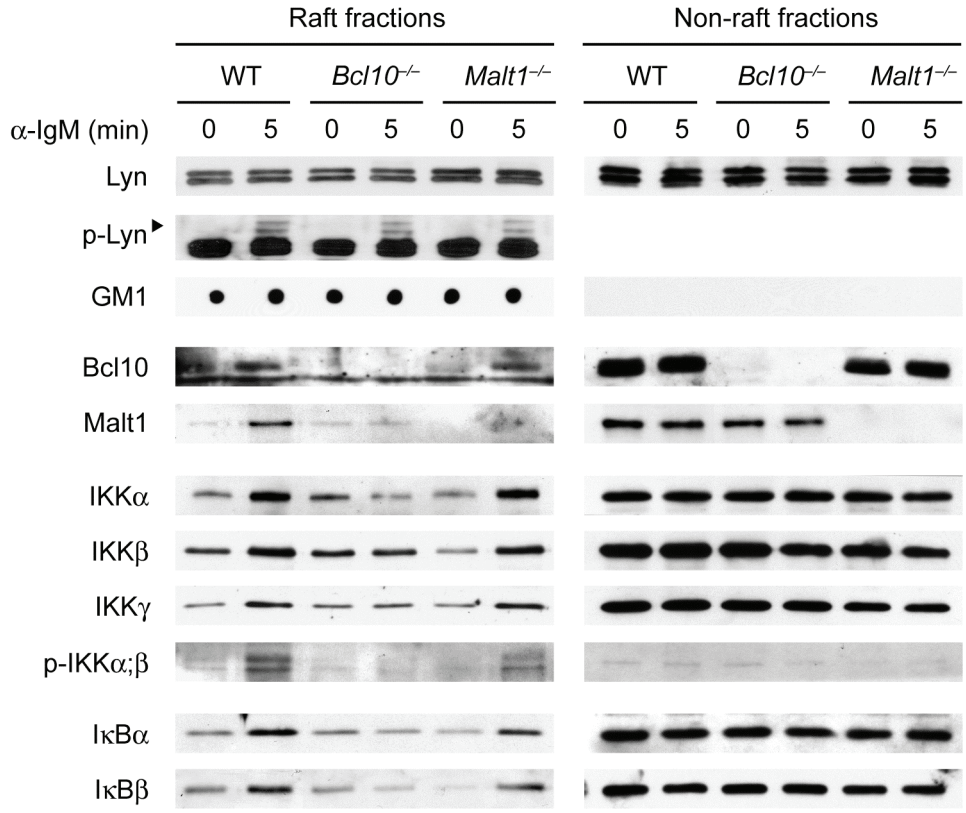

Figure 12. Differential control of BCR induced IKK activation by Bcl10 and Malt1. BCR-induced lipid raft recruitment and activation of key NF-κB regulators in B cells isolated and pooled and then left unstimulated (0) or stimulated for 5 min with anti-IgM (10 μg/ml; n = 4 mice per genotype); lysates were separated by sucrose-gradient ultracentrifugation to produce lipid raft preparations (Raft fractions) and non-lipid raft preparations (Non-raft fractions), followed by immunoblot analysis with antibodies to Lyn, phosphorylated Lyn (p-Lyn; arrowhead), Bcl10, Malt1, IKKα, IKKβ, IKKγ, phosphorylated IKKα and IKKβ (p-IKKα;β), IκBα and IκBβ. GM1, dot blots to confirm successful separation by ultracentrifugation. Data are one experiment representative of four.

IKK recruitment or IKK signalosome stabilization. To additionally test for IKK activation, immunoblot analysis was carried out, using antibodies that specifically recognize phosphorylated activation loop serine residues on IKK. Both catalytic subunits IKKα and IKKβ were selectively activated in the raft fractions but not in the non-raft fractions of wild-type and of *Malt1*$^{-/-}$ cells. In contrast, no BCR-induced IKK activation was detected in the absence of Bcl10. Finally, in coordination with IKK recruitment and activation, BCR signalling was found to enrich IκBα and IκBβ molecules in lipid rafts of wild-type and *Malt1*$^{-/-}$ cells. Again, according to the prior observed defects, Bcl10-deficient B cells failed to concentrate IκBα and IκBβ molecules within lipid rafts. These data collectively

indicate that Bcl10 controls in a Malt1-independent way the assembly of BCR induced lipid raft signalosomes that are needed to activate IKK and to corecruit IκBs. These mechanisms presumably bring active IKK molecules into proximity with their substrates to transduce BCR signals to canonical NF-κB activation.

3.4 Malt1 Couples the BCR Signal to c-Rel Activation

Although the initial experiments indicated that Malt1 is essential for optimal B cell responses, including BCR mediated Bcl-x_L induction, the molecular function of Malt1 in the BCR pathway remained elusive. Therefore, we next studied the NF-κB activating signalling events that follow initial formation of the IKK signalosome (Figure 13).

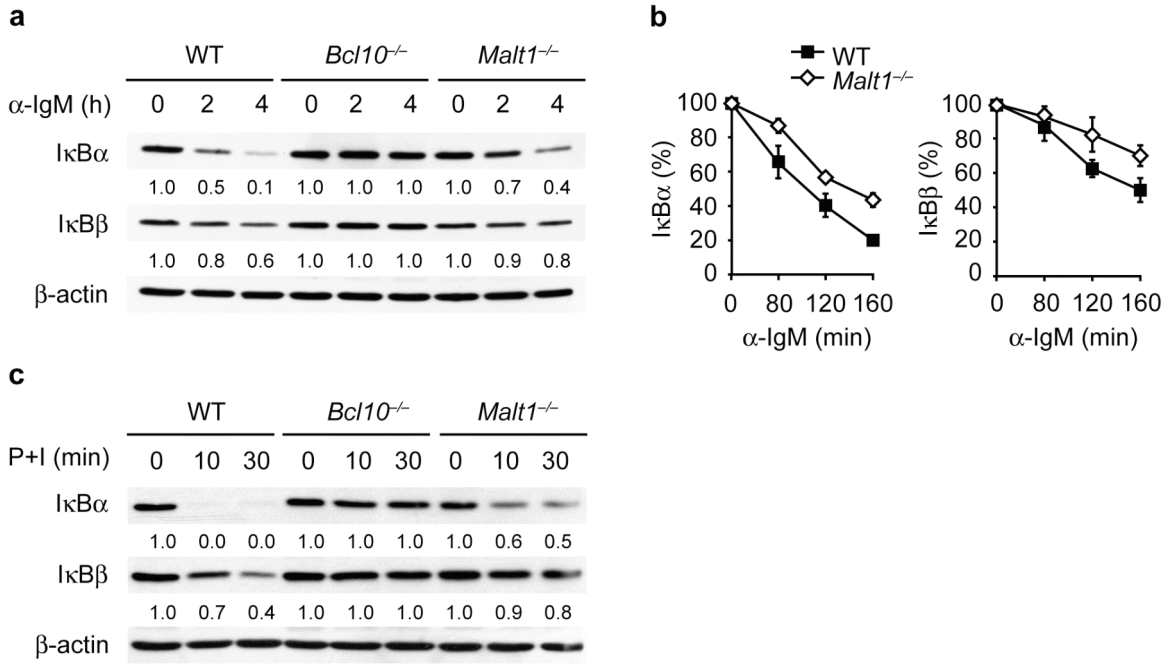

Figure 13. Bcl10- and Malt1-dependent regulation of IκB degradation. (**a**) BCR-induced degradation of IκBα and IκBβ in wild-type (WT), *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ B cells stimulated with anti-IgM (10 μg/ml), assessed by immunoblot analysis followed by densiometry. Below lanes, amounts normalized to those obtained for β-actin, presented relative to the amounts in unstimulated cells. (**b**) Immunoblot analysis (as described in **a**) of IκBα and IκBβ in wild-type and *Malt1*$^{-/-}$ B cells stimulated with anti-IgM (10 μg/ml), presented relative to that in unstimulated cells. (**c**) Degradation of IκBα and IκBβ in wild-type (WT), *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ B cells in response to PMA and ionomycin (P+I) treatment, assessed by immunoblot. Densiometric analysis as described in (**a**). Data are representative of eight (**a**, **b** (average ± s.e.m.)) or are one representative of three (**c**) independent experiments.

