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B cell-intrinsic functions of the MALT1 paracaspase

A dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Peishan Lee

Committee in charge:

Professor Robert Rickert, Chair
Professor Jack Bui, Co-Chair
Professor John Chang
Professor Michael David
Professor Guy Salvesen
Professor Dwayne Stupack

2016

The Dissertation of Peishan Lee is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2016

Dedication

This thesis is dedicated to my parents and siblings, whose love and support sustain me over the years and drive me to better myself.

"Basic research is what I'm doing
when I don't know what I'm doing."
– Wernher von Braun

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VITA

Education

2009-2016 **Doctor of Philosophy**, University of California, San Diego
Biomedical Sciences

2005-2007 **Master of Science**, National Taiwan University
Biochemical Sciences

2001-2005 **Bachelor of Science**, California Institute of Technology
Biology

Research experience

2008-2009 **Research technician**, Ludwig Institute for Cancer Research
Laboratory of Dr. Yao-Tseng Chen

2007-2008 **Research assistant**, Academia Sinica
Laboratory of Dr. Alice Lin-Tsing Yu

2004 **Summer intern**, Asahi Glass Company

2003 **Summer intern**, Jet Propulsion Laboratory

Teaching experience

Winter 2011 **Teaching assistant**, Laboratory in microbiology (BIMM21)
University of California, San Diego

Academic honors

2012 National Research Service Award F31 Predoctoral fellowship recipient

2011 Gastrointestinal Predoctoral Training Grant recipient
University of California, San Diego

Selected publications

Lee P, Zhu Z, Hachmann J, Nojima T, Kitamura D, Salvesen GS, Rickert RC. Differing requirements for MALT1 function in the survival and differentiation of peripheral B cell subsets. (Manuscript in Preparation)

Chen YT, Chadburn A, **Lee P**, Hsu M, Ritter E, Chiu A, Gnjatic S, Pfreundschuh M, Knowles DM, Old LJ. Expression of cancer testis antigen CT45 in classical Hodgkin lymphoma and other B-cell lymphomas. *Proc Natl Acad Sci U S A.* 107(7):3093-8 (2010)

Chang WW, Lee CH, **Lee P**, Lin J, Hsu CW, Hung JT, Lin JJ, Yu JC, Shao LE, Yu J, Wong CH, Yu AL. Expression of Globo H and SSEA3 in breast cancer stem cells and the involvement of fucosyltransferases 1 and 2 in Globo H synthesis. *Proc Natl Acad Sci U S A* 105(33):11667-72 (2008)

ABSTRACT OF THE DISSERTATION

B cell-intrinsic functions of the MALT1 paracaspase

by

Peishan Lee

Doctor of Philosophy in Biomedical Sciences

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Professor Robert Rickert, Chair

Professor Jack Bui, Co-Chair

During a T cell-dependent immune response, formation of the germinal center (GC) is essential for the generation of high-affinity plasma cells and memory B cells. The canonical NF-κB pathway has been implicated in the initiation of GC reactions and defects in this pathway have been linked to immune deficiencies. The paracaspase MALT1 plays an important role in regulating NF-κB activation upon triggering of antigen receptors. Although previous studies have reported that MALT1 deficiency

abrogates the GC response, the relative contribution of B cells and T cells to the defective phenotype remains unclear.

To this end, I utilized chimeric mouse models to demonstrate that there is a B cell-intrinsic requirement for MALT1 in the initiation of GC response. In Chapter 2, I show that MALT1 is critical for proliferation and survival of B cells in response to signaling specifically through the B cell receptor, which cannot be overcome by providing T cell help. In addition to the GC defect, MALT1-deficient B cells were impaired in differentiation to plasma cells, although proliferation to mitogens was unaffected. In Chapter 3, I show that in the presence of normal T cells, ectopic expression of Bcl-2 in MALT1-deficient B cells can partially rescue the GC phenotype by prolonging the lifespan of activated B cells, but plasma cell differentiation and antibody production remain defective. Chapter 4 presents work on the regulation of MALT1 proteolytic activity in B cells, including the application of the activity-based probe to monitor MALT1 activity *in situ* and exploration of mechanisms leading to induction of MALT1-mediated proteolysis. Collectively, the findings described in this dissertation uncover a previously underappreciated role of MALT1 in B cells and highlight its importance in humoral immunity.

Chapter 1. Introduction

The immune system protects the host organism from pathogens or toxins. In vertebrates, the immune system is divided into two branches: innate and adaptive. Innate immunity provides the body with rapid, nonspecific defense mechanisms against foreign objects, but does not lead to lasting immunity. In contrast, the adaptive immune system takes longer to develop, but is more effective at eliminating infections because of the specific recognition functions of lymphocytes. This is achieved by the highly variable antigen receptors on the surface, through which the lymphocytes recognize and bind antigens. During the course of the adaptive immune response, a subset of activated lymphocytes also differentiates into memory cells, which will readily differentiate into effector cells upon repeated exposure to the same antigen.

Adaptive immunity

Adaptive immunity can be further divided into the humoral and cell-mediated branches, which are carried out by different classes of lymphocytes called B cells and T cells, respectively. B cells and T cells express distinct types of antigen receptors and play different roles. B cells develop in the bone marrow and produce immunoglobulins (Igs) as both cell-surface antigen receptors and secreted antibodies. Free antigens can stimulate the antigen receptors of B cells, but most B cells require help from activated helper T cells for optimal antibody responses. Activated B cells secrete antibodies that circulate in the bloodstream and bind specifically to the foreign antigen that stimulated their production. Binding of antibody inactivates toxins and also marks invading

pathogens for destruction by phagocytic cells of the innate immune system. Unlike B cells, T cells mature in the thymus and recognize antigens in the form of processed peptides presented by major hisocompatibility (MHC) protein on infected cells or antigen-presenting cells (APCs). Depending on the T cell subsets, activated T cells can directly kill the infected host cells, activate other immune cell types through secretion of cytokines or display of co-stimulatory molecules, or dampen activation of other T cells.

Adaptive immunity is initiated when innate immunity fails to eliminate a new infection and activated APCs bearing pathogen antigens are delivered to the draining lymphoid tissues. Cells of the innate immunity express different pattern recognition receptors that recognize conserved features of pathogens and become quickly activated to help destroy the invaders. In contrast, clonal selection of lymphocytes is the central principle of adaptive immunity (1). Each lymphocyte differs from others in the specificity of its antigen receptor. Only those lymphocytes that encounter antigens to which their receptor binds will be activated to proliferate and differentiate into effector cells to secrete antibodies of the same specificity, therefore eliminating the infectious agent.

Overview of B cell development

The generation of B cells proceeds through various distinct stages (Figure 1-1). Like all blood cells, B cells derive from the hematopoietic stem cells (HSCs) that originate from the bone marrow. The specialized microenvironment of the bone marrow provides signals for the development of lymphocyte progenitors, as well as for

the subsequent differentiation of B cells. Stromal cells in the bone marrow form specific adhesive contacts with the developing B cells and provide soluble and membrane-bound cytokines and chemokines that control B cell proliferation and differentiation. Multipotent progenitor cells express the receptor kinase FLT3. Binding of FLT3 to its ligand on the stromal cells is required for differentiation to the common lymphoid progenitors (2). Binding of progenitor cells to stromal cells through integrins and other cell adhesion molecules promote the proliferation of B cell progenitors. The cytokine interleukin (IL)-7, secreted by the stromal cells, is also essential for the proliferation and survival of B cell progenitors and differentiation to the B lineage (3, 4). The common lymphoid progenitors give rise to pro-B cells, which are the earliest committed B-lineage cells.

During B cell development, diversity of the antibody repertoire is conferred by recombination-activating genes (RAG)-mediated V(D)J rearrangement and the program initiates at the pro-B stage (5). A successful rearrangement leads to the expression of the μ chain, which associates with the surrogate light chain composed of V-preB and λ5 to form the pre-B receptor on the surface (6). Signaling through the pre-BCR plus Igα and Igβ complex induces proliferation of large pre-B cells and activates the recombination of the Ig light chain (IgL) (7). As the large pre-B exits cell cycle and becomes small resting pre-B cells, RAG proteins are produced again and recombination of the IgL begins. The mechanism of allelic exclusion and isotype exlucion ensures that each B cell expresses a single species of antibodies with a unique specificity (8). Synthesis of the light chain results in assembly of the B cell receptor (BCR) that is expressed on the cell surface as a membrane-anchored IgM

molecule. The cell at this stage is defined as immature B cell. The BCR is tested for reactivity against autoantigens at the immature B cell stage and cells harboring receptors that are strongly reactive to self antigens are not allowed to mature.

Central tolerance is produced to ensure that the B cell population will be tolerant of self antigens. Immature B cells that undergo multivalent cross-linking of their surface IgM may undergo clonal deletion and die by apoptosis (9), or receptor editing to replace their antigen receptor with a new one that is not self-reactive (10). Induction of a permanent state of unresponsiveness, or anergy, occurs in immature B cells that encounter more weakly cross-linking self antigens of low valence (11). Immature B cells that pass the selection for auto-reactivity then leave the BM and migrate to the spleen, as they differentiate further into transitional and mature B cells (12). Transitional T1 B cells have yet to acquire the ability to recirculate and they are found in the BM and spleen. Transitional T2 B cells that have entered the follicles acquired surface IgD and CD23, and the ability to recirculate, but they still show markers of immaturity (13). Mature B cell is marked by the additional appearance of IgD on the cell surface made from alternatively spliced H-chain transcripts

Most of the immature B cells arriving in the spleen turn over rapidly due to competition among peripheral B cells to access the limited number of lymphoid follicles. The follicle provides signals necessary for B cell survival, the most important of which is the B cell activating factor (BAFF) that binds to the BAFF receptor (BAFF-R) on B cells (14). In the spleen, transitional B cells develop into either follicular (FO) or marginal zone (MZ) B cells and the cell fate decision depends on signaling through BCR, Notch2, BAFF-R, and the canonical nuclear factor-κB (NF-

κB) pathway, as well as signals for the retention of B cells in the MZ (15). FO B cells ($IgD^{hi}IgM^{lo}CD23^{hi}CD21^{lo}$) account for the majority of mature B cells; they recirculate and home mainly to B cell follicles in secondary lymphoid organs. MZ B cells ($IgD^{hi}IgM^{lo}CD23^{lo}CD21^{hi}CD1d^{hi}$) are sessile and reside in the marginal sinus of the white pulp in the spleen. MZ B cells are thought to function as innate-like lymphocytes that mount rapid defense against blood-borne antigens (16). Another subset of mature B cells that do not reside in B-cell follicles is the B-1 cells, which comprise about 5% of total B cell in mice and humans, and are found primarily in the pleural and peritoneal cavities. B-1 cells differ from conventional B cells (also known as B-2 cells) by their surface phenotype ($CD19^{hi}CD23^{-}CD43^{+}IgM^{hi}IgD^{variable}CD5^{\pm}$). Contrary to the continuous de novo development of B-2 cells, B-1 cells are produced mainly during fetal and neonatal life and B-1 cell pools are maintained mainly by self-renewal under steady state conditions. B-1 cells produce natural antibodies that are essential to defend against microbial infection and they also participate in tissue homeostasis (17).

Humoral immune response

During B cell development, the primary antibody repertoire is produced by a process involving proliferation accompanied by stringent selection. A second selection process occurs during the humoral immune response, generating a new antibody repertoire that is of higher affinity for the stimulating antigen than that of the antigen-specific B cells initiating the immune response (18). While B cells are capable of recognizing and responding to both soluble antigens and antigens on the surface of

APCs, the predominant form of antigens that mediates B cell activation in vivo is bound to a membrane surface (19). Activation of conventional B cells requires the transport of antigens by APCs into the B cell follicles of secondary lymphoid organs. Naïve B cell in the follicle encounters its specific antigen displayed on subcapsular sinus-lining macrophages or follicular dendritic cells (FDCs) (20). Upon binding of BCR to corresponding epitopes on the surface of antigens, a signal is first delivered to the interior of the B cell, followed by internalization of the bound BCR to deliver the antigen to intracellular sites, where it can be processed into peptides, returned to the surface and presented in the context of MHC class II molecules to T cells (21). Antigens that require help form T cells to induce antibody response are referred to as T-dependent (TD) antigens (Figure 1-2). The antigen-stimulated B cell have increased expression of chemokine receptor CCR7, which mediates its migration toward the boundaries between B cell the T cell areas within the secondary lymphoid tissues (22). When the peptide:MHC class II complexes are recognized by antigen-specific helper T cells, the T cells provide additional signals to activate the B cells. Most importantly, engagement of CD40 ligand (CD40L) on T cells with the costimulatory receptor CD40 on B cells helps to drive B cells into cell cycle, induces differentiation into Ig-secreting cells, as well as enhancing the APC capacity of B cells (23). Interaction between CD40-CD40L during T and B cell contact is essential for all events in TD immune response such as Ig production, isotype switching, somatic hypermutation, and induction of B cell memory (24). A given B cell can only be activated by helper T cells that respond to the same antigen. Antigen-stimulated B cells that fail to interact with cognate T cells die within a few hours (25, 26). Interactions between B and T

cells also play a role in the priming and clonal expansion of antigen-specific T cells (27). T cell also secretes cytokines, mostly notably interleukin-4 (IL-4), that regulate B cell proliferation and antibody production. Some microbial antigens can activate B cells directly in the absence of T cell help, by providing a second signal via recognition of a common microbial constituent or by extensive cross-linking of BCR (Figure 1-2). However, the antibodies induced by these T-independent (TI) antigens tend to have lower affinity and be less functionally versatile than those generated from a TD immune response.

Germinal center

In the spleen, antigen-stimulated B cells that have received T cell help migrate from follicle border to the border between the T cell area and the red pulp, where they establish a primary focus of clonal expansion. In the primary focus, some of the proliferating B cells differentiate into plasmablasts, which have begun to secrete antibodies but are still dividing and express co-stimulatory and MHC class II molecules, allowing them to interact with T cells. However, the antibodies produced by plasmablasts are mostly of the IgM isotype and are of low affinity. In order to establish a more effective antibody response and long-term memory, B cells have to undergo extensive rounds of proliferation, somatic hypermutation, and affinity maturation in the germinal center (GC). Guided by the orphan G-protein-coupled-receptors, EBI2, activated B cells move back to the lymphoid follicle (28, 29), where they continue to proliferate and form the nascent GC (30). Shortly after formation, GC resolves into distinguishable areas called light zone and dark zone. Densely packed,

proliferating cells in the dark zone are called centroblasts and the less denstly packed light zone contains the centrocytes. During proliferation in the GC, secondary diversification of Ig genes occurs via activation-induced cytidine deaminase (AID)-mediated somatic hypermutation, which introduces single nucleotide exchanges into the variable region genes of the BCR (31, 32). This process generates a large pool of closely related B cells expressing antibodiess of various affinity and specificity, from a single antigen-activated B cells. When the centroblast progresses into centrocyte, it increases the expression of surface BCR (33, 34) and moves to the light zone, where its ability to bind antigen on the FDCs is tested. Being able to bind antigen will also allow the centrocyte to take up, process, and present antigen to Tfh cells, which will then provides survival signals to the centrocyte via ICOS:ICOSL and CD40L:CD40 interactions. B cells harboring mutations that have a negative impact on the ability of BCR to bind to the original antigen were eliminated by apoptosis (35, 36). Competition for T cell help also governs the selection of GC B cells (37). The few variants with high-affinity receptors are efficiently selected and expanded, and some eventually downregulate the transcription factors paired box protein 5 (Pax5) and B cell lymphoma 6 (Bcl-6) and upregulates B lymphocyte-induced maturation protein 1 (Blimp-1), differentiating into plasma cells that produce large amount of antibodies (38). A portion of GC-derived plasma cells migrate to the bone marrow where they become long-lived. Other GC B cells differentiate into memory B cells, which exhibit a lower threshold for antigenic stimulation and enter cell cycle more rapidly (39, 40).

The primary antibody repertoire is composed of low-affinity IgM and IgD antibodies. During the GC reactions, B cells undergo Ig class-switching from IgM to

IgG, IgE, or IgA, a process that requires the induction of AID (31, 32). As a result, high affinity IgG, IgD, and IgE antibodies that constitute the secondary antibody repertoire are produced. Antibody isotype is determined by the constant region of the heavy chain and is important for determining the antibody's effector function (41). While naïve B cells have the potential to switch to any isotype, cytokines secreted by T cells direct class switch recombination to distinct classes.

BCR signaling

Signals generated via the surface BCR play a pivotal role in life and death decisions of B cells by activating multiple downstream pathways (Figure 1-3). Upon encounter with antigen, mature B cells undergo activation and proliferation, leading to immune response against harmful pathogens. In contrast, during development of immature B cells and GC reaction, BCR crosslinking in the absence of survival signals, such as CD40 or IL-4, induces anergy or apoptosis (42). Mature B cells also depend upon the expression of a functional BCR for their persistence, as the ablation of BCR results in cell death (43).

The BCR is a complex consisting of a membrane bound IgM non-covalently associated with an Igα/Igβ heterodimer, whose cytoplasmic tails contain immunoreceptor tyrosine activation motifs (ITAMs). Crosslinking of BCR by antigen initially leads to activation of both the protein tyrosine kinase (PTK) of the Src, Syk, and Tec-families. Src-family PTKs, essentially Lyn, Blk, and Fyn, phosphorylate the tyrosine residues in the ITAMs of Igα and Igβ, creating docking sites for proteins with Src homology-2 (SH2) domains (44). Phosphorylated ITAMs recruit and activate Syk

by the SH2 domain, resulting in allosteric activation of Syk and engaging a positive feedback loop that further amplifies BCR-induced signal transduction (45). Syk phosphorylates the adaptor molecule known as B cell linker (BLNK) and the modification allows BLNK to bind and recruit phospholipase Cγ (PLC-γ) and the Tec-family PTK Bruton's tyrosine kinase (Btk) to Syk (46). Syk and Btk then phosphorylates PLC-γ, thereby activates it to cleave phosphatidylinositol 4,5-bisphosphate (PIP2) to secondary messengers inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), provoking the release of intracellular Ca2+ and the activation of protein kinase C (PKC) (47, 48). PKCβ is essential for the downstream activation of the nuclear factor κB (NF-κB) transcription complex, which will be described in details below.

The mitogen-activated protein kinase (MAPK) pathway is also activated after BCR ligation, leading to activation of MAPKs including extracellular signal-regulated kinases (ERK), p38 MAPK and c-Jun NH2-terminal kinase (JNK), and subsequent induction of gene transcription (49). Activation of ERK requires membrane localization of a guanine nucleotide exchange factor (GEF) such as RasGRP3, which activates the small guanosine triphosphatase (GTPase) Ras to trigger the Raf1/MEK/ERK pathway (50). JNK and p38 activation is controlled by Vav-mediated activation of GTPases Rac-1 and is dependent on PLC-γ signals (51). Vav acts as a guanine nucleotide exchange factor for small GTPases and becomes phosphorylated shortly after BCR engagement (52). BLNK and the adaptor protein Grb2 cooperate to localize Vav into the membrane rafts, which is necessary for the optimal activation Rac1, which subsequently mediates activates MAPK kinase (MEKK) upstream of

both JNK and p38 (53). Following activation of the MAPK pathway, different sets of transcription factors are phosphorylated: Elk-1 and c-Myc by ERK, c-Jun and ATF-2 by JNK, and ATF-2, MAX by p38 (54). Elevated cytosolic Ca2+ level also triggers the activation of the calcineurin phosphatase, which in turn dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT) to allow its nuclear translocation and induction of specific gene transcription (55).

Another important BCR signal cascade is the phosphatidylinositol-3-kinase (PI3K) pathway, which requires the phosphorylation of CD19 and the adaptor molecule termed B cell adaptor molecule for PI3K (BCAP). CD19 is a transmembrane protein expressed throughout B cell development and exists as a component of the CD21/CD19/CD81 B cell coreceptor complex that positively modulates BCR signal transduction (56). Phosphorylated CD19 and BCAP mediate the recruitment and subsequent activation of PI3K to the plasma membrane, where it converts PIP2 to phosphatidylinositol-(3,4,5)-triphosphate (PIP3) (57). There is evidence that Vav also contributes to the PI3K signaling pathway, presumably via activation of Rac1, which binds to PI3K and potentiates its catalytic activity (58). PIP3 provides binding sites for effectots that contain a pleckstrin homology (PH) domain, including serine/threonine kinase (Akt), 3-phosphoinositide-dependent protein kinase (PDK1), and Btk (59). On one hand, PIP3 production promotes formation of a signalosome containing Btk, BLNK, and PLCγ, leading to hydrolysis of PIP2 into DAG and IP3 and therefore enhancing a variety of signaling pathways (60). A second outcome is the recruitment of Akt and PDK1. PI3K exerts its main physiological function via Akt, to modulate downstream transcription factors that promote proliferation and survival in B cells.

Following activation of the PI3K pathway, Akt is recruited to the plasma membrane and becomes fully activated through dual phosphorylation of threonine 308 by PDK1 (61) and of serine 473 by the mammalian target of rapamycin 2 (mTORC) complex (62). Fully activated Akt leads to phosphorylation of additional substrates, most notably the Forkhead Box Subgroup (Foxo) transcription factors, which undergo nuclear export, leading to repression of transcription of target genes that are involved in cell cycle arrest, quiescence, and apoptosis (63). Akt also promotes mTORC1 activation, triggering biosynthetic pathways essential for cell growth and proliferation via mTORC1-mediated phosphorylation of substrates including the ribosomal S6 kinase (S6K) and the eIF4E binding proteins (4EBP) (64).

NF-κB activation in B cells

NF-κB is a family of transcriptional factors, composed of p50, p52, RelA (or p65), c-Rel, and RelB, that control cell activation, proliferation, and survival. All five subunits share a conserved N-terminal Rel homology domain that encompasses sequences essential for DNA binding, dimerization, and nuclear import. However, only RelA, c-Rel, and RelB contain the c-terminal transcription activation domains, which enable them to bind as dimers to κB sites in the promoters and enhance regions to regulate target gene transcription (65). While NF-κB subunits may form virtually any homo- or heterodimer, the common complexes present in B cells constitute heterodimers of p50/RelA and p50/Rel subunits (66). In quiescent B cells, NF-κB dimer is sequestered in the cytoplasm through physical interaction via the Rel homology domain with inhibitory proteins known as inhibitor of the κB (IκB) (67).

The NF-κB pathway can be activated by a multitude of stimuli that converge on phosphorylation and subsequent degradation of IκB, a process that is mediated by the IκB kinase (IKK) complex (Figure 1-4). The IKK complex is consisted of 2 catalytically active kinases IKKα and IKKβ and a regulatory scaffold protein IKKγ (or NEMO). While IKKβ and IKKγ are responsible for the activation of the canonical NF-κB pathway involving RelA and c-Rel, IKKα alone controls the non-canonical NF-κB pathway leading to RelB activation.

Upon BCR engagement, PKCβ phosphorylates serine 668 in the linker region of the adaptor protein caspase-recruitment domain membrane-associated guanylate kinase protein 1 (CARMA1), resulting in conformation change of CARMA1 and allows for recruitment of B-cell lymphoma 10 (BCL10) and mucosal associated lymphoid tissue 1 (MALT1) (68, 69). BCL10 is constitutively associated with MALT1 and recent evidence suggests that CARMA1 induces oligomerization of BCL10 into helical filamentous structures to trigger signal downstream events in the NF-κB pathway (70). The exact mechanism by which the CARMA-1-BCL10-MALT1 (CBM) complex induces IKK activity has not been fully elucidated. One plausible scheme is that MALT1 recruits TNF receptor-associated factor (TRAF) 6-contianing ubiquitin ligase complexes and results in K63-linked ubiquitnation of TRAF6 and IKK-γ, the catalytically inactive subunit of the IKK complex (71). The macromolecule complex then recruits TAK1 through TAB2/3, leading to autophosphorylation of TAK1 and subsequent recruitment of IKK complex (72). Phosphorylation of IKK-β by TAK1 results in activation of IKK, which then phosphorylates IκB, leading to its ubiquitination and degradataion. As a consequence, the bound NF-κB dimers can be

released from IκB and are free to translocate to the nucleus and activates gene expression.

The NF-κB family can also be activated via an alternative, non-canonical pathway involving NF-κB2 (or p100), which is activated by a subset of receptor and ligand pairs belonging to the tumor necrosis factor (TNF) family (Figure 1-4). Among these is B cell activating factor of the TNF family (BAFF) produced by macrophages, monocytes, and dendritic cells, and BAFF receptor (BAFFR) whose expression is restricted to B cells (14). In this pathway, NF-κB2 acts as IκB and sequesters iself and RelB in the cytoplasm. Non-canonical NF-κB signaling is controlled by TRAF3, which associates with the protein kinase NF-κB-inducing kinase (NIK) under resting conditions and mediates its ubiquitination and proteasomal degradation. Stimulation leads to degradation of TRAF3, thereby resulting in stabilization of NIK (73). NIK acts with IKKα to induce phosphorylation and limited proteolysis of NF-κB2 to p52 and facilitates translocation of p52/RelB dimers to the nucleus (74, 75). BAFF-induced NF-κB2 signaling is crucial to survival and maturation of peripheral B cells, generation of antibody response, and the maintenance of plasma cells (76). Ablation of BAFF dramatically reduces the number of peripheral B cells, which do not progress beyond the transitional stage T1 (77, 78). Simultaneous stimulation of BCR and BAFF has syngergistic effect on B cell proliferation and maintenance (79). The mechanisms that account for this could be the sequential induction of one pathway by the other in a positive feedback loop. Alternatively, the two pathways could be directly linked to one another with one providing essential signaling intermediates for the other (80).

In response to TD antigens, B cell requires co-stimulatory signaling from the T helper cells expressing CD40L and cytokines like IL4. The engagement of CD40 by CD40L also leads to activation of both the canonical and non-canonical NF-κB pathway by recruitment of the TRAF proteins, namely TRAF2, TRAF3, and TRAF6 (81-84). CD40 has the ability to directly activate the canonical NF-κB1 through TRAF2 and TRAF6, which leads to enhanced production of p100 (85). In addition, CD40 regulates the posttranslational processing of p100 by disrupting the TRAF2/TRAF3/cIAP complex, resulting in accumulation of NIK and downstream activation of IKKα (73, 86).

Unlike TD antigens, TI antigens have the intrinsic ability to activate B cell response in the absence of T cell help. In such cases, B cell response is more dependent on BCR or Toll-like receptor (TLR)-induced signaling. B cells express TLRs that can detect microbial pathogens and play a direct role in B cell activation and optimal antibody responses to TD antigens (87). Ligand binding to TLRs also lead to activation of canonical IKK complexes, degradation of IκB, and liberation of RelA- and c-Rel-containing NF-κB dimers. TLR-induced NF-κB signaling can be achieved via either MyD88-dependent or TRIF-dependent pathways, both converge on IKK activation through TRAF proteins (88).