BCR ligation in wild-type cells induced a progressive degradation of IκBα and IκBβ (Figure 13a,b), whereas IκBε was not degraded (data not shown). Consistent with their absent IKK activation and their failure to corecruit IκB molecules into lipid rafts, *Bcl10*$^{-/-}$ cells did not demonstrate degradation of IκBα or IκBβ (Figure 13a). In contrast, BCR signalling in *Malt1*$^{-/-}$ cells induced the degradation of both IκBα or IκBβ (Figure 13a). However, densiometric quantification of the remaining IκBα and IκBβ showed that the extent of IκB degradation was lower than that of wild-type cells (Figure 13a,b). Qualitatively similar but quantitatively stronger and faster effects were obtained by stimulating cells with phorbol 12-myristate 13-acetate (PMA) and ionomycin as a strong pharmacological mimic of antigen receptor triggering (Figure 13c). As PMA and ionomycin directly activate PKC isoforms, these findings further indicate that deficiency in Bcl10 or Malt1 does not affect BCR proximal events.

To directly test BCR-induced nuclear translocation and DNA binding of NF-κB, we carried out gel shift assays of nuclear lysates from stimulated cells (Figure 14a).

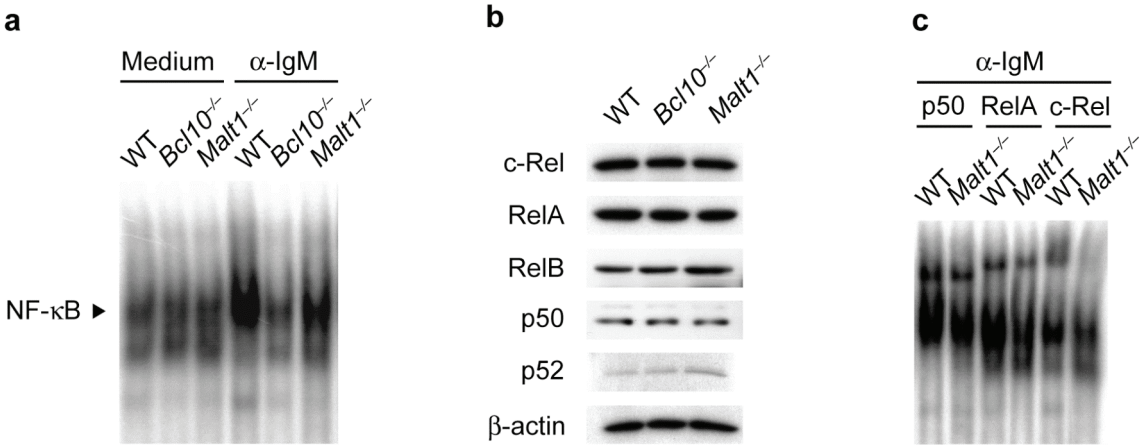

Figure 14. Bcl10- and Malt1-dependent regulation of NF-κB-DNA-binding. (**a**) Gel shift assay of BCR-induced NF-κB activation in purified wild-type (WT), *Bcl10*$^{-/-}$ or *Malt1*$^{-/-}$ B cells stimulated for 4 h with anti-IgM (10 μg/ml); nuclear lysates were analysed by electrophoretic mobility-shift assay for NF-κB DNA binding activity. (**b**) Immunoblot analysis of c-Rel, RelA, RelB, NF-κB p50 and NF-κB p52 expression in resting wild-type, Bcl10$^{-/-}$ and Malt1$^{-/-}$ B cells. 10 mice per genotype were analysed, depicted is a representative experiment. (**c**) BCR-induced activation of p50, RelA and c-Rel in wild-type (WT) or *Malt1*$^{-/-}$ B cells stimulated for 4 h with anti-IgM (10 μg/ml); nuclear lysates prepared (same as those in **a**) underwent gel supershift analysis with anti-p50, anti-RelA and anti-c-Rel. Data are one representative out of 6 (**a, c**) or one representative out of 10 (**b**) independent experiments.

In line with the immunoblot analyses of the degradation IκBα and IκBβ (Figure 13a,b) and consistent with published results (Ruland, Duncan et al. 2001; Ruland, Duncan et al. 2003; Xue, Morris et al. 2003), we noted a robust induction of NF-κB DNA binding activity in BCR stimulated wild-type B cells but no increase above baseline in *Bcl10*$^{-/-}$ cells (Figure 14a). *Malt1*$^{-/-}$ B cells, in contrast, activated NF-κB considerably, but with less intensity than wild-type cells did. This impairment was not due to a lower abundance of NF-κB proteins, as RelA, RelB, c-Rel, p50 and p52 all had equal expression in wild-type, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ cells (Figure 14b). To test whether Malt1 quantitatively determines the strength of the BCR-to-NF-κB signal or whether it qualitatively controls distinct canonical NF-κB subunits, NF-κB supershift assays were carried out (Figure 14c). Similar degrees of p50 and RelA DNA binding activity were detected in BCR-stimulated wild-type and *Malt1*$^{-/-}$ cells. However, complexes containing c-Rel were induced only in BCR stimulated wild-type cells, not in those lacking Malt1. Thus, Malt1 is specifically required to transduce BCR signals to c-Rel activation.

The expression of the c-Rel protein itself is induced by BCR signalling (Bajpai, Zhang et al. 2000). Therefore, the possibility had to be considered that the shown lack of c-Rel activation in *Malt1*$^{-/-}$ B cells could have been due to a defective *Rel* transactivation. However, this possibility could be excluded as BCR triggering induced similar accumulation of c-Rel protein in wild-type and *Malt1*$^{-/-}$ cells (Figure 15).

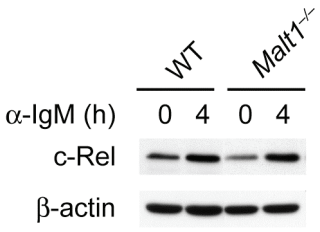

Figure 15. Lack of Malt1 does not interfere with BCR-induced upregulation of c-Rel. Immunoblot analysis of the BCR-induced upregulation of c-Rel in wild-type and *Malt1*$^{-/-}$ B cells left unstimulated or stimulated with anti-IgM (10 μg/ml). Data are one representative of three individual experiments.

Hence, the failure of *Malt1*$^{-/-}$ B cells to activate c-Rel might have arisen from either a defective nuclear translocation or an absent induction of NF-κB DNA binding activity in the nucleus. To address this issue, signal induced nuclear translocation of c-Rel and RelA was subsequently monitored and quantified by immunofluorescence microscopy (Figure 16a,b). For this, cells were stimulated in parallel via the BCR or with LPS.

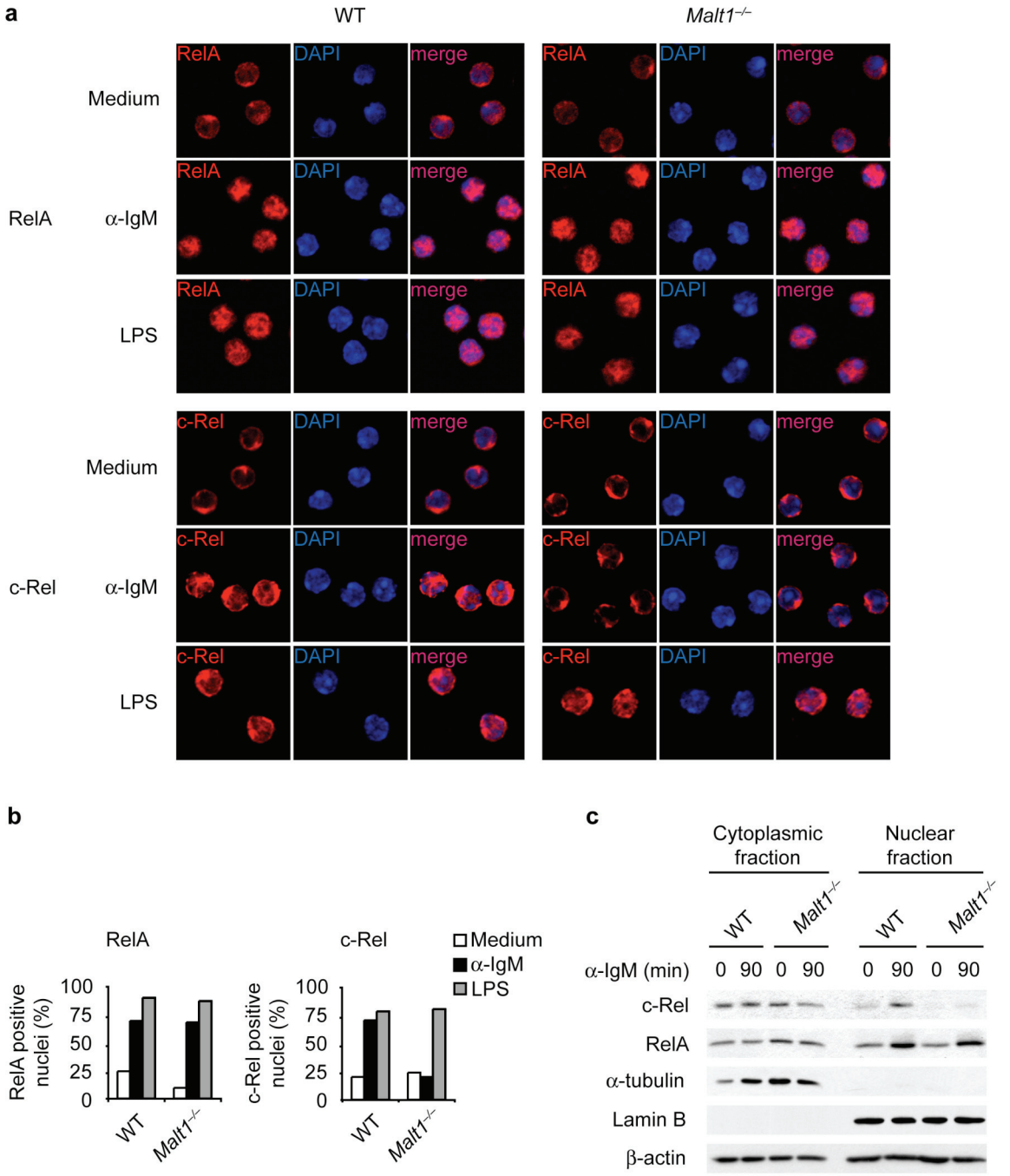

Figure 16. Malt1 controls BCR mediated nuclear translocation of c-Rel. (**a**) Immunofluorescence microscopy of the translocation of RelA (red; top three rows) and c-Rel (red; bottom three rows) into the nucleus (DAPI staining, blue; co-localization (Merge), pink). Original magnification, x630. (**b**) Quantification of the translocation in **a** by calculation of the of RelA+ or c-Rel+ nuclei in at least 100 individual cells. (**c**) Cell fractionation analysis of the nuclear translocation of c-Rel by immunoblot analysis of the cytoplasmatic and nuclear abundance of c-Rel and RelA in extracts of B cells left untreated or stimulated for 90 min with anti-IgM (10 μg/ml). Below, α-tubulin (cytoplasmic marker) and lamin B (nuclear marker) serve as controls to confirm successful separation into cytoplasmic and nuclear extracts. β-actin, loading control. Data are representative of three (**a**,**b**) or four (**c**) individual experiments.

Consistent with normal LPS-induced cell proliferation, *Malt1*$^{-/-}$ B cells showed normal nuclear translocation of both NF-κB subunits in response to ligation of TLR4 (Figure 16a,b). In addition, BCR signalling resulted in a similar RelA translocation in wild-type and *Malt1*$^{-/-}$ cells. However, efficient BCR mediated c-Rel translocation was only detected in wild-type cells, not in those lacking Malt1. To additionally address the translocation of these two NF-κB subunits, cell fractionation and immunoblot analysis of cytoplasmic and nuclear extracts with c-Rel and RelA specific antibodies were carried out (Figure 16c). In wild-type cells, BCR crosslinking led to a robust increase in the abundance of both nuclear RelA and c-Rel. In contrast, but consistent with the data presented above, BCR ligation in *Malt1*$^{-/-}$ cells induced a substantial increase in only nuclear RelA, but not c-Rel. This allows the conclusion that a Malt1-dependent pathway regulates the translocation of c-Rel into the nucleus.

3.5 Malt1 Signalling Controls the Release of c-Rel from IκB

To test whether the impairment of c-Rel translocation in *Malt1*$^{-/-}$ cells was caused by a defect in c-Rel release from IκBs, IκBα or IκBβ were coimmunoprecipitated with RelA and c-Rel before and after cell stimulation (Figure 17). In these experiments we used the strong stimulus PMA and ionomycin as a `surrogate´ of antigen receptor signalling. In resting B cells from wild-type, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mice, both RelA and c-Rel were bound to IκBα and to IκBβ (Figure 17a). In line with the results presented in (Figure 13a-c), signal-induced IκBα and IκBβ degradation was blocked in *Bcl10*$^{-/-}$ cells (Figure 17a). Consistent with those findings, both RelA and c-Rel were still bound to IκBs in *Bcl10*$^{-/-}$ cells. Furthermore, the extent of IκBα and IκBβ degradation was again lower in *Malt1*$^{-/-}$ cells compared to the wild-type (Figure 17b). However, after cell stimulation, both wild-type and *Malt1*$^{-/-}$ cells liberated similar amounts of RelA from IκBα (Figure 17b). In contrast, whereas c-Rel was efficiently released from both IκB molecules in activated wild-type cells, c-Rel remained bound to IκBα and to IκBβ in *Malt1*$^{-/-}$ B cells. Thus, Malt1 specifically directs BCR signals to the degradation of IκB molecules that are in complex with c-Rel.

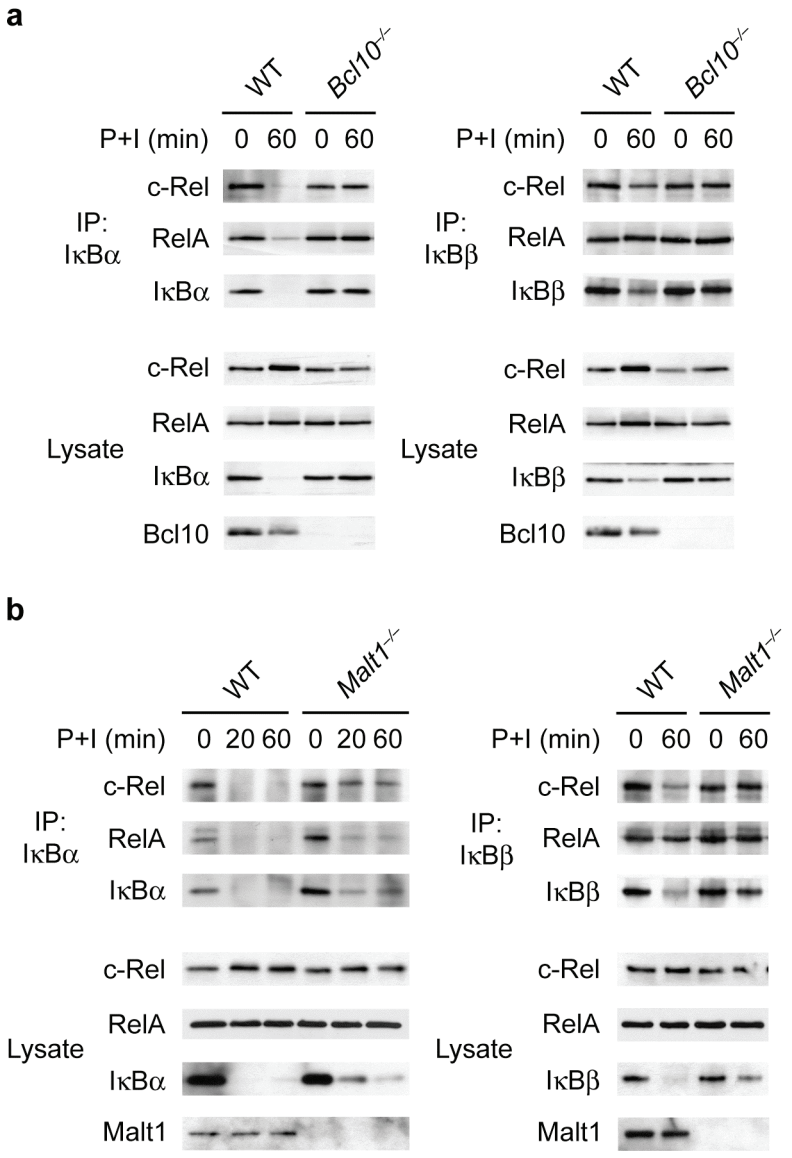

Figure 17. Malt1 controls the degradation of IκB molecules that are bound to c-Rel. (**a**) Release of RelA and c-Rel from IκB in wild-type and *Bcl10*$^{-/-}$ B cells left untreated or stimulated for 60 min with PMA plus ionomycin (P+I; 10 nM each); lysates were analysed by immunoblot for c-Rel, RelA, Bcl10, and IκBα or IκBβ content (bottom) or were immunoprecipitated (IP) with anti-IκBα or anti-IκBβ, followed by immunoblot for c-Rel, RelA, and IκBα or IκBβ (top). (**b**) Release of c-Rel from IκBα and IκBβ in wild-type and *Malt1*$^{-/-}$ B cells stimulated with PMA plus ionomycin (P+I; 10 nM each; time, above lanes); lysates were analysed by immunoblot for c-Rel, RelA, Malt1, and IκBα or IκBβ content (bottom) or were immunoprecipitated (IP) with anti-IκBα or anti-IκBβ and then analysed by immunoblot for c-Rel, RelA, and IκBα or IκBβ (top). Experiments were repeated three (**a**) or four times (**b**) with similar results.