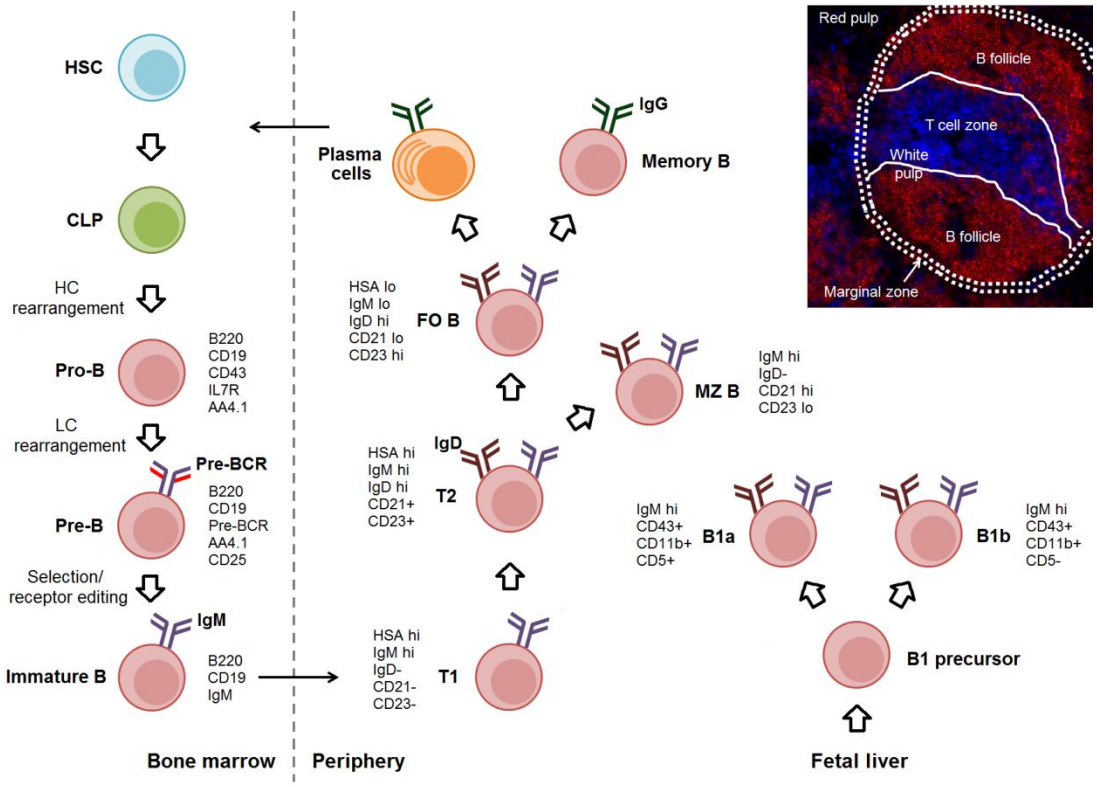


Figure 1-1. Summary of B cell development. Conventional B-2 cells originate from HSCs in the bone marrow and progress through pro-B, pre-B, and immature B cells stages. Cells successfully passing the selection checkpoints leave the bone marrow, proceeds through two transitional stages before maturing into FO or MZ B cells and colonize the follicles or MZ of the spleen white pulp, respectively. Following an immune response, antigen-specific-FO B cells further differentiate into antibody-secreting plasma cells or memory B cells. The B-1 lineage develops primarily from fetal liver-derived precursors. B-1 B cells predominate the peritoneal and pleural cavities. Similar to MZ B cells, B-1 B cells respond rapidly to TI antigens and produce low-affinity antibodies with broad reactivity.

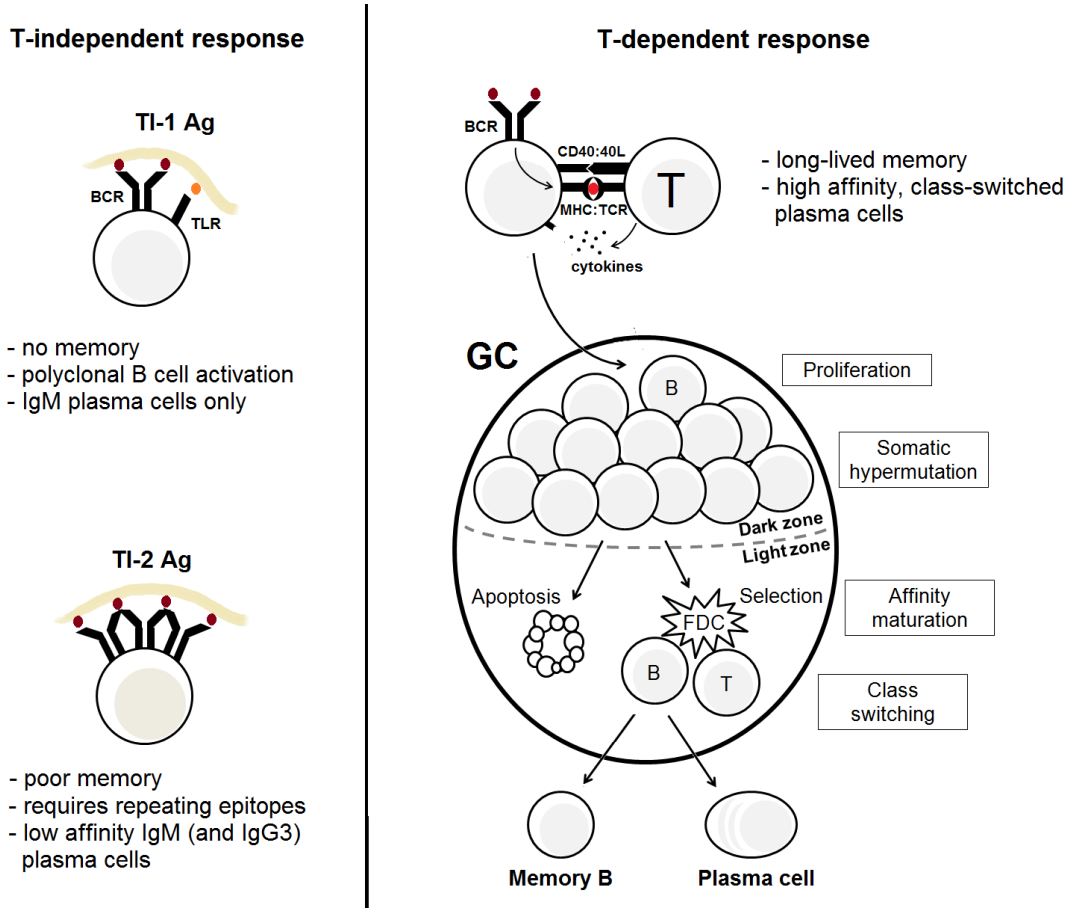


Figure 1-2. B cell activation by TI or TD antigens. Activation of B cells requires accessory signals in addition to the first signal delivered through the BCR. In TI-1 responses, B cells receive the second signals through receptors of the innate immune system, such as TLRs, and become activated regardless of their specificity. In contrast, TI-2 antigens display highly repetitious epitopes, thereby leading to extensive crosslinking of the BCRs and triggering B cell activation directly. Response to TD antigen requires contact- and cytokine-mediated interactions between antigen-stimulated specific B and T cells. GC reaction is induced as a result and in which, clonally expanded B cells undergo somatic hypermutation, affinity maturation, and class switching, and the activated B cells eventually differentiate into effector plasma cells or memory B cells.

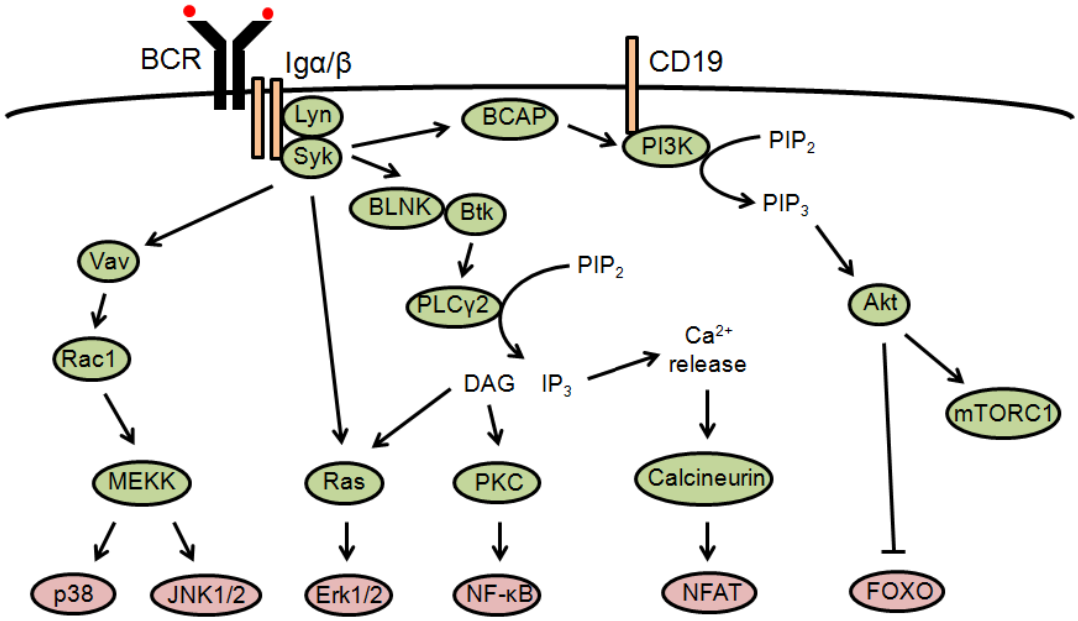


Figure 1-3. Simplified scheme of BCR signaling pathways. Upon engagement of BCR by antigens, signals are initiated by the SRC family kinases, propagated by various signaling intermediates and secondary messengers, leading to activation of transcription factors. The complexity of BCR signaling permits several outcomes, including survival, proliferation, and differentiation, as determined by the maturation state of the cell, the nature of the antigen, as well as the magnitude and duration of the signal.

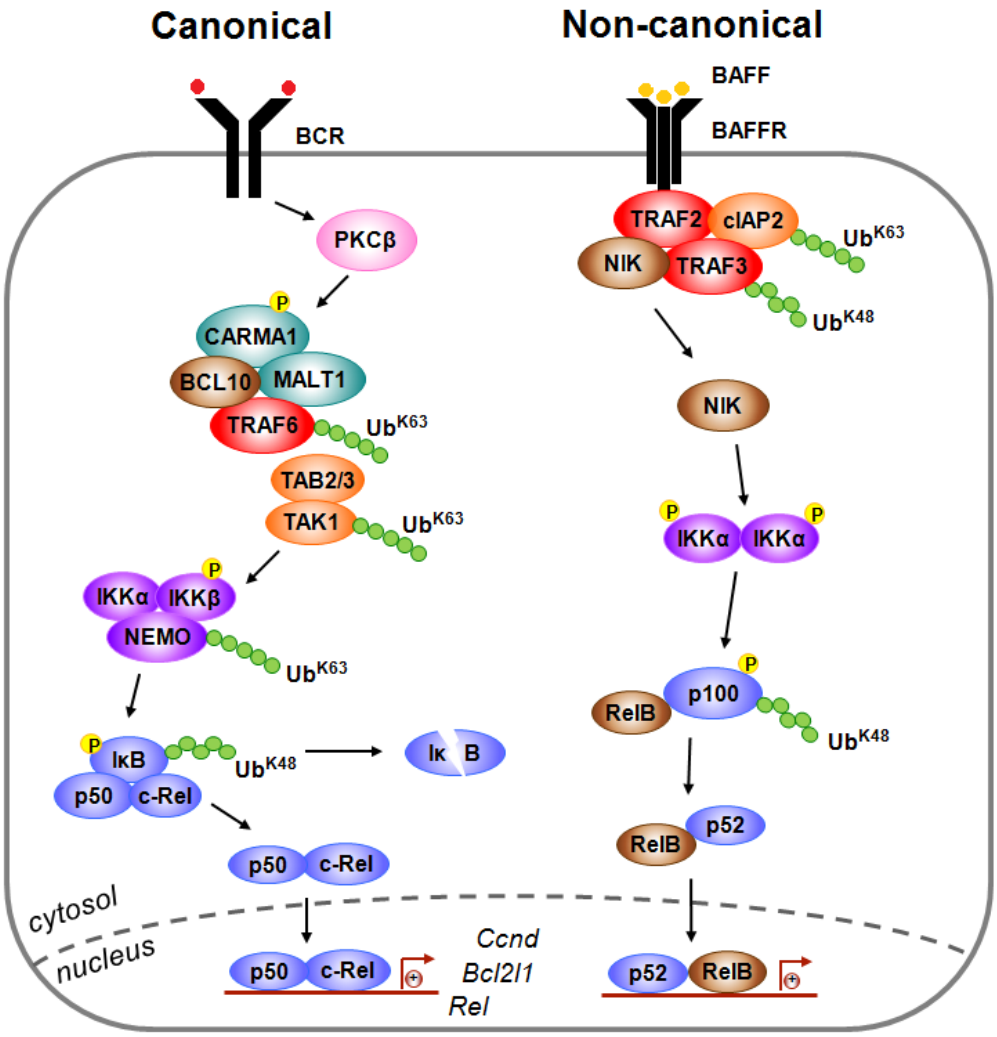


Figure 1-4. NF-κB signaling pathways in B cells. Stimulation through the BCR leads to activation of PKCβ, which in turn leads to activation of the canonical NF-κB1 pathway through CBM complex. By contrast, BAFF preferentially activates the non-canonical NF-κB2 pathway via NIK accumulation.

Chapter 2. B cell-intrinsic requirement of MALT1 in germinal center response

Introduction

Activation of NF-κB has emerged as one of the most crucial steps in mounting an effective immune response, regulating a wide panel of genes essential for immune cell survival and function. The canonical NF-κB pathway becomes transiently activated after BCR engagement via assembly of the signaling platform composed of scaffold proteins CARMA-1 and BCL-10, and the paracaspase MALT1 (termed CBM complex), which relays signals from the BCR proximal kinases and adaptors to the core IKK complex. Gene-targeting studies have revealed that mice deficient in any of the components of CBM complex show defective B cell and T cell activation and impaired adaptive immune response (89-92). Particularly, *Malt1*$^{-/-}$ mice exhibit a reduction in MZ and B1 B cell compartment, decreased serum IgM and IgG3 levels, as well as impaired antibody responses to both TD and TI antigens (91, 92). A subsequent study unraveled a new role for MALT1 in mediating BAFF-induced non-canonical NF-κB signaling specifically in MZ B cells, where MALT1 deficiency resulted in diminished BAFF-induced p100 processing and survival (93). While the detailed mechanism has not been completely elucidated, the defect in MALT1-deficient B cells has been further pinpointed to the selective nuclear translocation of c-Rel upon triggering of BCR (94). In addition to its scaffold role in promoting NF-κB activation, MALT1 also possesses a functional caspase-like domain that shows

activity toward a number of substrates and it is thought that MALT1 fine-tunes antigen receptor-induced NF-κB signaling through proteolysis of its substrates (95).

GCs are specialized microenvironments within the secondary lymphoid tissues that are formed at the height of an ongoing immune response and are critical for the optimization of TD humoral immune responses. As described in Chapter 1, antigen-activated B cells entering GC proliferate rapidly and undergo somatic hypermutation and affinity maturation, ultimately leading to generation of memory B cells and plasma cells producing high-affinity antibodies. This process requires intricate interactions among antigen-specific B and T cells, and FDCs (96). The initiation of GC reactions is also dependent on a multitude of B cell-intrinsic factors modulating the BCR signal and notably, deletion or mutation of NF-κB family members often abrogates GC formation (97). For example, c-Rel-deficient mice show severe impairment in GC formation upon immunization, due to its critical role in regulating B cell survival and cell cycle progression (98, 99). Immunofluorescent staining of the spleens in *Malt1*$^{-/-}$ mice immunized with TD antigen also revealed the complete absence of GC formation (91). Furthermore, MALT1-deficient animals are devoid of spontaneously formed GC B cells in the Peyer's patches (100), which are chronically exposed to antigens because of their anatomical location. The lack of GC B cells also correlate with a severe reduction in Tfh cells (100), since it has been shown that GC B cells are required to sustain the Tfh phenotype (101).