4. DISCUSSION

In the present study BCR-mediated signal transduction to NF-κB activation was investigated. Particular focus was directed on the involvement of the scaffold proteins Bcl10 and Malt1. To date, the interaction partners Bcl10 and Malt1 have been considered to constitute a module with inseparable functions, controlling a linear pathway to canonical NF-κB activation.

Using primary B cells derived from genetic mouse models, the present study demonstrates that Bcl10 and Malt1 fulfil both common and distinct functions in B cell signalling and identifies a previously unrecognized bifurcation in the BCR-induced canonical signalling pathway at the level of Bcl10 and Malt1. Hence, two distinct pathways, one commonly controlled by Bcl10 and Malt1, in contrast to a second Malt1-independent pathway, individually mediate the activation of the NF-κB subunits c-Rel and RelA to transduce BCR signals to specific functional outcomes.

4.1 Bcl10 and Malt1 Mediate Tonic Signalling to NF-κB

Accumulating evidence provides support for the existence of ligand-independent or tonic signalling via the mature BCR (Monroe 2006). Tonic BCR signals are critically required for the survival of mature lymphocytes in the absence of antigen and, according to that, for the maintenance of a broad receptor repertoire, given that each B cell clone represents a distinct BCR specifity. Although the mechanistic basics are still very poorly understood, activation of NF-κB has been implicated in the transmission of resting B cell survival, as B cells deficient in NF-κB1 (p50) or B cells doubly deficient in RelA and c-Rel show an enhanced `passive death´ (Grumont, Rourke et al. 1998; Grossmann, O'Reilly et al. 2000). To address a putative function for Bcl10 and Malt1 in the signal transduction to NF-κB, mature B cells derived from *Bcl10*- and *Malt1*-knockout mice were cultured in the absence of specific stimuli. Resting, mature *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ B cells were found to die more rapidly than wild-type control cells. No notable differences in survival could be detected between *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ B cells, what was in line with the current view of the cooperative action of these two molecules. The observed phenotype was most similar to that of PKC-β-deficient B cells or

Carma1-deficient B cells (Su, Guo et al. 2002; Hara, Wada et al. 2003), indicating a linear PKC-β-Carma1-Bcl10-Malt1 pathway that contributes to the homeostatic survival of B lymphocytes.

4.2 Ligand Induced BCR-Signalling: the Canonical NF-κB Pathway is Bifurcated

For ligand-induced BCR signalling, which is molecularly distinct from tonic signalling (Monroe 2006), this study provides evidence for a bifurcation of the NF-κB pathway at the level of Bcl10 and Malt1 and demonstrates distinct mechanisms for RelA and c-Rel control.

4.2.1 Bcl10 is Essential for Recruitment and Activation of IKK in Lipid Rafts

The dependency of BCR-mediated induction of NF-κB DNA binding activity on Bcl10 has been reported previously (Ruland, Duncan et al. 2001; Xue, Morris et al. 2003). However, the molecular function of Bcl10 in BCR signalling to NF-κB remained unclear. The present study now provides evidence that Bcl10 orchestrates lipid raft-associated IKK signalosome formation.

The importance for lipid rafts in spatial organization of BCR-mediated signal transduction has been well established by biochemical preparation of these detergent-resistant microdomains as well as by live cell imaging (Simons and Toomre 2000; Pierce 2002; Dykstra, Cherukuri et al. 2003; Tolar, Sohn et al. 2005; Sohn, Tolar et al. 2006). Regarding the function of Bcl10, initial signalosome formation in lipid raft microdomains is intact in $Bcl10^{-/-}$ B cells as monitored by phosphorylation of the constitutively raft-associated kinase Lyn. An unaffected signal initiation is further supported by similar receptorproximal tyrosine phosphorylation and ERK activation shown in cytoplasmic extracts of BCR-stimulated $Bcl10^{-/-}$ B cells.

By contrast, recruitment of essential complex components `downstream´ of Bcl10 into lipid rafts was severely affected. Most importantly, the subunits of the IKK complex and their substrates, the IκB proteins, could not be detected in detergent-resistant microdomains of BCR-stimulated Bcl10-deficient B cells. In wild-type B

cells, BCR-mediated IKK activation as monitored by phosphorylation occurred exclusively in lipid raft but not in non-raft fractions. This result is in line with previous findings (Su, Guo et al. 2002) and further underlines the meaning of compartmentalisation into membrane microdomains for complex stabilization. Bcl10 was critical for BCR-induced recruitment of IKK into lipid rafts and no activation of IKK could be shown in Bcl10-deficient B cells. Collectively, this allows the conclusion that Bcl10 mediates the recruitment of IKK signalosome components or the stabilization of the formed multiprotein receptor complex within lipid rafts. This membrane microdomain-associated signalosome assembly is a prerequisite for IKK activation and downstream signalling.

A most recent study addressed the role of Bcl10 in IKK phosphorylation and activation in AgR-stimulated lymphocytes (Shambharkar, Blonska et al. 2007). In this work, a Bcl10-independent IKK phosphorylation has been reported that is not sufficient for the induction of IKK kinase activity. As these results mainly rely on investigation of T cell lines, it will be necessary to address this in physiological settings.

4.2.2 Bcl10 is Essential for the Activation of RelA and c-Rel

In Bcl10-deficient B cells, IKK could not be activated upon stimulation with anti-IgM or P+I. IκBα and IκBβ were not phosphorylated and degraded. Therefore Bcl10$^{-/-}$ B cells entirely failed to release RelA and c-Rel containing dimers from cytoplasmic inhibition and showed no induction of NF-κB after BCR engagement. Consequently, the upregulation of all tested NF-κB target genes was defective. This comprised the activation of a number of important anti-apoptotic genes of the *Bcl2* family encoding Bcl-x_L (Chen, Edelstein et al. 2000) and A1 (Vigorito, Gambardella et al. 2005), as well as c-Flip (Schram and Rothstein 2003). Subsequently, Bcl10$^{-/-}$ B cells died rapidly in response to BCR ligation. In general, B cell fate is assumed to be the balance between survival and death signals initiated through the BCR (Niiro and Clark 2002). The enhanced death of Bcl10-deficient cells reflects the pivotal role of NF-κB in mediating survival upon BCR stimulation. As induction of apoptosis can be abrogated by transgenic overexpression of BCL2, the intrinsic apoptosis pathway seems to be predominant in this scenario, but c-Flip, which interferes mainly with extrinsic apoptosis

pathways, probably contributes to NF-κB-dependent B cell survival in additional physiological settings (Schram and Rothstein 2003; Budd, Yeh et al. 2006; Golks, Brenner et al. 2006). In addition to these severe survival defects, Bcl10-deficient B cells fail to proliferate upon BCR engagement. This may be due to the broad impairment in the induction of various NF-κB-mediated cell cycle regulators. In particular, cyclins that control progression through various cell cycle checkpoints, have been shown to be NF-κB regulated (Solvason, Wu et al. 2000; Joyce, Albanese et al. 2001). Among the group of cyclins, the induction of cyclin D2, D3 and E was profoundly affected in BCR-stimulated Bcl10$^{-/-}$ B cells. To exclude the possibility that the involved failure of Bcl10$^{-/-}$ B cells to proliferate was caused by rapid cell death, this survival defect was rescued by transgenic overexpression of Bcl2. This allowed the discrimination between the requirement for Bcl10-signalling in either mediating survival or proliferation. Thus, independent from survival, signal transduction via Bcl10 has been shown to be essential for BCR-regulated proliferation.

Collectively, Bcl10-signalling is critical for the induction of both survival and proliferation upon BCR engagement. These functional defects are consistent with a molecular role of Bcl10 in IKK signalosome formation to mediate cytoplasmic release of RelA and c-Rel. Hence, the presented results assign Bcl10 to act as a `central gatekeeper´ in AgR-induced B cell maintenance and clonal expansion.