For the initiation and progression of GC response, T cell help is crucial. T cells can either participate through the engagement of CD40 on B cells or via signals delivered through costimulatory molecules and cytokines (102, 103). It has been

consistently reported that *Malt1*$^{-/-}$ T cells exhibit impairment in activation, proliferation, and IL-2 production in response to T cell receptor (TCR) ligation, as well as in NF-κB activation induced by TCR (91, 92, 104). However, it remains unclear whether the inability of the *Malt1*$^{-/-}$ animals to mount GC reactions can be entirely attributed to the defective T cell response. Moreover, the role of MALT1 in B cell activation remains controversial, as the first two papers describing MALT1-defiicient animals presented conflicting results and could not agree on the requirement of MALT1 in BCR-stimulated proliferative response and NF-κB activation in B cells (91, 92). Despite of the established link to c-Rel translocation, *Malt1*$^{-/-}$ animals do not fully phenocopy mice with c-Rel deletion, suggesting that MALT1 may regulate B cell functions via an unknown mechanism.

Loss-of-function mutations in MALT1 have also been discovered in human patients with combined immunodeficiency disorders, resulting in abnormal T cell proliferation and failure to activate NF-κB following treatment with the direct PKC activator 12-myristate 13-acetate (PMA) (105, 106). Yet, the contribution of B cells to the disease manifestation in these patients is still unclear. On the other hand, deregulated expression of MALT1 has been implicated in B cell lymphomagenesis, and a recent study demonstrated the oncogenic potential of MALT1 in driving B cell lymphopoiesis (107). Consequently, understanding the role of MALT1 in B cells is crucial, from a clinical perspective, for the management of diseases including immunodeficiency and lymphoma.

In this chapter, I provide the first evidence that MALT1 is required in B cells for GC reactions. MALT1 is critical for efficient proliferation and survival in response

to signaling specifically through the BCR. In addition, T-cell derived help fails to rescue the proliferative and survival defects in MALT1-deficient B cells. Consequently, there is reduced entry of B cells into the GC in the absence of MALT1.

Materials and Methods

Mice

The *Malt1*$^{-/-}$ mice have been previously described (91) and were provided by Dr. Vishva Dixit (Genentech). Littermates controls of *Malt1*$^{+/+}$ or *Malt1*$^{+/-}$ genotypes were generated along with *Malt1*$^{-/-}$ mice by interbreeding heterozygous animals. For generation of B cell specific-wildtype (WT) and *Malt1*$^{-/-}$ mice, μMT mice (The Jackson Laboratory) were sublethally irradiated (5-Gy dose) and reconstituted with bone marrow from *Malt1*$^{+/+}$ or *Malt1*$^{-/-}$ mice. Mixed chimeras were generated as follows: μMT animals were exposed to a lethal dose (10-Gy) of irradiation and reconstituted with a 1:1 mix of bone marrow from CD45.1 and *Malt1*$^{-/-}$ (CD45.2) mice. Bone marrow cells from a single donor were divided among four to six recipient animals. The OT-II mice were generously provided by the laboratory of Dr. Linda Bradley (Sanford Burnham Prebys). The mice were housed in pathogen-free environment in the animal facility at the Sanford Burnham Prebys Medical Discovery Institute. All experiments conformed to the ethical principles and guidelines approved by Institutional Animal Care and User Committee.

Immunizations

Citrated sRBCs (Colorado Serum Company) were washed twice with PBS and resuspended in PBS to a final concentration of 10% (vol/vol). Animals were injected intraperitoneally with 0.1 mL of sRBCs suspensions and sacrificed 7 days later for analysis by flow cytometry and histology. Sera were collected on day 0 and day 7

post-immunization for measuring the levels of SRBC-specific IgM and IgG1 levels. For studying TD immune response, animals were administered intraperitoneally with 100 µg of NP_{25}-KLH (Biosearch Technologies) precipitated in alum and analyzed on day 14 post-immunization. Sera were collected on day 0 and day 14.

Flow cytometry

Single-cell suspensions prepared from spleens and Peyer's patches were blocked with anti-CD16/32 (clone 2.4G2) (BD Biosciences) and stained with the indicated combination of conjugated antibodies for 30 minutes on ice. Live cells were assessed by forward and side scatter profiles. All cells were acquired on a FACSCanto flow cytometer using the FACSDiva software (BD Biosciences) and data were analyzed using the FlowJo software (Tree Star). The following antibodies were obtained from eBioscience: anti-B220 (RA3-6B2), -CD3e (145-2C11), -CD21/35 (8D9), -CD23 (B3B4), -CD45.1 (A20), -CD4 (RM4-5), -IgD (11-26), -CD86 (PO3.1), -PD1 (J43), -ICOS (7E.17G9), -IgM (II/41), -CD5 (53.753), -CD62L (MZL-14), -CD86 (PO3.1), -MHC class II (M5/114.15.2), -CD25 (PC61.5), and -CD69 (H1.2F3). Anti-GL7, -FAS (JO2), -CD45.2 (104), -CD138 (281-2), -IgG1 (A85.1), and -CD80 (16-10A1) antibodies were purchased from BD Biosciences.

In vitro proliferation, cell cycle, and apoptosis assays

B cells were isolated from splenocytes by negative magnetic-based sorting of cells labeled with CD43 microbeads (Miltenyi Biotec). For the proliferation assay,

purified B cells were labeled with eFluor670 (eBioscience) according to the manufacturer's protocol and cultured in 96-well plate at the density of 10^6 cells/mL in complete RPMI 1641 (Corning Cellgro) supplemented with 10% fetal bovine serum (FBS) (Sigma), 1x Penicilin/Streptomycin (Corning Cellgro), 2mM GlutaGro (Corning Cellgro), 1x MEM non-essential amino acids (Corning Cellgro), 1 mM sodium pyruvate (Corning Cellgro), and 50µM β-Mercaptoethanol (Gibco), with or without various stimuli. The following stimuli were used at the indicated concentrations: 25 ng/mL recombinant murine BAFF (R&D Systems), 10 ng/mL recombinant IL4 (eBioscience), 5 µg/mL αCD40 (eBioscience), 10 µg/mL αIgM $F(ab')_2$, (Jackson Immuno Research), and 10 µg/mL LPS (Sigma). To evaluate the effect of caspase inhibition, B cells were stimulated in the presence of IDN-6556 at 10 µM (generously provided by the laboratory of Dr. Guy Salvesen). For inhibition of IL4-induced JAK signaling, B cells were stimulated in the presence of 10 µM of tofacitinib.

For cell cycle analysis, splenic B cells were stimulated with the indicated stimuli and duration, and pulsed with 10 µM of 5-bromo-2'-deoxyuridine (BrdU) (Invitrogen) during the last hour of incubation prior to harvest. The cells were fixed with 70% ice-cold ethanol, treated with 2M HCl to denature the DNA, following by washing with 0.1 M sodium tetraborate (pH8.5) to neutralize the acid. The cells were then incubated with αBrdU antibody (Invitrogen) and resuspended in a cocktail containing RNase at 100 µg/mL and 7-aminoactinomycin D (7-AAD) at 5 µg/mL, and analyzed by flow cytometry. For combined apoptosis and proliferation assay,

eFluor670-labeled B cells were cultured as indicated and stained with Annexin-V-FITC (BioVision) according to the manufacturer's instructions.

Hisology

Spleens were embedded in Tissue-TEK OCT compound (Sakura Finetek) and frozen in -80 ^{0}C. Frozen tissue blocks were sectioned, mounted on Superfrost/Plus slide (Fisher Scientific), fixed in ice-cold acetone, and blocked with PBS plus 5% FBS. The sections were stained with the following reagents: αIgD, αCD45.1 (clone A20) (eBioscience), αCD45.2 (clone 104) (BD Biosciences), αKi67 (clone S01A15) (eBioscience), and peanut agglutinin (Vector Labs). Imaging was acquired on a Zeiss Axio ImagerM1 microscope (Zeiss) using the Slidebook software (Intelligent Imaging Innovations). GNU Image Manipulation Program was used for overlaying images.

In vitro GC differentiation

CD43-depleted naïve B cells were cultured in the presence of 40LB cells (108) in complete RPMI1640 supplemented with IL-4. On day 5, the cells were harvested and analyzed by flow cytometry or immunoblotting.

Measuremnt of antibody titer

For sRBC immunization, the levels of sRBC-specific IgM and IgG1 were determined using a flow cytometry-based approach. Briefly, sRBCs were incubated

with varying dilutions of the serum samples, washed and detected with fluorochrome-conjugated antibodies against either IgM or IgG. Mean fluorescence intensities of the sRBC-bound αIgM and αIgG1 antibodies were plotted against the dilution factors and the values in the linear range were used for comparing the relative antibody titers.

For immunization with NP-KLH, the antibody titer was measured by enzyme-linked immunosorbent assay (ELISA). Coaster EIA/RIA plates (Corning) were coated with NP23-BSA (Biosearch Technologies) in PBS containing 0.05% sodium azide, followed by blocking with 0.25% BSA in PBS. Serial dilutions of serum samples were added and detected using αIgM or αIgG antibodies coupled to alkaline phosphatase (Southern Biotech). After addition of the substrate p-nitrophenylphosphates (Sigma), absorbance values at 405 nm were measured using an ELx808 plate reader with KC4 software (BioTech Instruments).

Antigen presentation assay

T cells were isolated from the spleens and lympho nodes of OT-II mice by first labeling the cells with biotinylated anti-B220 (RA3-6B2), -CD11b (M1/70), and -CD11c (N418) antibodies (eBioscience), followed by negative magnetic based sorting using the anti-biotin microbeads (Miltenyi Biotec). T cells were labeled with the eFluor670 proliferation dye and cocultured with CD43-depleted B cells from control or *Malt1*$^{-/-}$ spleens at a density of 0.75 x 10^6 cells/mL (T cells) and 1 x 10^6 cells/mL (B cells) in complete RPMI1640 in the presence of OVA peptides 323-239 (500 ng/mL) for 3 days.

Quantitative real-time polymerase chain reaction (qRT-PCR)

CD43-depleted splenic B cells were rested for at least 60 minutes and stimulated with 1 µg/ml αIgM F(ab')$_2$ for the indicated times at 37 ^{0}C. Total RNA was isolated from B cells using Trizol (Invitrogen) and cDNA was prepared using he MMLV Reverse Transcriptase cDNA Advantage kit (Clontech), according to the manufacturers' instructions. qRT-PCR was performed with the SsoAdvanced SYBR Green Supermix (Bio-Rad) on a CFX384 Touch Real-Time PCR Detection system (Bio-Rad). The primers used were designed with the help of PrimerBank (109) and the sequences are listed below:

Bcl2
Forward: ATGCCTTTGTGGAACTATATGGC
Reverse: GGTATGCACCCAGAGTGATGC

Bcl2l1
Forward: GACAAGGAGATGCAGGTATTGG
Reverse: TCCCGTAGAGATCCACAAAAGT

Bcl2a1a
Forward: GGCTGAGCACTACCTTCAGTA
Reverse: TGGCGGTATCTATGGATTCCAC

Rel
Forward: AGACTGCGACCTCAATGTGG
Reverse: GCACGGTTGTCATAAATTGGGTT

Irf4
Forward: TCCGACAGTGGTTGATCGAC
Reverse: CCTCACGATTGTAGTCCTGCTT

Gapdh
Forward: CATGGCCTTCCGTGTTCCTA

Reverse: CCTGCTTCACCACCTTCTTGAT

E2f3
Forward: AAACGCGGTATGATACGTCCC
Reverse: CCATCAGGAGACTGGCTCAG

The expression level of each target gene was normalized to that of *Gapdh* and the changes were calculated using the ΔΔ cycle threshold method.

Immunoblotting

Freshly isolated B cells were cultured in complete RPMI 1640 with or without stimuli. After 24 hours, cells were collected and lysed with RIPA buffer (PBS, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 10 mM EDTA) plus 10 mM sodium fluoride and complete protease inhibitor (Roche). Protein content of cleared lysates was measured using the BCA Protein Assay kit (Thermo Scientific). Lysates were resolved on 4-12% or 10% polyacrylamide Bis-Tris gel (Bio-Rad or Invitrogen) and transferred onto polyvinylidene fluoride membrane (EMD Millipore). The membrane was probed for the indicated proteins. The following antibodies were purchased from Cell Signaling: anti-MALT1, -Bcl-2 (D17C4), -β-actin (13E5), -phospho-Rb (Ser807/811) (D20B12), -cyclin D3 (DCS22), -c-Myc (D84C12), -survivin (71G4B7), and -total Akt. Anti-Mcl-1 was purchased from Rockland Immunochemicals, anti-cyclin D2 (M-20) from Santa Cruz, and anti-Bcl-xL from BD Biosciences. Primary antibodies were then detected with horseradish peroxidase-labeled donkey anti-rabbit or anti-mouse antibodies (Jackson ImmunoResearch) and developed with the SuperSignal West Pico chemiluminescence kit (Thermo Scientific).

Statistics

Bar graphs represent mean values and the error bars represent standard deviation (SD). Statistical significance was calculated using two-tailed unpaired Student t test or one way ANOVA using GraphPad Prism (GraphPad Software). Only significant differences were indicated in the figures by asterisks as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p<0.0001$.

Results

Characterization of MALT1-deficient animals

The phenotypes of the *Malt1*$^{-/-}$ animals were first verified by FACS staining. In line with the previously published data (100), non-immunized *Malt1*$^{-/-}$ mice showed a drastic reducton in GL7^{+}FAS^{+} GC B cells, as well as CD4^{+}PD1^{+}ICOS^{+} Tfh cells, in the Peyer's patches, while the percentage of total B220^{+} B cells were similar to that of WT controls (Figure 2-1A). In the spleens, the *Malt1*$^{-/-}$ animals displayed a modest and statistically insignificant decrease in total B cell pool, relatively normal frequencies of CD21^{lo}CD23^{hi} FO B cells, and significantly reduced CD21^{hi}CD23^{lo} MZ B subsets as previously reported (91, 92) (Figure 2-1B). Comparing the IgD versus IgM profiles, an accumulation of IgM^{hi}IgD^{hi} population and a concomitant decline in IgM^{lo}IgD^{hi} population was noted in the MALT1-deficient animals (Figure 2-1C, left panel). However, the IgM^{hi}IgD^{hi} cells in the *Malt1*$^{-/-}$ mice express lower level of heat stable antgen (HSA) on their surface, suggesting that they are not transitional B cells (Figure 2-1C, right panel). These restuls confirmed the impaired GC response and MZ B cell differentitation that have been described in MALT1-deficient animals.

B cell-intrinsic MALT1 function is required for the GC response.

While the importance of MALT1 for GC formation and subsequent antibody production is undisputed (91, 92, 100), it remains unclear whether the defects associated with MALT1 deficiency arise from perturbed B cell or T cell activation or a combination thereof. To resolve this issue, chimeric animals were generated by

reconstituting sublethally irradiated B cell-deficient μMT mice, with bone marrow from WT or *Malt1*$^{-/-}$ mice. The recipients, bearing B cells that are MALT1-sufficient (μMT/WT), or -deficient (μMT/KO) were immunized with sRBCs. At 7 days post-immunization, the presence of GC B cells in both the spleens and Peyer's patches was assessed by flow cytometry. Similar to the defect observed in *Malt1*$^{-/-}$ mice, μMT/KO mice exhibited a ~7-fold reduction in B220^{+}GL7^{+}Fas^{+} GC B cells (Figure 2-2A). The reduction in MZ B cells was also recapitulated in μMT/KO mice (Figure 2-2B). Interestingly, μMT/KO mice displayed a modest but significant reduction in FO B cells, which we did not observe in intact *Malt1*$^{-/-}$ mice (Figure 2-2B). Consistent with the flow cytometry data, histology of spleen and Peyer's patches sections from the μMT/KO mice revealed that there were very few PNA^{+} cells in the follicles, despite the presence of organized FDC networks (Figure 2-2C). The defect in GC reactions in μMT/KO animals is also consistent with the impaired production of sRBC-specific antibodies (Figure 2-2D). These findings indicate that B cells intrinsically require MALT1 for both the GC response and differentiation into MZ B cells.

Malt1$^{-/-}$ *B cells fail to initiate GC reactions*

One explanation for the lack of GCs in μMT mice reconstituted with *Malt1*$^{-/-}$ bone marrow is a failure of B cells to induce or respond to T cell help. To address this possibility, a congenic mixed chimera approach was employed, by injecting a 1:1 ratio of WT (CD45.1) to *Malt1*$^{-/-}$ (CD45.2) bone marrow into lethally irradiated μMT mice (μMT/mixed). Following reconstitution, control and *Malt1*$^{-/-}$ cells can be tracked by

flow cytometry using antibodies specific for CD45.1 or CD45.2, respectively. This model also enabled a direct comparison of the competitive fates of antigen-activated control versus *Malt1*$^{-/-}$ B cells in the identical environment. Transplantation of pure CD45.1 or *Malt1*$^{-/-}$ bone marrow was performed as controls (yielding µMT/CD45.1 and µMT/CD45.2 animals, respectively).

When the µMT/mixed chimeras were immunized with TD antigens sRBCs or NP-KLH, B cells derived from the CD45.1 donor accounted for more than 80% of the splenic GC compartment in the recipients (Figure 2-3A). While the distribution of CD45.1^{+} and CD45.2^{+} populations in total splenocytes were roughly equal among all the µMT/mixed chimeras, some variation in total B220^{+} B cells was observed. To account for these differences, a more fair comparison would be to first gate on the B220^{+} B cells and analyzed for CD45.1/CD45.2 status, then the frequency of GC in each population was determined. Using this gating strategy, a 5-fold reduction in GC B cells was seen within the CD45.2^{+} *Malt1*$^{-/-}$ population in the spleen, and at least 10-fold reduction in this population in the Peyer's patches (Figure 2-3B, top panels). Moreover, characterization of splenic B cell subsets revealed an almost complete absence of MZ B cells derived from the *Malt1*$^{-/-}$ hematopoietic precursors (Figure 2-3B, bottom panels). Both µMT/WT and µMT/mixed mice, but not µMT/KO mice, were capable of mounting antibody response against NP-KLH (Figure 2-3D).

When examining the spleen sections obtained from the immunized µMT/mixed chimeric mice, it was noted that the GC region, defined by PNA positivity, was dominated by CD45.1^{+} cells, as evidenced by the diffuse red staining with sporadic green cells (Figure 2-4). Since GCs are sites of B cell clonal expansion,

the proliferation marker Ki67 was included to detect cycling B cells. Ki67 expression mainly co-localized with CD45.1-stained cells within the GC (Figure 2-4). Collectively, these results indicate that the only a few *Malt1*$^{-/-}$ cells were able to enter GC reactions and they were not actively proliferating.

The reduction in GC B cells observed at the peak of the response could have resulted from either reduced entry of B cells into the GC, or a failure of the early GC cells to properly expand and differentiate. To distinguish between these possibilities, I utilized an analytic approach that had been previously developed (110), involving immunization with sRBC, which induces a robust TD response with well-defined kinetics, and examination of the progression of GC B cell differentiation in the μMT/mixed chimera setting. First, PNA was used as a marker to encompass all stages of GC B cell development. After immunization, the proportion of PNA^{+} B cells that were of the *Malt1*$^{-/-}$ (CD45.2) origin was diminished (Figure 2-5A, upper left panel). Next, GL7 and IgD staining was incorporated to resolve GC B cell maturation which proceeds from an IgD^{+}GL7^{-} to an IgD^{-}GL7^{+} stage (110). Focusing on the CD45.1^{+} population, there was an expansion of IgD^{-}GL7^{+} GC B cells, accompanied by a decrease in IgD^{+}GL7^{-} early GC B cells (Figure 2-5B). In contrast, the majority of the PNA^{+} B cells within the CD45.2^{+} gate retained the IgD^{+}GL7^{-} phenotype and less than 10% of the cells matured into IgD^{-}GL7^{+} GC B cells. The defect in GC initiation paralleled the reduced frequency of *Malt1*$^{-/-}$-derived B220^{lo}CD138^{+} plasma cells on days 7 and 10 (Figure 2-5A, upper right panel). Interestingly, I also observed a decreased Tfh population of *Malt1*$^{-/-}$ origin on day 7, suggesting that MALT1-deficient T cells harbor an intrinsic block in Tfh differentiation (Figure 2-5A, lower left panel).

The defect in MZ B cell differentiation observed in *Malt1*$^{-/-}$ animals was again successfully recapitulated (Figure 2-5A, lower right panel). Collectively, these results suggest that GC initiation is defective in *Malt1*$^{-/-}$ animals and that the few PNA^{+}IgD^{-} GL7^{+} early GC B cells that are present also fail to mature and expand.

Malt1$^{-/-}$ *B cells can be induced into GC phenotype in vitro*

The inability to mount GC response translates into failure in generating memory B cells and long-lived plasma cells. To identify how extensive the defect in GC formation is, I adopted an *in vitro* culture system to propagate GC phenotype B cells (108). When naïve B cells were cultured on the 40LB feeder cells, which are BALB/c 3T3 fibroblasts stably transfected with CD40L and BAFF, they underwent massive expansion and acquired the GL7^{+}FAS^{+} GC phenotype, termed induced GC B (iGB) cells (Figure 2-6A left panels). Strikingly, I was able to efficiently induce the *Malt1*$^{-/-}$ B cells to differentiate into GL7^{+}FAS^{+} GC-like B cells after 5 days in culture with 40LB cells. Supplementing the culture with IL4 also induced BCR class switching from IgM to IgG1 (Figure 2-6A, middle panels). Similar to the iGB cells derived from WT B cells, approximately 50% of the *Malt1*$^{-/-}$ iGB cells expressed IgG1, indicating that the class-switching machinery in MALT1-deficient B cells remains intact. While a small fraction of the *Malt1*$^{-/-}$ iGB cells differentiated into B220^{lo}CD138^{+} plasmablastss, it was noted that this population was significantly reduced relative to WT iGB cells (Figure 2-6A, right panels). Immunoblotting analysis of WT and *Malt1*$^{-/-}$ day 5 iGCs revealed no appreciable difference in the level of

various proteins associated with the regulation of apoptosis or proliferation (Figure 2-6B). Furthermore, both WT and *Malt1*$^{-/-}$ iGCs were capable of undergoing at least 1 round of division after 24 hours of stimulation with αIgM F(ab')$_2$ or αIgG F(ab')$_2$ (Figure 2-6C). Thus, *Malt1*$^{-/-}$ follicular B cells proliferate normally to T cell-derived stimuli to adopt a GC B cell phenotype and retain the capacity to class switch, but are impaired in maturation towards the plasma cell lineage.

BCR-activated Malt1$^{-/-}$ *B cells are less proliferative and more apoptotic*

Although the response of *Malt1*$^{-/-}$ B cells to various mitogens havs been previously examined, the role of MALT1 in B cell proliferation remains elusive due to conflicting results presented by several groups (91, 92, 94, 111). To rule out the effect of reduced MZ B cell compartment, FO B cells were enriched from the spleens of WT and *Malt1*$^{-/-}$ animals by magnetic depletion of CD43$^+$ non-B cells and CD9$^+$ MZ B cells (Figure 2-7A). Purified B cells were then stimulated with a panel of stimuli for 3 days. *Malt1*$^{-/-}$ B cells exhibited robust proliferation, comparable to that of WT B cells, in response to LPS or αCD40 plus IL4, but were hyporesponsive to αIgM stimulation (Figure 2-7B). Interestingly, costimulation with IL4 restored the proliferative response to αIgM in *Malt1*$^{-/-}$ B cells. The combination of αIgM plus αCD40 also partially rescued the proliferative defect, albeit to a lesser extent compared to what was observed in WT B cells. However, BAFF costimulation showed minimal improvement on αIgM-induced proliferation in *Malt1*$^{-/-}$ B cells.

To investigate the nature of the proliferative defect to αIgM in *Malt1*$^{-/-}$ B cells, cell cycle analysis was performed using BrdU incorporation in combination with the DNA dye 7-AAD. I observed a significant reduction in S phase entry of *Malt1*$^{-/-}$ B cells at 24 hours after αIgM treatment (Figure 2-8A). However, by 48 hours post-stimulation, the percentage of *Malt1*$^{-/-}$ B cells in S phase increased to level comparable with WT B cells. Normal B cells induce expression of both cyclins D2 and D3 upon stimulation with αIgM (112). The two D-type cyclins activate the G1 kinases and target the retinoblastoma gene product (Rb) for phosphorylation, thus freeing the E2F protein to drive transcription of genes required for the transition from G1 to S phase. In line with the cell cycle analysis, upregulation of cyclins D2 and D3, and subsequent induction of phosphor-Rb (pRb) were observed in *Malt1*$^{-/-}$ B cells upon αIgM treatment (Figure 2-8B). Taken together, these results suggest that there is a delay, rather than lack of proliferative response of *Malt1*$^{-/-}$ B cells to BCR crosslinking.

The finding that the proliferation machinery remains functional in *Malt1*$^{-/-}$ B cells raises the possibility that owing to their survival disadvantage, *Malt1*$^{-/-}$ B cells undergo apoptosis before they have a chance to divide. Consisten with this notion, there was a significant reduction in viability when *Malt1*$^{-/-}$ B cells were cultured in media alone or stimulated with αIgM F(ab')$_2$ (Figure 2-9A). While the addition of BAFF or αCD40 to αIgM treatment was able to enhance viability of WT B cells, it failed to achieve the same effect on *Malt1*$^{-/-}$ B cells. LPS- or αCD40-induced proliferation remained intact in *Malt1*$^{-/-}$ B cells and in line with these results, *Malt1*$^{-/-}$ B cells survived just as well as WT B cells when treated with either stimulus, suggesting that both the proliferative and survival defects are restricted to signaling

through the BCR. Although BAFF costimulation failed to increase viability of αIgM-stimulated *Malt1*$^{-/-}$ B cells, compared to cells in media, treatment with BAFF alone clearly improved the survival of *Malt1*$^{-/-}$ B cells. Altogether, these findings raise the interesting possibility that in the absence of MALT1, activation of the BCR induces a dominant pro-apoptotic signal that cannot be rescued by BAFF- or CD40-induced signaling.

To examine more closely the interplay of proliferation and survival, combined proliferation dye and Annexin V labeling was used to track cell division and apoptosis induced by αIgM F(ab')$_2$ treatment. I noted that in the case of the *Malt1*$^{-/-}$- B cells, the majority of the Annexin V$^+$ apoptotic cells did not show dilution of the proliferation dye, suggesting that the cells underwent apoptosis before cell division took place (Figure 2-9B). Consistently, the addition of IL4, but not BAFF, was able to induce proliferation of BCR-activated *Malt1*$^{-/-}$ B cells and increase their viability. LPS alone was shown to provide strong mitogenic signal to both WT and *Malt1*$^{-/-}$ B cells and I did not see any difference in proliferation and survival when LPS was administered in conjunction with αIgM. In contrast, WT and *Malt1*$^{-/-}$ B cells responded differently to combined αIgM and αCD40 treatment. Whil αCD40 costimulation was able to rescue BCR-induced proliferation in *Malt1*$^{-/-}$ B cells to some extent, there were fewer rounds of divisions compared to WT B stimulated in the same condition and there were far fewer viable Annexin-V$^-$ *Malt1*$^{-/-}$ B cells.

It has been reported that BAFF-induced survival is reduced specifically in the MZ B cell subset in *Malt1*$^{-/-}$ mice (93). I attempted to verify this observation by FACS-sorting FO and MZ B cells according to their surface markers

($B220^{+}CD21^{lo}CD23^{hi}$ and $B220^{+}CD21^{hi}CD23^{lo}$, respectively) and cultured them in the presence of αIgM $F(ab')_2$ or BAFF. Contrary to the findings made by Tusche *et al* (93), I noted that the defect in BAFF-induced survival response applied to both *Malt1*$^{-/-}$ FO and MZ B cell subsets (Figue 2-10A). However, viability of *Malt1*$^{-/-}$ FO and MZ B cells were increased by BAFF treatment compared to cells cultured in media alone. In addition, the MZ B population of both WT and *Malt1*$^{-/-}$ animals were susceptible to αIgM $F(ab')_2$ stimulation and died within 24 hours in culture. Also consistent with the survival assay described above, *Malt1*$^{-/-}$ FO B cells were more prone to αIgM $F(ab')_2$-induced death. Immunoblotting analysis also revealed that processing of p100 can be induced in *Malt1*$^{-/-}$ B cells by combined αIgM and BAFF treatment (Figure 2-10B). Therefore, the fact that BAFF failed to restore the proliferation response and increase survival of *Malt1*$^{-/-}$ B cells to αIgM treatment suggests that the defect in canonical NF-κB may be dominant over BAFF-induced signaling.