4.2.3 Malt1 Directs Canonical NF-κB Signalling Selectively to c-Rel

First insights into the role of Malt1 in B cell signalling were gained by the analysis of primary B cells derived from two *Malt1*-knockout mouse strains (Ruefli-Brasse, French et al. 2003; Ruland, Duncan et al. 2003). A direct comparison of Bcl10$^{-/-}$ and Malt1$^{-/-}$ primary B cells revealed a differential requirement for Bcl10 and Malt1 in B cell activation (Ruland, Duncan et al. 2003). The present study shows that Malt1 regulates a BCR-induced Bcl10 subprogram, which controls B cell survival. On the molecular level, this subprogram could be attributed to c-Rel control. Malt1-mediated activation of c-Rel containing dimers was essential for Bcl-x_L expression and optimal upregulation of A1 and c-Flip at early time points of BCR ligation. However, Malt1 signalling was not required for BCR-induced activation of RelA. This correlated with a largely normal upregulation of cyclin D2, D3 and E and

accounted for the ability to proliferate. These observations are in line with the fact that distinct NF-κB dimers have been proposed to activate selected target genes (Baldwin 1996; Leung, Hoffmann et al. 2004; Sanjabi, Williams et al. 2005). Although the precise regulation of c-Flip is not well understood, c-Rel is known to contribute to the induction of Bcl-x_L and A1 after BCR ligation (Grumont, Rourke et al. 1999; Chen, Edelstein et al. 2000); however, the phenotype of *Malt1*$^{-/-}$ B cells does not mimic that of B cells homozygous for c-Rel deficiency, as the latter show a complete block in A1 induction as well as defective proliferative responses to BCR ligation or LPS stimulation (Kontgen, Grumont et al. 1995; Grumont, Rourke et al. 1999). Yet, genetic deficiency in c-Rel not only blocks signal-mediated c-Rel induction but completely abolishes constitutive c-Rel activity as well. This distinction is very important, as c-Rel dimers are strongly upregulated during B cell differentiation and they contribute to a large extent to the constitutive NF-κB activity of normal mature B cells, which might set the threshold for BCR responses (Liou, Sha et al. 1994; Miyamoto, Schmitt et al. 1994). *Malt1*$^{-/-}$ cells have normal amounts of c-Rel in the resting state. Thus, the presented results indicate that constitutive c-Rel activity together with a strong Malt1-independent RelA activation signal is sufficient to drive the division of surviving BCR-stimulated cells.

4.2.4 Molecular Model of BCR-Mediated NF-κB Activation

Based on the presented biochemical results the previous model of BCR-mediated signal transduction to NF-κB can be extended with respect to the functions of Bcl10 and Malt1 as suggested in the following (Figure 18). Proximal signals leading to activation of PKC-β induce Carma1 oligomerization and interaction with Bcl10. Oligomerization of Carma1 involves a preferential recruitment into lipid rafts and induction of an oligomerization cascade. This results in the formation of a raft associated multiprotein complex consisting of Malt1 and IKK subunits together with IκB molecules. As Bcl10 is not known to directly bind to IKK or IκB molecules, the interaction of Bcl10 with Carma1 might stabilize Carma1 signalosomes in membrane microdomains and prevent dissociation of complex components. Prolonged persistency in lipid rafts would allow the recruitment of IKK and cofactors either directly or indirectly to activate IKK in close proximity to IκBα and IκBβ for `downstream´ signalling to RelA and c-Rel. Malt1 is not critical for

signalosome stabilization and the overall IKK recruitment and activation events, however specifically mediates the degradation of IκB molecules that are in complex with c-Rel, but not of those bound to RelA. Thus, two types of BCR-induced subcomplexes are conceivable, which either depend on the presence of Bcl10: one that contains Malt1 as an `essential organizer´ to bring active IKK into close proximity with IκB-bound c-Rel molecules, and a second distinct type that contains IKK in the vicinity of IκB-bound RelA molecules.

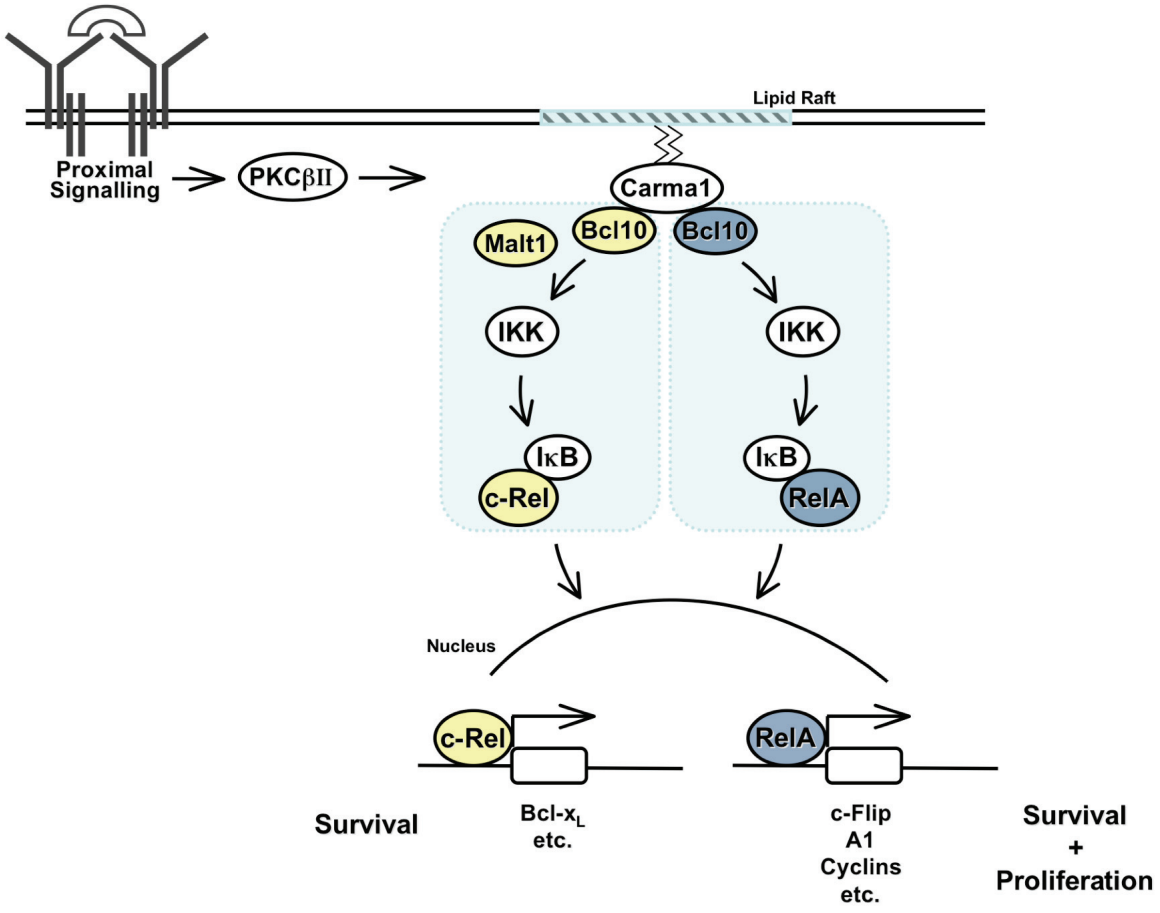

Figure 18. Molecular model of BCR-mediated NF-κB activation. Proximal signals leading to activation of PKC-β induce Carma1 oligomerization and interaction with Bcl10, involving a preferential recruitment into lipid rafts. A raft associated multiprotein complex is formed consisting of Malt1 and IKK subunits together with IκB molecules. Unlike Malt1, Bcl10 is critical for the stabilization of signalosome components in membrane microdomains. The distinction between the release of RelA and c-Rel is mediated via two subcomplexes (shaded in blue), which either depend on Bcl10: one that contains Malt1 to direct active IKK to IκB-bound c-Rel molecules (left), and a second distinct type leading IKK to IκB-bound RelA molecules (right). The Bcl10-Malt1-c-Rel pathway is critical in mediating sustained survival, while acute survival as well as proliferation are regulated by the Malt1-independent Bcl10-RelA pathway.

Although it is possible that the precise control of Bcl10 and Malt1 signalling could vary among cellular contexts and change during B cell maturation, this model explains the phenotypes of mature splenic *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ B cells.

4.3 Implications for the Presented Model in Other Cellular Contexts

The presented model proposing the existence of a bifurcated signal transduction to the activation of either RelA or c-Rel containing NF-κB dimers might explain the observation that RelA and c-Rel exhibit divergent oncogenic activities. Of note, among the NF-κB family members, solely c-Rel is related to a viral Rel oncoprotein, and only c-Rel has been consistently shown to be able to malignantly transform cells in culture (Gilmore, Kalaitzidis et al. 2004). Moreover, genomic amplifications of *REL* (the gene encoding C-Rel) but not of *RelA* are frequently detected in various types of human B cell lymphoma (Karin, Cao et al. 2002; Gilmore, Kalaitzidis et al. 2004; Jost and Ruland 2007). *Bcl10* and *MALT1* were identified from chromosomal translocations and are also deregulated by other mechanisms in B cell malignancies (Ngo, Davis et al. 2006; Jost and Ruland 2007). The presented experimental evidence suggests existence of a specific BCR-engaged Bcl10-Malt1-c-Rel survival pathway. Hence, it seems that an aberrant activation of this specific cascade through various mechanisms might have a more dominant function in lymphomagenesis than the Malt1-independent RelA activation pathway.