Next, I sought to determine if rescuing survival can overcome the impairment in proliferative response to αIgM stimulation in *Malt1*$^{-/-}$ B cells. When treated with the broad spectrum caspase inhibitor IDN-6556 (113), viability of *Malt1*$^{-/-}$ B cells, in media alone or stimulated with αIgM $F(ab')_2$, was increased to comparable level to that of WT B cells (Figure 2-11A, left panels). Moreover, in the presence of IDN-6556, *Malt1*$^{-/-}$ B cells exhibited a similar proliferative profile to αIgM $F(ab')_2$ as WT B cells (Figure 2-11A, right panels). In comparison, the rescue effect of IL4 requires an intact JAK-STAT signaling pathway, since the addition of tofacitinib, a JAK3 inhibitor (114), completely abrogated proliferation induced by αIgM and IL4 (Figure 2-11B). The levels of various proteins were also examined by immunoblotting and it was noted

the combined αIgM and IL4 treatment resulted in reduction of Bax and Bim, and subsequently less cleaved caspase-3, and this protective mechanism was inhibited by tofacitinib (Figure 2-11C). Collectively, these findings indicate that the delay in cell cycle entry and increased propensity to caspase-dependent apoptosis account for the impaired proliferative response of *Malt1*$^{-/-}$ B cells to BCR stimulation.

T cell-derived help fails to rescue defects in BCR-activated Malt1$^{-/-}$ *B cells*

To rule out the possibility that *Malt1*$^{-/-}$ B cells are simply anergic to BCR crosslinking, the levels of various activation markers on cell surface were assessed by flow cytometry. When stimulated with αIgM F(ab')$_2$, *Malt1*$^{-/-}$ B cells upregulated CD5, CD86, MHC II, CD69 and CD80, to comparable level as WT B cells did (Figure 2-12A). The only notable differences were downregulation of CD62L and induction of CD25, both of which occurred to a lesser extent in *Malt1*$^{-/-}$ B cells.

Profiling of activation makers on BCR-activated *Malt1*$^{-/-}$ B cells suggest that they retain the ability to prime T cells. To verify such possibility, I examined whether MALT1-deficient B cells could successfully stimulate antigen-specific T cells to expand clonally. Splenic B cells from WT or *Malt1*$^{-/-}$ animals were isolated and cultured with T cells from OT-II mice, which express a transgenic TCR that is specific for the chicken ovalbumin (OVA) 323-339 epitope (115). Strikingly, in the presence of OVA peptide, *Malt1*$^{-/-}$ B cells induced OT-II T cells to undergo multiple rounds of cell division (Figure 2-12B). In addition, the T cells cultured with *Malt1*$^{-/-}$ B cells displayed similar induction of CD25 and CD69 to those cultured with control B cells

(Figure 2-12B). These results indicate that even in the absence of MALT1, B cells are able to effectively collaborate with T cells.

During the TD immune response, antigen-activated B cells migrate to the B zone-T zone border to seek help form cognate T cells. Accessory signals, notably CD40 and CD40L interactions, are necessary to drive the initial B cell proliferation and induction of GC formation. While I found that the combination of αIgM and αCD40 stimuli were able to restore αIgM-induced proliferation defect in *Malt1*$^{-/-}$ B cells to some extent, *Malt1*$^{-/-}$ B cells did not survive well under the combined stimulation. These findings prompted the examination of fate of *Malt1*$^{-/-}$ B cell upon receiving T cell help. I mimicked the events in GC response by pre-treating B cells with αIgM, followed by αCD40 stimulation (Figure 2-13A). It has been shown that washing out αIgM after 24 hours did not prevent the first division, but most of the divided cells died instead of completing further divisions (116). However, subsequent engagement of CD40 was sufficient to induce further proliferation of WT B cells and rescue cell death (Figure 2-13B and C). In contrast, *Malt1*$^{-/-}$ B cells failed to proliferate and were more prone to cell death, suggesting that CD40L signals from the T cells cannot correct the proliferation and survival defects in BCR-activated *Malt1*$^{-/-}$ B cells. Interestingly, *Malt1*$^{-/-}$ B cells primed with αCD40 first were able to undergo one round cell division upon subsequent αIgM stimulation, but their viability was also markedly reduced compared to that of WT B cells (Figure 2-13B and C). Thus, the inability of CD40 signaling to rescue the impairment in BCR-induced proliferative and survival response may explain why GC initiation was defective in *Malt1*$^{-/-}$ B cells.

Expressions of Bcl-2 and Bcl-xL are reduced in Malt1$^{-/-}$ B cells

PKCβ is situated upstream of the CBM complex and controls assembly of the complex via phosphorylation of CARMA1 upon BCR engagement (68). PKCβ-deficient animals share many of the defects observed in *Malt1*$^{-/-}$ mice. In particular, BCR-dependent proliferation and survival in *Pkcb*$^{-/-}$ B cells were impaired due to defective induction of Bcl-2 and Bcl-xL (117, 118). Thus, the levels of various anti-apoptotic proteins in BCR-activated WT and *Malt1*$^{-/-}$ B cells were examined. While WT B cells upregulated Bcl-xL and survivin when stimulated with αIgM alone or in combination with BAFF or αCD40, I did not observe comparable induction of either protein in *Malt1*$^{-/-}$ B cells after stimulation (Figure 2-14A). Intriguingly, there was a general decrease in Bcl-2 protein level in *Malt1*$^{-/-}$ B cells under all the conditions tested. In contrast, the level of Mcl-1 protein was similar between WT and *Malt1*$^{-/-}$ B cells. *Malt1*$^{-/-}$ B cells also induced c-Myc expression upon stimulation, indicating that they were responding to the treatment.

Both *Bcl2* and *Bcl2l1* genes have been reported as targets of the transcription factor c-Rel (119), which was found to be selectively activated by MALT1 (94). To investigate whether the reduction of Bcl-2 and Bcl-xL can be attributed to defective c-Rel activation, I monitored the transcript levels of several known c-Rel target genes in WT versus *Malt1*$^{-/-}$ B cells after αIgM treatment by time-course real-time PCR. With the exception of *Bcl2l1*, the profiles of all the other genes in response to αIgM F(ab')$_2$ stimulation were strikingly similar between WT and MALT1-deficient B cells (Figure 2-14B). While αIgM induced an increase in *Bcl2l1* mRNA level that is evident at 4 hours in WT B cells, *Bcl2l1* became downregulated in *Malt1*$^{-/-}$ B cells. However, when

the expression of each gene was measured at 4 hours post-stimulation, the time point at which I saw the greatest induction in the assay, and normalized to that of WT, significant reductions in the levels of both *Bcl2* and *Bcl2l1* were observed (Figure 2-14C). These data suggest that the impaired survival in BCR-activated *Malt1*$^{-/-}$ B cells is due to reduced levels of Bcl-2 and Bcl-xL.

Discussion

MALT1 has been recognized as an essential component of antigen receptor signaling pathway, regulating NF-κB activity via both scaffolding and protease functions (95). However, its specific role in B cells has not been fully characterized. In this chapter, several lines of evidence were provided for the cell-intrinsic role of MALT1 in regulating B cell immune function. The μMT/mixed chimeras gave the most direct comparison of MALT1-sufficient and MALT1-deficine B cells in the same setting with identical stimuli. Using chimeric mouse models, I demonstrated that there is a B cell-intrinsic requirement of MALT1 for GC formation and MZ differentiation. Central to BCR-induced NF-κB signaling is the kinase Btk, which is primarily responsible for the activation of PLCγ2 and subsequently PKCβ, leading to assembly of the CBM complex. Mutation or inhibition of Btk has been shown to result in a compromised GC response (120, 121). In accordance with the BCR-Btk pathway, deficiency in any of the downstream signaling proteins also led to impaired TD humoral response (89, 91, 92, 117, 122, 123). It has been accepted that NF-kB is dispensable for GC B proliferation, since GC B cells fail to express most NF-kB target genes and NF-kB pathway components (124). This notion is supported by a later study, which showed that BCR signaling is dampened in GC B cells, due to phosphatase activity, and heightens again in the light zone (125). A recent study dissecting the differential roles of the NF-κB subunits RelA and c-Rel in GC response also revealed that c-Rel is required for GC maintenance, whereas activation of RelA is indispensable at later stages of the GC response for the generation of plasma cells (126). Thus, BCR-

induced NF-κB activation is more associated with GC initiation, as a consequence of antigen activation. Consistently, the findings presented here indicate that the block in *Malt1*$^{-/-}$ animal occurs at the early stage of GC reactions, before the proliferative expansion of antigen-activated B cells takes place.

The members of the canonical NF-κB family exhibit distinct expression profiles; while p50, p65, and p52 are found in all cell types and pre-B cells, c-Rel and RelB are specifically present in lymphoid tissues (66, 127). Although prior studies have established that Rela and c-Rel are individually dispensable for B cell development (128-131), it was shown that the combined loss of Rela and c-Rel results in an arrest at the immature HAS^{hi}B220^{lo}IgM^{hi}IgD^{hi} stage and a complete absence of mature, long-live IgM^{lo}IgD^{hi} population (132). In contrast, the absence of MALT1 did not have a deleterious effect on B cell development and impacted only the MZ and B1 compartment, which are known to be particularly sensitive to pertubratons in NF-κB activation (133). Likewise, B cell development was largely normal in mice lacking other components of the CBM complex (134). These findings suggest the existence of a route alternative of CBM signalocome for NF-κB activation during B lymphopoiesis.

Humoral responses to TI antigens were also found to be defective in *Malt1*$^{-/-}$ animals (91, 92), most likely due to the compromised B1 and MZ compartment (135). It has been shown that MZ B cells proliferate better than FO B cells to LPS stimulation, presumably because MZ B cells express higher level of TLR4 (136). Moreover, MZ B cells are essential to the initiation of TI immune response by differentiating into plasmablasts upon interactions with other antigen presenting cells (137). Intriguingly, unlike mice with mutations in components of the BCR-Btk

pathway which exhibit a loss of FO B cells but normal MZ structure (138, 139), deletion of any of the CBM complex members results in substantial decrease in MZ B cells (91, 92, 123, 140). While canonical NF-κB signaling plays a essential role in lymphocyte development, it has been shown that c-Rel or Rela deficiency only partially affected MZ B cell development (141). In contrast, MZ B development is dependent on BAFF-induced signaling, but such requirement for BAFF can be substituted by activating canonical NF-κB signaling (142). These data suggest an involvement of CBM complex in BAFF-induced canonical NF-κB signaling, which is distinct from the BCR-Btk pathway, that mediates the differentiation of MZ B cells.

Intriguingly, characterization of the *Malt1*$^{-/-}$ spleens revealed an accumulation of IgM^{hi}IgD^{hi} population of intermediate maturity, despite their HSA^{lo} status. Whether these cells truly resemble immature B cells would necessitate further investigation. However, a minor reduction in the differentiation of T2 B cells into follicular B cells has also been noted in both CARMA1- and BCL10-defcient mice (89, 123), thus highlighting the requirement of the CMB complex in the proper maturation of FO B cells during the later stages of B cell development. An early study indicated that c-Rel-deficient mature B cells closely resemble the characteristics of normal immature B cells in that they exhibit hypersensitivity to BCR-induced growth arrest and apoptosis, as a consequence of impaired NF-κB activation (143). Both CARMA1- and BCL10-deficient B cells showed impaired proliferation and reduced viability after BCR activation (89, 94). Therefore, the current study provides additional evidence that an intact CBM complex is required for BCR-induced survival signaling that is primarily mediated by c-Rel acitivty, and that disruption of any of the component of the CBM

complex would lead to an immature B cell-like phenotype. Further studies are needed to identify the factors, mostly likely c-Rel target genes, that are involved in the differentiation of transitional B cells into mature B cells.

B cells lacking RelA show impaired proliferative responses to a panel of stimuli including the lectin concanavalin A, LPS, αIgM, and PMA plus calcium ionophore (129). c-Rel-deficient B cells are also hyporesponsive to αIgM, LPS, or αCD40 stimuli (99). In comparison, the proliferative defect in *Malt1*$^{-/-}$ B cells appears to be restricted to stimulation through BCR crosslinking and is not due to a decrease in MZ B subset, since sorted *Malt1*$^{-/-}$ FO B cells also failed to proliferate in response to BCR crosslinking. A similar defect in BCR-induced proliferation was seen in mice deficient in CARMA1, BCL10, Btk, or PKCβ, thus supporting the requirement of the CBM signalosome along the BCR-Btk axis (89, 90, 144, 145). Stimulation with αCD40 has been shown to synergize with αIgM and potentiated proliferation of *Rel*$^{-/-}$ B cells (99). While a partial rescue in proliferative response was observed in *Malt1*$^{-/-}$ B cells treated with αIgM and αCD40, the extent of cell division, as well as viability, were drastically reduced in comparision to WT B cells cultured under the same conditions. Co-stimulation with BAFF has also been reported to augment αIgM-induced proliferation (146-148), but such synergistic effect was absent in MALT1-deficient B cells. Contrary to the findings made by Tusche et al. (93), I noted that BAFF-induced processing of p100 remains intact in the absence of MALT1. Despite of the modest decrease in viability compared to WT B cells, treatment of BAFF did improve the survival of both *Malt1*$^{-/-}$ MZ and FO B cells. These results suggest that in MALT1-deficient B cells, triggering of the BCR emits a dominat death signal that

cannot be overcome by CD40 or BAFF stimulation. One likely mechanism that accounts for the intersction of BCR and BAFF signaling is that sustained activation of the non-canonical NF-κB pathway requires continued production of p100, which is regulated by c-Rel at the transcription level (149). Consequently, when BCR signaling becomes perturbed, as in the case of MALT1 deficiency, this in turn affects BAFF function and lead to improper B cell survival. Strikingly, co-treatment of IL4 reversed the proliferative and survival defects in MALT1-deficient B cells to αIgM stimulation, similar to what have been reported in *Rel*$^{-/-}$ B cells (99). The restoration by IL4 is dependent on the JAK-STAT signaling and possibly involves the transduction of additional signaling pathways to heighten the responsiveness of B cells to BCR activation (150, 151).