Apart from potential distinct roles in B lymphocyte transformation, it is tempting to speculate about the meaning of the Malt1-dependent bifurcated signalling in other cellular contexts. AgR mediated NF-κB activation is considered to be highly analogous in B and T lymphocytes. However, independent studies of primary T cells derived from the two distinct *Malt1*-knockout mouse strains reported a complete block in TCR mediated NF-κB activation (Ruefli-Brasse, French et al. 2003; Ruland, Duncan et al. 2003). According to this, the demonstrated function for the scaffold protein Malt1 in directing the signal selectively to c-Rel seems to be B cell-specific. But the discovery of a new strategy that allows via distinct activation of RelA and c-Rel the translation of a receptor signal into diverse

functional outcomes might have implications beyond lymphocyte biology. Many signalling systems engage the canonical pathway in immune and non-immune cells to induce IKK-dependent activation of RelA and c-Rel (Hayden and Ghosh 2004). For most scenarios, it is unclear how these subunits are selectively controlled. Based on the presented results, it is conceivable that different scaffolds that are functionally similar to Malt1 could also act at the level of IKK-containing signalosomes to `decide´ in other cellular contexts between c-Rel and RelA induction.

ABBREVIATIONS

A1	Bcl2-Related Protein A1
AgR	Antigen Receptor
AP-1	Activator-Protein-1
API2	Apoptosis Inhibitor 2
B Cell	B Lymphocyte
BAFF	B Cell Activating Factor
Bcl10	B Cell Lymphoma 10
Bcl2	B Cell Lymphoma 2
Bcl-x_L	B Cell Lymphoma 2-like 1
BCR	B Cell Receptor
BIR	Baculovirus IAP Repeat
BM	Bone Marrow
Btk	Bruton Agammaglobulinemia Tyrosine Kinase
CARD	Caspase Recruitment Domain
Carma1	Caspase Recruitment Domain Family, Member 11
cDNA	Complementary Deoxyribonucleic Acid
c-Flip	Cellular FLICE-like Inhibitory Protein
CFSE	5(6)-Carboxyfluorescein Diacetate N-Succinimidyl Ester
CLD	Caspase-like Domain
c-Rel	v-Rel Reticuloendotheliosis Viral Oncogene Homolog C
DAG	Diacylglycerol
DAPI	4,6-Diamidino-2-Phenylindole
DD	Death Domain
ERK	Extracellular Signal Regulated Protein Kinases
GALT	Gut-Associated Lymphoid Tissue
GC	Germinal Center
GEF	Guanine-Nucleotide Exchange Factor
GM1	Monosialotetrahexosylganglioside 1
GTP	Guanosine Triphosphate
h	Hour
Ig	Immunoglobulin

IκB	Inhibitor of κB
IKK	Inhibitor of κB Kinase
IP	Immunoprecipitation
IP3	Inositol-1, 4, 5-Trisphosphate
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
Jnk	c-Jun N-Terminal Kinases
LN	Lymph Node
LPS	Lipopolysaccharide
Lyn	Yamaguchi Sarcoma Viral-Related Oncogene Homolog
M Cell	Mature B Lymphocyte
MALT	Gut-Associated Lymphoid Tissue
Malt1	Mucosa Associated Lymphoid Tissue Lymphoma Translocation Gene 1
MAP	Mitogen-Activated-Protein Kinase
MHC	Major Histocompatibility Complex
min	Minute
MIRR	Multichain Immune Recognition Receptor
MZB cell	Marginal Zone B Lymphocyte
NF-κB	Nuclear Factor-kB
P+I	Phorbol-12 Myristate 13-Acetate Ester and Calcium Ionophore
p50	NF-κB1
p52	NF-κB2
PC	Plasma Cell
PI	Propidium Iodide
PKC	Protein Kinase C
PLC	Phospholipase C
p	Phospho-
qPCR	Quantitative Polymerase Chain Reaction
REL	v-Rel Reticuloendotheliosis Viral Oncogene Homolog
RelA	v-Rel Reticuloendotheliosis Viral Oncogene Homolog A
RelB	v-Rel Reticuloendotheliosis Viral Oncogene Homolog B
RHD	REL-Homology Domain
RNA	Ribonucleic Acid
SH2	Src-Homology Domain 2

Src	v-Src Sarcoma Viral Oncogene Homolog
Syk	Spleen Tyrosine Kinase
TAD	Transactivation Domain
TAK1	TGF-β-Associated Kinase
TCR	T Cell Receptor
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF-Receptor-Associated Factor
vav	Vav Guanine Nucleotide Exchange Factor

CITATION INDEX

Akagi, T., M. Motegi, et al. (1999). "A novel gene, MALT1 at 18q21, is involved in t(11;18) (q21;q21) found in low-grade B-cell lymphoma of mucosa-associated lymphoid tissue." Oncogene **18**(42): 5785-94.

Alizadeh, A. A., M. B. Eisen, et al. (2000). "Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling." Nature **403**(6769): 503-11.

Bajpai, U. D., K. Zhang, et al. (2000). "Bruton's tyrosine kinase links the B cell receptor to nuclear factor kappaB activation." J Exp Med **191**(10): 1735-44.

Baldwin, A. S., Jr. (1996). "The NF-kappa B and I kappa B proteins: new discoveries and insights." Annu Rev Immunol **14**: 649-83.

Berland, R. and H. H. Wortis (2002). "Origins and functions of B-1 cells with notes on the role of CD5." Annu Rev Immunol **20**: 253-300.

Bonizzi, G. and M. Karin (2004). "The two NF-kappaB activation pathways and their role in innate and adaptive immunity." Trends Immunol **25**(6): 280-8.

Budd, R. C., W. C. Yeh, et al. (2006). "cFLIP regulation of lymphocyte activation and development." Nat Rev Immunol **6**(3): 196-204.

Casey, P. J. (1995). "Protein lipidation in cell signaling." Science **268**(5208): 221-5.

Chau, V., J. W. Tobias, et al. (1989). "A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein." Science **243**(4898): 1576-83.

Chen, C., L. C. Edelstein, et al. (2000). "The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L)." Mol Cell Biol **20**(8): 2687-95.

Chen, Z. J., L. Parent, et al. (1996). "Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination-dependent protein kinase activity." Cell **84**(6): 853-62.

Cheng, P. C., M. L. Dykstra, et al. (1999). "A role for lipid rafts in B cell antigen receptor signaling and antigen targeting." J Exp Med **190**(11): 1549-60.

Cory, S. and J. M. Adams (2002). "The Bcl2 family: regulators of the cellular life-or-death switch." Nat Rev Cancer **2**(9): 647-56.

Dierlamm, J., M. Baens, et al. (1999). "The apoptosis inhibitor gene API2 and a novel 18q gene, MLT, are recurrently rearranged in the t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue lymphomas." Blood **93**(11): 3601-9.

Dykstra, M., A. Cherukuri, et al. (2003). "Location is everything: lipid rafts and immune cell signaling." Annu Rev Immunol **21**: 457-81.

Egawa, T., B. Albrecht, et al. (2003). "Requirement for CARMA1 in antigen receptor-induced NF-kappa B activation and lymphocyte proliferation." Curr Biol **13**(14): 1252-8.

Gaide, O., B. Favier, et al. (2002). "CARMA1 is a critical lipid raft-associated regulator of TCR-induced NF-kappa B activation." Nat Immunol **3**(9): 836-43.

Gaide, O., F. Martinon, et al. (2001). "Carma1, a CARD-containing binding partner of Bcl10, induces Bcl10 phosphorylation and NF-kappaB activation." FEBS Lett **496**(2-3): 121-7.

Ghosh, S. and M. Karin (2002). "Missing pieces in the NF-kappaB puzzle." Cell **109 Suppl**: S81-96.

Ghosh, S., M. J. May, et al. (1998). "NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses." Annu Rev Immunol **16**: 225-60.

Gilmore, T. D. (1999). "Multiple mutations contribute to the oncogenicity of the retroviral oncoprotein v-Rel." Oncogene **18**(49): 6925-37.

Gilmore, T. D., D. Kalaitzidis, et al. (2004). "The c-Rel transcription factor and B-cell proliferation: a deal with the devil." Oncogene **23**(13): 2275-86.

Golks, A., D. Brenner, et al. (2006). "The c-FLIP-NH2 terminus (p22-FLIP) induces NF-kappaB activation." J Exp Med **203**(5): 1295-305.

Grossmann, M., L. A. O'Reilly, et al. (2000). "The anti-apoptotic activities of Rel and RelA required during B-cell maturation involve the regulation of Bcl-2 expression." Embo J **19**(23): 6351-60.

Grumont, R. J., I. J. Rourke, et al. (1999). "Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis." Genes Dev **13**(4): 400-11.

Grumont, R. J., I. J. Rourke, et al. (1998). "B lymphocytes differentially use the Rel and nuclear factor kappaB1 (NF-kappaB1) transcription factors to regulate cell cycle progression and apoptosis in quiescent and mitogen-activated cells." J Exp Med **187**(5): 663-74.