Analysis of cell surface markers ruled out the possibility that MALT1-deficient B cells are simply anergic to BCR stimulation. In response to αIgM treatment, *Malt1*$^{-/-}$ B cells exhibited similar induction of co-stimulatory molecules CD80 and CD86, which has been shown to not rely on c-Rel activity (99). The notion that *Malt1*$^{-/-}$ B cells retain the capacity to prime T cells is supported by the finding that *Malt1*$^{-/-}$ B cells can present OVA peptide to OT-II T cells and induce robust proliferation of the antigen-specific T cells. What also remained functional in MALT1-deficient B cells was the ability to undergo class switch recombination when stimulated with αCD40 and IL4, which highlights an intact response to CD40-driven proliferation and differentiation (152, 153).

The increase propensity of BCR-activated *Malt1*$^{-/-}$ B cells to undergo apoptosis is likely due to reduced expression of Bcl-2 and lack of Bcl-xL regulation, on both

mRNA and protein levels. The essential role for c-Rel in protection of B cells from BCR-mediated apoptosis has been widely accepted, partly via induction of the Bcl-2 homologs Bcl-xL and A1 (99, 154). However, similar induction of A1 transcripts were detected in both WT and *Malt1*$^{-/-}$ B cells upon BCR crosslinking, despite the fact that A1 is a known critical survival factor in BCR activation (155). While Mcl-1 was shown to be required for GC response (156), MALT1-deficient B cells exhibited similar level of Mcl-1 protein relative to WT B cells. The precise mechanism of how the CBM complex selectively leads to induction of Bcl-xL and maintaenance of Bcl-2 awaits further investigation. Interestingly, reduction of Bcl-2 protein level has also been noted in *Plcg2*$^{-/-}$ B cells in response to αIgM stimulation, leading to enhanced BCR-induced apoptosis (157). Together with the findings in *Malt1*$^{-/-}$ B cells, these results illustrate the importance of the BCR-Btk-PLCγ2 pathway in regulating Bcl-2 expression.

In summary, the findings presented in this chapter indicate that upon triggering of BCR, B cells require MALT1 to ensure survival and efficient entry into cell cycle, thereby leading to proper initiation of GC reaction. In addition, BCR-induced proliferative and survival defects that are associated with MALT1 deficiency cannot be compensated for by T cell-derived help.

Chapter 2 contains work modified from the submitted manuscript titled "Differing requirements for MALT1 function in the survival and differentiation of peripheral B cell subsets". The co-authors are Zilu Zhu, Janna Hachmann, Takuya

Nojima, Daisuke Kitamura, Guy Salvesen, and Robert Rickert. The author of this dissertation is the primary investigator and author of the manuscript.

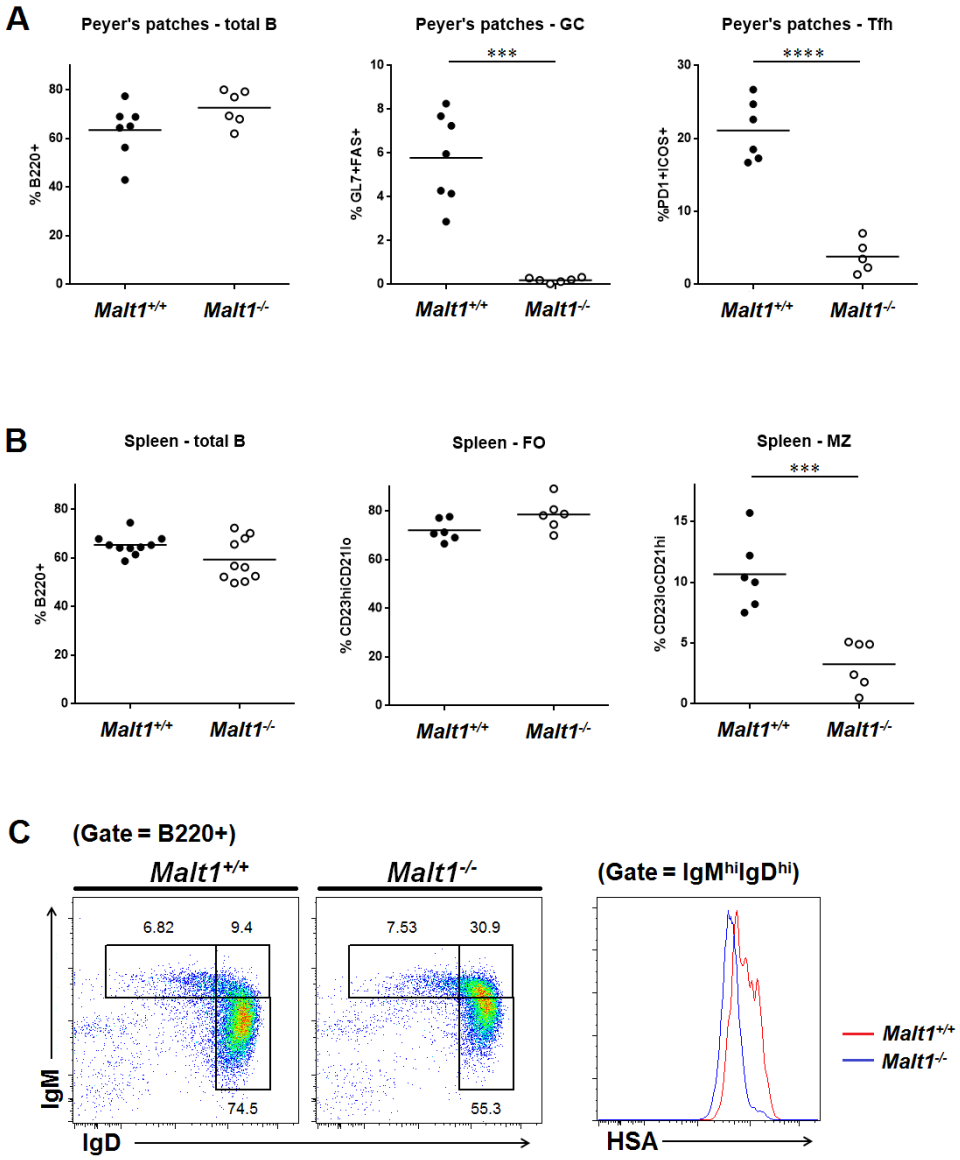
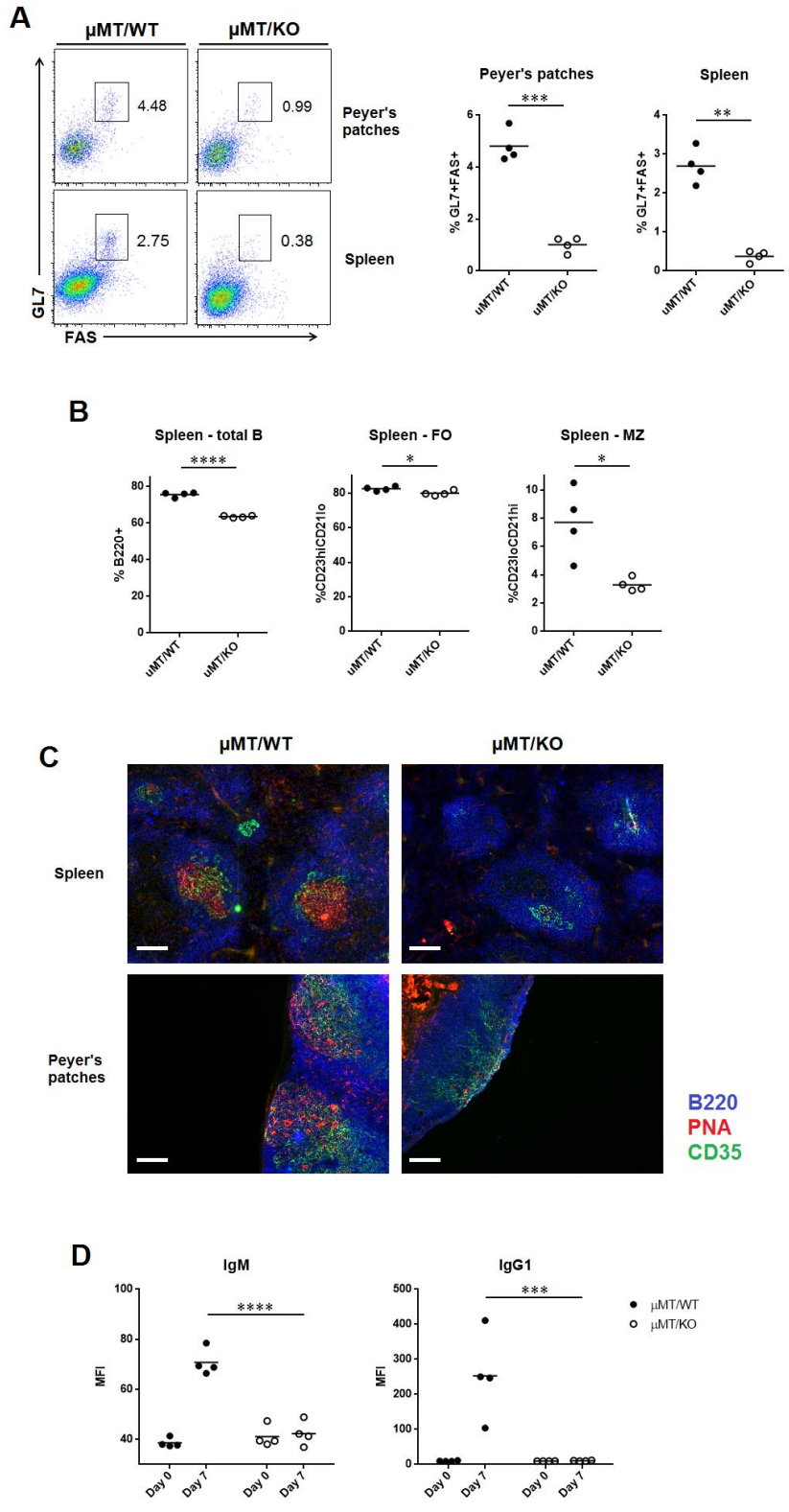


Figure 2-1. Characterization of *Malt1*$^{-/-}$ mice. (A) Frequencies of total B cells (B220^{+}), GC B cells (B220^{+}GL7^{+}FAS^{+}), and Tfh cells (CD3^{+}CD4^{+}PD1^{+}ICOS^{+}) in the Peyer's patches of WT and *Malt1*$^{-/-}$ mice. (B) Frequencies of total B cells (B220^{+}), FO B cells (CD21^{lo}CD23^{hi}), and MZ B cells (CD21^{hi}CD23^{lo}) in the spleen of WT and *Malt1*$^{-/-}$ mice. (C) Representative FACS plots showing the surface IgD versus IgM profiles of splenice B cells in WT and *Malt1*$^{-/-}$ mice. The right panel is the overlay of histrograms showing the expression of HSA in the IgM^{hi}IgD^{hi} gate.

Figure 2-2. B cells intrinsically require MALT1 for GC formation and MZ B differentiation. Sublethally irradiated µMT mice were reconstituted with *Malt1*$^{+/+}$ (µMT/WT) or *Malt1*$^{-/-}$ (µMT/KO) bone marrow, immunized with sRBCs, and analyzed on day 7 (n = 4 per group). (A) Representative FACS plots (left panel) and the frequencies of GC B cells as a percentage of total B cells (right panel) in both the spleens and Peyer's patches are shown. (B) Immunofluorescent staining of the spleens and Peyer's patches performed using antibodies specific for B220 (APC) and CD35 (FITC), as well as PNA (Cy3). The images are shown at 10x magnification and the scale bars represent 100 µm. (C) The frequencies of total B cells, FO B cells ($CD21^{lo}CD23^{hi}$), and MZ B ($CD21^{hi}CD23^{lo}$) cells in the spleen were also assessed by flow cytometry. (D) Serum levels of sRBC-specific IgM and IgG1 antibodiess on day 0 and day 7 post-immunization were determined by flow cytometry. Mean fluorescent intensities were plotted.


A
μMT/WT
μMT/KO
4.48
0.99
2.75
0.38
Peyer's patches
Spleen
GL7
FAS
Peyer's patches
Spleen
% GL7+FAS+
***
**
B
Spleen - total B
Spleen - FO
Spleen - MZ
% B220+
%CD23hiCD21lo
%CD23loCD21hi
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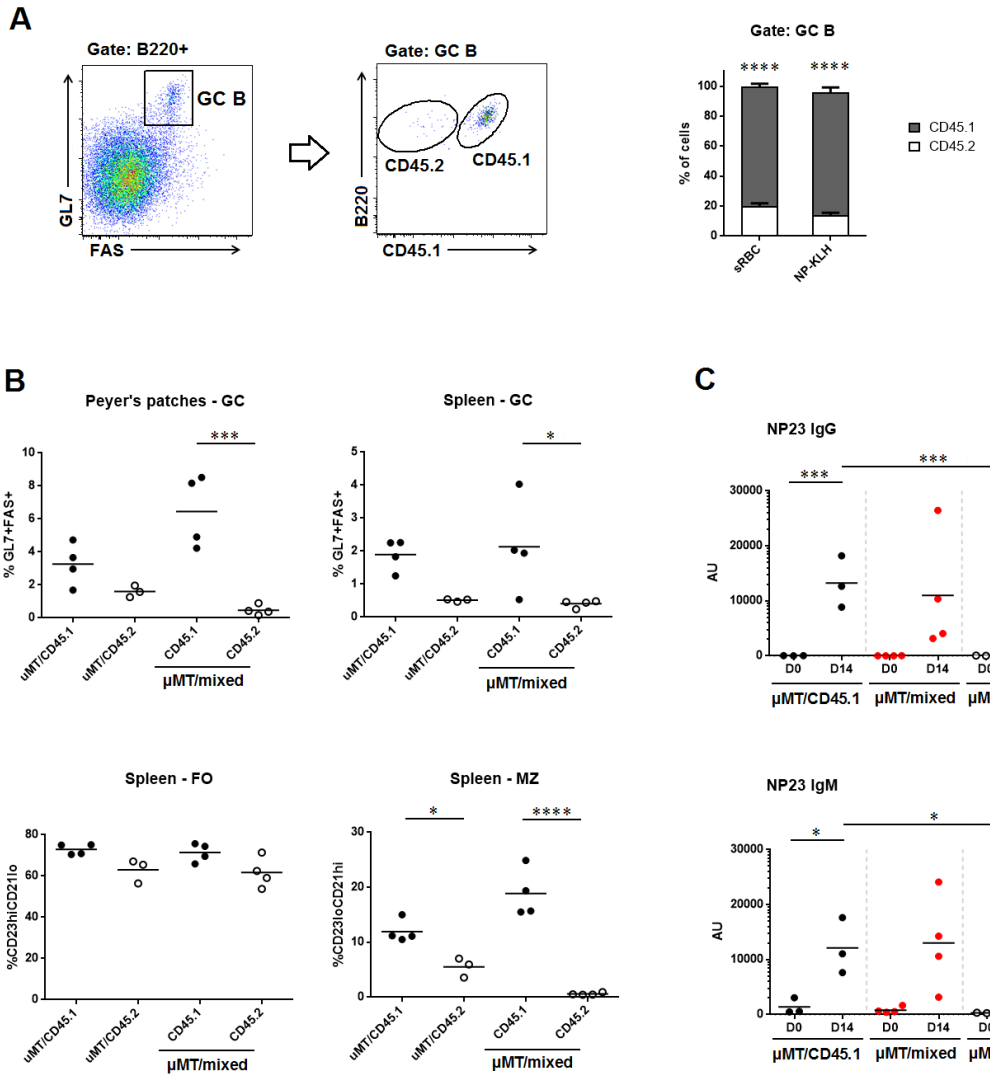