Guo, B., T. T. Su, et al. (2004). "Protein kinase C family functions in B-cell activation." Curr Opin Immunol **16**(3): 367-73.

Hara, H., T. Wada, et al. (2003). "The MAGUK family protein CARD11 is essential for lymphocyte activation." Immunity **18**(6): 763-75.

Hayden, M. S. and S. Ghosh (2004). "Signaling to NF-kappaB." Genes Dev **18**(18): 2195-224.

Hsu, B. L., S. M. Harless, et al. (2002). "Cutting edge: BLyS enables survival of transitional and mature B cells through distinct mediators." J Immunol **168**(12): 5993-6.

Isaacson, P. G. and M. Q. Du (2004). "MALT lymphoma: from morphology to molecules." Nat Rev Cancer **4**(8): 644-53.

Janeway, C. A., Jr. and K. Bottomly (1994). "Signals and signs for lymphocyte responses." Cell **76**(2): 275-85.

Jost, P. J. and J. Ruland (2007). "Aberrant NF-kappaB signaling in lymphoma: mechanisms, consequences, and therapeutic implications." Blood **109**(7): 2700-7.

Joyce, D., C. Albanese, et al. (2001). "NF-kappaB and cell-cycle regulation: the cyclin connection." Cytokine Growth Factor Rev **12**(1): 73-90.

Karin, M., Y. Cao, et al. (2002). "NF-kappaB in cancer: from innocent bystander to major culprit." Nat Rev Cancer **2**(4): 301-10.

Kontgen, F., R. J. Grumont, et al. (1995). "Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression." Genes Dev **9**(16): 1965-77.

Kurosaki, T. (2002). "Regulation of B-cell signal transduction by adaptor proteins." Nat Rev Immunol **2**(5): 354-63.

Langlet, C., A. M. Bernard, et al. (2000). "Membrane rafts and signaling by the multichain immune recognition receptors." Curr Opin Immunol **12**(3): 250-5.

Leung, T. H., A. Hoffmann, et al. (2004). "One nucleotide in a kappaB site can determine cofactor specificity for NF-kappaB dimers." Cell **118**(4): 453-64.

Liou, H. C., W. C. Sha, et al. (1994). "Sequential induction of NF-kappa B/Rel family proteins during B-cell terminal differentiation." Mol Cell Biol **14**(8): 5349-59.

Loder, F., B. Mutschler, et al. (1999). "B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals." J Exp Med **190**(1): 75-89.

Lucas, P. C., M. Yonezumi, et al. (2001). "Bcl10 and MALT1, independent targets of chromosomal translocation in malt lymphoma, cooperate in a novel NF-kappa B signaling pathway." J Biol Chem **276**(22): 19012-9.

Mak, T. W., J. M. Penninger, et al. (2001). "Knockout mice: a paradigm shift in modern immunology." Nat Rev Immunol **1**(1): 11-9.

Martin, F. and J. F. Kearney (2000). "B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a "natural immune memory"." Immunol Rev **175**: 70-9.

Martin, F. and J. F. Kearney (2002). "Marginal-zone B cells." Nat Rev Immunol **2**(5): 323-35.

Martin, F., A. M. Oliver, et al. (2001). "Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens." Immunity **14**(5): 617-29.

Matsumoto, R., D. Wang, et al. (2005). "Phosphorylation of CARMA1 plays a critical role in T Cell receptor-mediated NF-kappaB activation." Immunity **23**(6): 575-85.

Miyamoto, S., M. J. Schmitt, et al. (1994). "Qualitative changes in the subunit composition of kappa B-binding complexes during murine B-cell differentiation." Proc Natl Acad Sci U S A **91**(11): 5056-60.

Monroe, J. G. (2006). "ITAM-mediated tonic signalling through pre-BCR and BCR complexes." Nat Rev Immunol **6**(4): 283-94.

Moreno-Garcia, M. E., K. M. Sommer, et al. (2006). "Proximal signals controlling B-cell antigen receptor (BCR) mediated NF-kappaB activation." Adv Exp Med Biol **584**: 89-106.

Ngo, V. N., R. E. Davis, et al. (2006). "A loss-of-function RNA interference screen for molecular targets in cancer." Nature **441**(7089): 106-10.

Niiro, H. and E. A. Clark (2002). "Regulation of B-cell fate by antigen-receptor signals." Nat Rev Immunol **2**(12): 945-56.

Ogilvy, S., D. Metcalf, et al. (1999). "Constitutive Bcl-2 expression throughout the hematopoietic compartment affects multiple lineages and enhances progenitor cell survival." Proc Natl Acad Sci U S A **96**(26): 14943-8.

Pierce, S. K. (2002). "Lipid rafts and B-cell activation." Nat Rev Immunol **2**(2): 96-105.

Rawlings, D. J., K. Sommer, et al. (2006). "The CARMA1 signalosome links the signalling machinery of adaptive and innate immunity in lymphocytes." Nat Rev Immunol **6**(11): 799-812.

Rolink, A., P. Ghia, et al. (1995). "In-vitro analyses of mechanisms of B-cell development." Semin Immunol **7**(3): 155-67.

Rothwarf, D. M., E. Zandi, et al. (1998). "IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex." Nature **395**(6699): 297-300.

Ruefli-Brasse, A. A., D. M. French, et al. (2003). "Regulation of NF-kappaB-dependent lymphocyte activation and development by paracaspase." Science **302**(5650): 1581-4.

Ruland, J., G. S. Duncan, et al. (2001). "Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure." Cell **104**(1): 33-42.

Ruland, J., G. S. Duncan, et al. (2003). "Differential requirement for Malt1 in T and B cell antigen receptor signaling." Immunity **19**(5): 749-58.

Ruland, J. and T. W. Mak (2003). "Transducing signals from antigen receptors to nuclear factor kappaB." Immunol Rev **193**: 93-100.

Sanchez-Izquierdo, D., G. Buchonnet, et al. (2003). "MALT1 is deregulated by both chromosomal translocation and amplification in B-cell non-Hodgkin lymphoma." Blood **101**(11): 4539-46.

Sanjabi, S., K. J. Williams, et al. (2005). "A c-Rel subdomain responsible for enhanced DNA-binding affinity and selective gene activation." Genes Dev **19**(18): 2138-51.

Sasaki, Y., E. Derudder, et al. (2006). "Canonical NF-kappaB activity, dispensable for B cell development, replaces BAFF-receptor signals and promotes B cell proliferation upon activation." Immunity **24**(6): 729-39.

Schiemann, B., J. L. Gommerman, et al. (2001). "An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway." Science **293**(5537): 2111-4.

Schram, B. R. and T. L. Rothstein (2003). "NF-kappa B is required for surface Ig-induced Fas resistance in B cells." J Immunol **170**(6): 3118-24.

Shambharkar, P. B., M. Blonska, et al. (2007). "Phosphorylation and ubiquitination of the IkappaB kinase complex by two distinct signaling pathways." Embo J **26**(7): 1794-805.

Shipp, M. A., K. N. Ross, et al. (2002). "Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning." Nat Med **8**(1): 68-74.

Siebenlist, U., K. Brown, et al. (2005). "Control of lymphocyte development by nuclear factor-kappaB." Nat Rev Immunol **5**(6): 435-45.

Simons, K. and D. Toomre (2000). "Lipid rafts and signal transduction." Nat Rev Mol Cell Biol **1**(1): 31-9.

Snipas, S. J., E. Wildfang, et al. (2004). "Characteristics of the caspase-like catalytic domain of human paracaspase." Biol Chem **385**(11): 1093-8.

Sohn, H. W., P. Tolar, et al. (2006). "Fluorescence resonance energy transfer in living cells reveals dynamic membrane changes in the initiation of B cell signaling." Proc Natl Acad Sci U S A **103**(21): 8143-8.

Solvason, N., W. W. Wu, et al. (1998). "Transgene expression of bcl-xL permits anti-immunoglobulin (Ig)-induced proliferation in xid B cells." J Exp Med **187**(7): 1081-91.

Solvason, N., W. W. Wu, et al. (2000). "Cyclin D2 is essential for BCR-mediated proliferation and CD5 B cell development." Int Immunol **12**(5): 631-8.

Sommer, K., B. Guo, et al. (2005). "Phosphorylation of the CARMA1 linker controls NF-kappaB activation." Immunity **23**(6): 561-74.

Streubel, B., A. Lamprecht, et al. (2003). "T(14;18)(q32;q21) involving IGH and MALT1 is a frequent chromosomal aberration in MALT lymphoma." Blood **101**(6): 2335-9.

Su, T. T., B. Guo, et al. (2002). "PKC-beta controls I kappa B kinase lipid raft recruitment and activation in response to BCR signaling." Nat Immunol **3**(8): 780-6.

Su, T. T., B. Guo, et al. (2004). "Signaling in transitional type 2 B cells is critical for peripheral B-cell development." Immunol Rev **197**: 161-78.

Sun, L., L. Deng, et al. (2004). "The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes." Mol Cell **14**(3): 289-301.

Sun, Z., C. W. Arendt, et al. (2000). "PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes." Nature **404**(6776): 402-7.

Thome, M. (2004). "CARMA1, BCL-10 and MALT1 in lymphocyte development and activation." Nat Rev Immunol **4**(5): 348-59.

Tolar, P., H. W. Sohn, et al. (2005). "The initiation of antigen-induced B cell antigen receptor signaling viewed in living cells by fluorescence resonance energy transfer." Nat Immunol **6**(11): 1168-76.

Turner, M., A. Gulbranson-Judge, et al. (1997). "Syk tyrosine kinase is required for the positive selection of immature B cells into the recirculating B cell pool." J Exp Med **186**(12): 2013-21.

Uren, A. G., K. O'Rourke, et al. (2000). "Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma." Mol Cell **6**(4): 961-7.

Vigorito, E., L. Gambardella, et al. (2005). "Vav proteins regulate peripheral B-cell survival." Blood **106**(7): 2391-8.

Wang, C., L. Deng, et al. (2001). "TAK1 is a ubiquitin-dependent kinase of MKK and IKK." Nature **412**(6844): 346-51.

Wang, L., Y. Guo, et al. (2001). "Card10 is a novel caspase recruitment domain/membrane-associated guanylate kinase family member that interacts with BCL10 and activates NF-kappa B." J Biol Chem **276**(24): 21405-9.

Wegener, E., A. Oeckinghaus, et al. (2006). "Essential role for IkappaB kinase beta in remodeling Carma1-Bcl10-Malt1 complexes upon T cell activation." Mol Cell **23**(1): 13-23.

Willis, T. G., D. M. Jadayel, et al. (1999). "Bcl10 is involved in t(1;14)(p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types." Cell **96**(1): 35-45.

Wu, C. J., D. B. Conze, et al. (2006). "Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]." Nat Cell Biol **8**(4): 398-406.

Xue, L., S. W. Morris, et al. (2003). "Defective development and function of Bcl10-deficient follicular, marginal zone and B1 B cells." Nat Immunol **4**(9): 857-65.

Yamaoka, S., G. Courtois, et al. (1998). "Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation." Cell **93**(7): 1231-40.

Zhang, Q., R. Siebert, et al. (1999). "Inactivating mutations and overexpression of BCL10, a caspase recruitment domain-containing gene, in MALT lymphoma with t(1;14)(p22;q32)." Nat Genet **22**(1): 63-8.

Zhang, W., R. P. Trible, et al. (1998). "LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation." Immunity **9**(2): 239-46.

Zhong, H., M. J. May, et al. (2002). "The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1." Mol Cell **9**(3): 625-36.

Zhou, H., I. Wertz, et al. (2004). "Bcl10 activates the NF-kappaB pathway through ubiquitination of NEMO." Nature **427**(6970): 167-71.

PUBLICATIONS

Oeckinghaus A., Wegener E., Welteke V., **Ferch U**., Arslan SC., Ruland J., Scheidereit C., Krappmann D. (2007). "Malt1 ubiquitination triggers NF-kappaB signaling upon T-cell activation." *EMBO J*. 14;26(22):4634-45.

Ferch U., Meyer zum Büschenfelde C., Gewies A., Wegner E., Rauser S., Peschel C., Krappmann D., Ruland J. (2007). "Malt1 directs B cell receptor induced canonical NF-κB signaling selectively to the c- Rel subunit." *Nat. Immunol.* 8(9):984-91.

Misra RS., Russel JQ., Koenig A., Hinshaw-Makepeace JA., Wen R., Wang D., Huo H., Littmann DR., **Ferch U**., Ruland J., Thome M., Budd RC. (2007). "Caspase-8 and c-FLIPL associate in lipid rafts with NF-kappa B adaptors during T cell activation." *J Biol Chem.* 6;282(27):19365-74.

Rueda D., Gaide O., Ho L., Lewkowicz E., Niedergang F., Hailfinger S., Rebeaud F., Guzzardi M., Conne B., Thelen M., Delon J., **Ferch U**., Mak TW., Ruland J., SchwallerJ., Thome M. (2007). "Bcl10 controls TCR- and FcγR-induced actin polymerization." *J Immunol.* 1;178(7):4373-84.

Jost PJ., Weiss S., **Ferch U**., Gross O., Mak TW., Peschel C., Ruland J. (2007). “Bcl10/Malt1 signaling is essential for TCR-induced NF-kappaB activation in thymocytes but dispensable for positive or negative selection.” *J Immunol.* 15;178(2):953-60.

Wegener E., Oeckinghaus A., Papadopoulou N., Lavitas L., Schmidt- Supprian M., **Ferch U**., Mak T.W., Ruland J.,Heissmeyer V., Krappmann D. (2006). “Essential role for IkappaB kinase beta in remodelling Carma1-Bcl10-Malt1 complexes upon T cell activation.” *Mol Cell* 7;23(1):13-23.

CURRICULUM VITAE

Name	Uta Ferch
Geburtsdatum	11.04.1979
Geburtsort	Bad Karlshafen
Familienstand	ledig

Promotion

10 / 03- heute An der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der TU München;
Arbeitsgruppe PD Dr. Jürgen Ruland, Abteilung Hämatologie/Onkologie, Klinikum rechts der Isar

Studium

08 / 02- 07 / 03 Diplomarbeit an der medizinischen Fakultät der Georg-August-Universität Göttingen;
Arbeitsgruppe Prof. Dr. Heidi Hahn, Abteilung Molekulare Entwicklungsgenetik, Institut für Humangenetik

- Titel: »Genexpressionsanalysen in *Patched*-assoziierten Tumoren«

06 / 02 Diplomhauptprüfung

- Hauptfach Biochemie
- Nebenfächer Humangenetik, Organische Chemie

10/ 98- 06/ 02 Studium der Biologie an der Georg-August-Universität Göttingen

Schule

08/ 89- 07/ 98 Oberstufengymnasium Hofgeismar, Abitur
08/ 89- 07/ 96 Gymnasialzweig Gesamtschule Hofgeismar
08/ 85- 07/ 89 Grundschule Trendelburg

DANKSAGUNG

Diese Arbeit wäre ohne vielfältige Mithilfe und Unterstützung undenkbar gewesen. Ich möchte daher an dieser Stelle all jenen danken, die zum Erstellen meiner Arbeit beigetragen haben, möchte jedoch einigen Menschen meinen besonderen Dank aussprechen.

Prof. Dr. Wolfgang Wurst danke ich für die Übernahme dieser Dissertation in die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt.

Herzlich möchte ich mich bei Prof. Dr. Christian Peschel bedanken. Das wissenschaftliche Umfeld in der III. Medizinischen Klinik hat maßgeblich zum Gelingen dieser Arbeit beigetragen.

Mein ganz besonderer Dank gilt PD. Dr. Jürgen Ruland für die umfassende wissenschaftliche Ausbildung und die Bereitstellung eines so viel versprechenden Projektes. Er hat mein Interesse und mein wissenschaftliches Arbeiten ganz wesentlich geprägt.

Besonders bedanken möchte ich mich bei Dr. Andreas Gewies. Sein Wissen und seine ständige Hilfsbereitschaft haben wesentlich zum Erfolg dieser Arbeit beigetragen.

Konstanze Pechloff danke ich für die kritische Durchsicht dieser Arbeit und wertvolle Korrekturvorschläge.

Ganz herzlich möchte ich mich bei allen weiteren Mitgliedern der Arbeitsgruppe Ruland, Kristina Brunner, Mercedes María Castiñeiras-Vilariño, Katrin Finger, Olaf Groß, Christina Grupp, Nicole Hannesschläger, Philipp Jost, Stefanie Klemm, Stephanie Leeder, Katja Meiners, Thomas Patzelt, Dominik Straßer, Stefan Wanninger, Stefanie Weiß und Stephanie Zimmermann, für die freundliche und produktive Arbeitsatmosphäre bedanken.

Den Arbeitsgruppen Dyster und Bernhard sowie den Mitarbeitern des Instituts für Pathologie danke ich für die freundliche und gute Zusammenarbeit.

Ganz besonders möchte ich mich bei Christian bedanken. Seine permanente Diskussionsbereitschaft und seine tagtägliche Unterstützung haben diese Arbeit erst möglich gemacht.

Zum Schluss möchte ich meiner Mutter und Reinhard ganz herzlich danken. Ihr Interesse, ihre Anteilnahme und ihr Verständnis für mich und meine Arbeit haben mir nicht nur in den letzten Jahren sehr geholfen. Daher möchte ich ihnen diese Arbeit widmen.