Figure 2-3. GC defect associated with MALT1 deficiency cannot be rescued in the presence of WT B cells. Lethally irradiated µMT mice were reconstituted with CD45.1 (µMT/CD45.1) or *Malt1*$^{-/-}$ (µMT/CD45.2) bone marrow, or a 1:1 mix of the two (µMT/mixed), immunized with either sRBCs or NP-KLH, and analyzed on day 7 or day 14, respectively (n = 3-4 per group). (A) Distribution of GC B cells in the spleens of µMT/mixed animals based on their origin. The gating strategy is depicted in the left panel and the summary is plotted in the right panel. (B) B220^{+} populations were first analyzed for CD45.1 status and the frequency of GC B cells (in both spleens and Peyer's patches), as well as FO and MZ B cells in the spleens were determined by flow cytometry. Results from sRBC and NP-KLH immunizations were similar and only the data from the NP-KLH experiment is shown here. (C) NP-specific IgG and IgM titers in the sera from NP-KLH immunized recipients were quantified by ELISA.

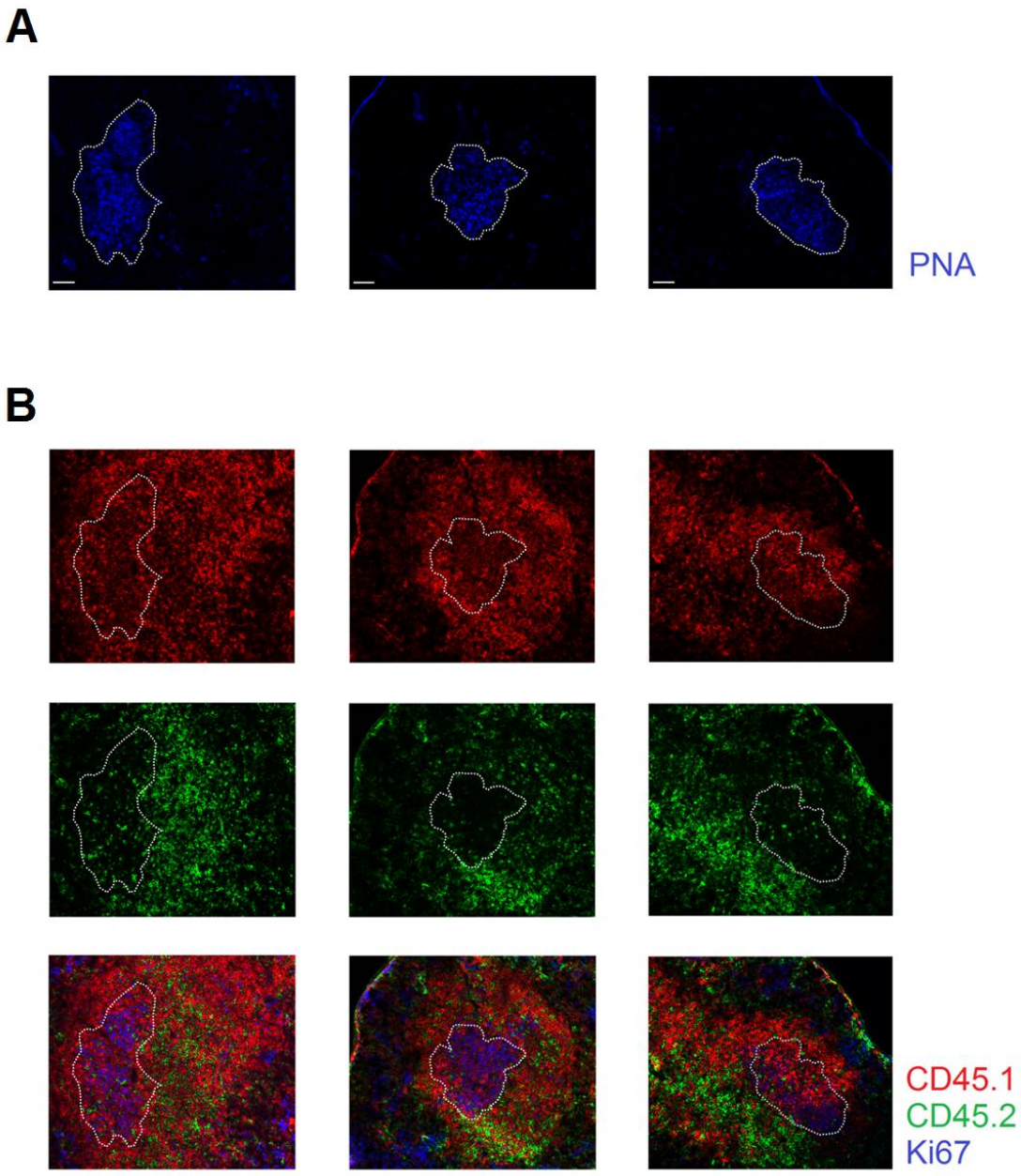


Figure 2-4. *Malt1*$^{-/-}$ B cells were excluded from the GC region in the immunized µMT/mixed chimeras. (A) Consecutive spleen sections from sRBC-immunized µMT/mixed chimeras were first stained with PNA to visualize the GC region, which is outlined by the white line. (B) The distributions of WT- and *Malt1*$^{-/-}$-derived cells within the GC were determined by staining with antibodies specific for CD45.1 (PE) and CD45.2 (FITC), respectively. Proliferating cells were identified by Ki67^{+} (APC) staining. The images were taken at 10x magnification. Scale bars represent 50 µm.

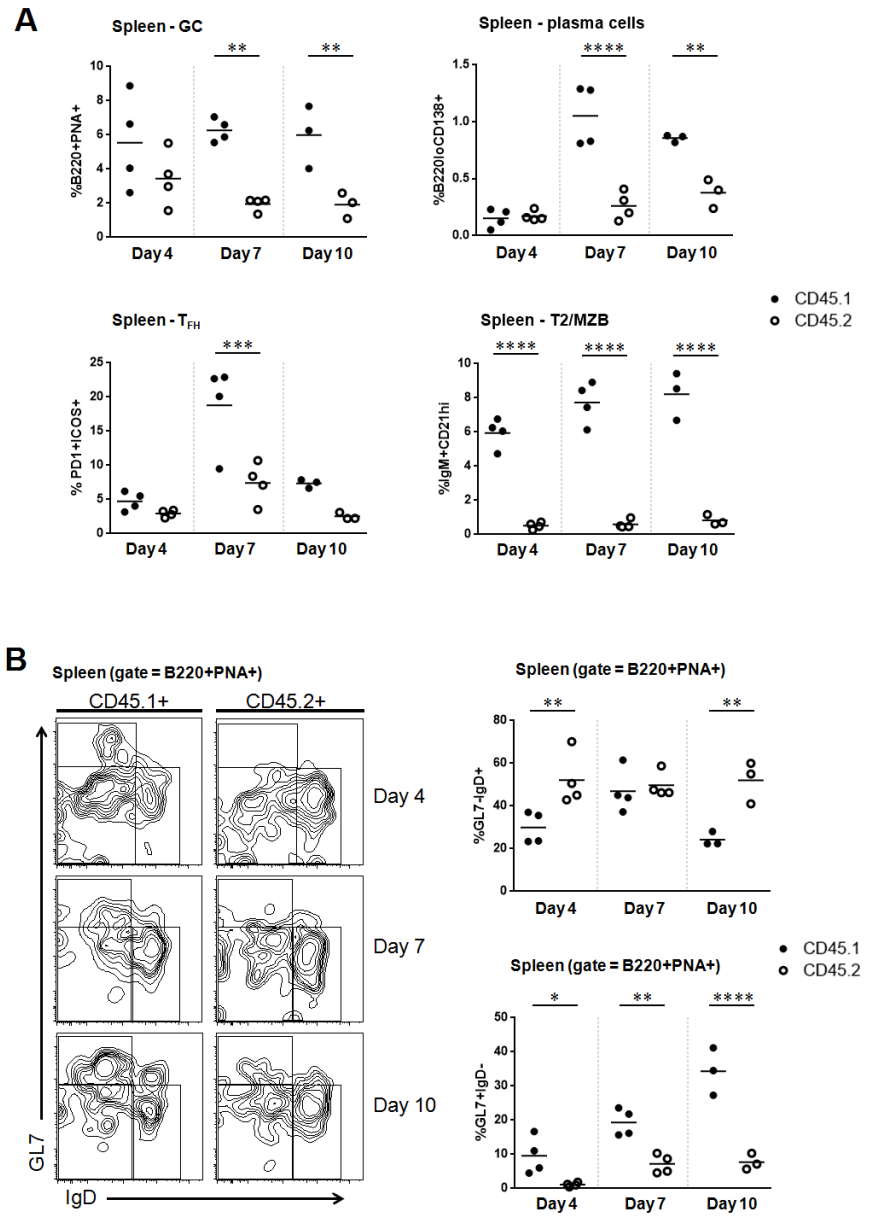


Figure 2-5. B cells intrinsically require MALT1 to initiate GC reactions. Lethally irradiated μMT mice were reconstituted with a 1:1 mix of CD45.1 and *Malt1*$^{-/-}$ bone marrow, immunized with sRBCs, and analyzed at various time points (n = 3-4 per group). (A) B220^{+} B cells or CD3^{+}CD4^{+} T cells were first analyzed for CD45.1 status and frequency of GCs, plasma cells, T2/MZB, and Tfh in the spleens within each CD45.1 or CD45.2 population was determined by flow cytometry. (B) Representative FACS plots showing the progression of GCs in the B220^{+}PNA^{+} populations (left panel). The frequencies of early GL7^{-}IgD^{+} and mature GL7^{+}IgD^{-} GC B cells were plotted (right panels).

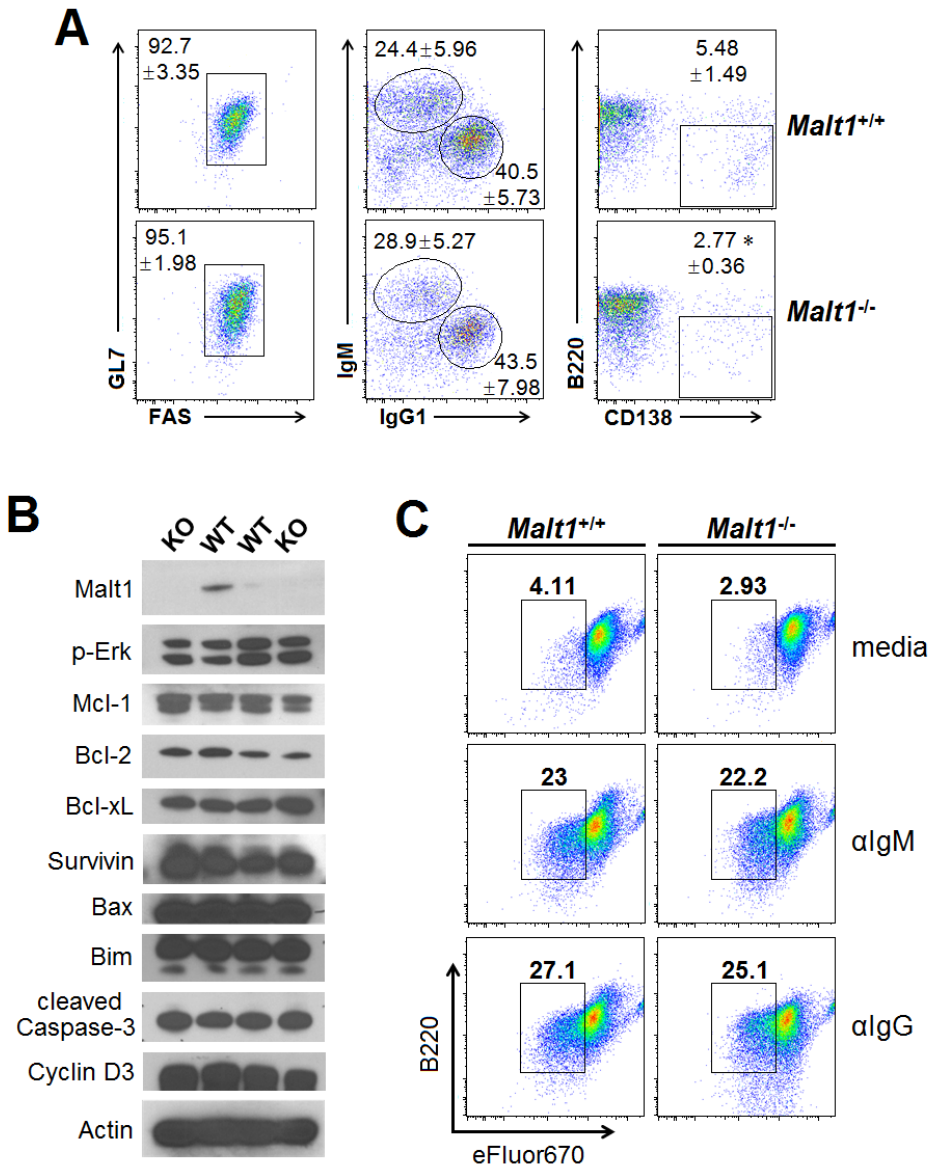
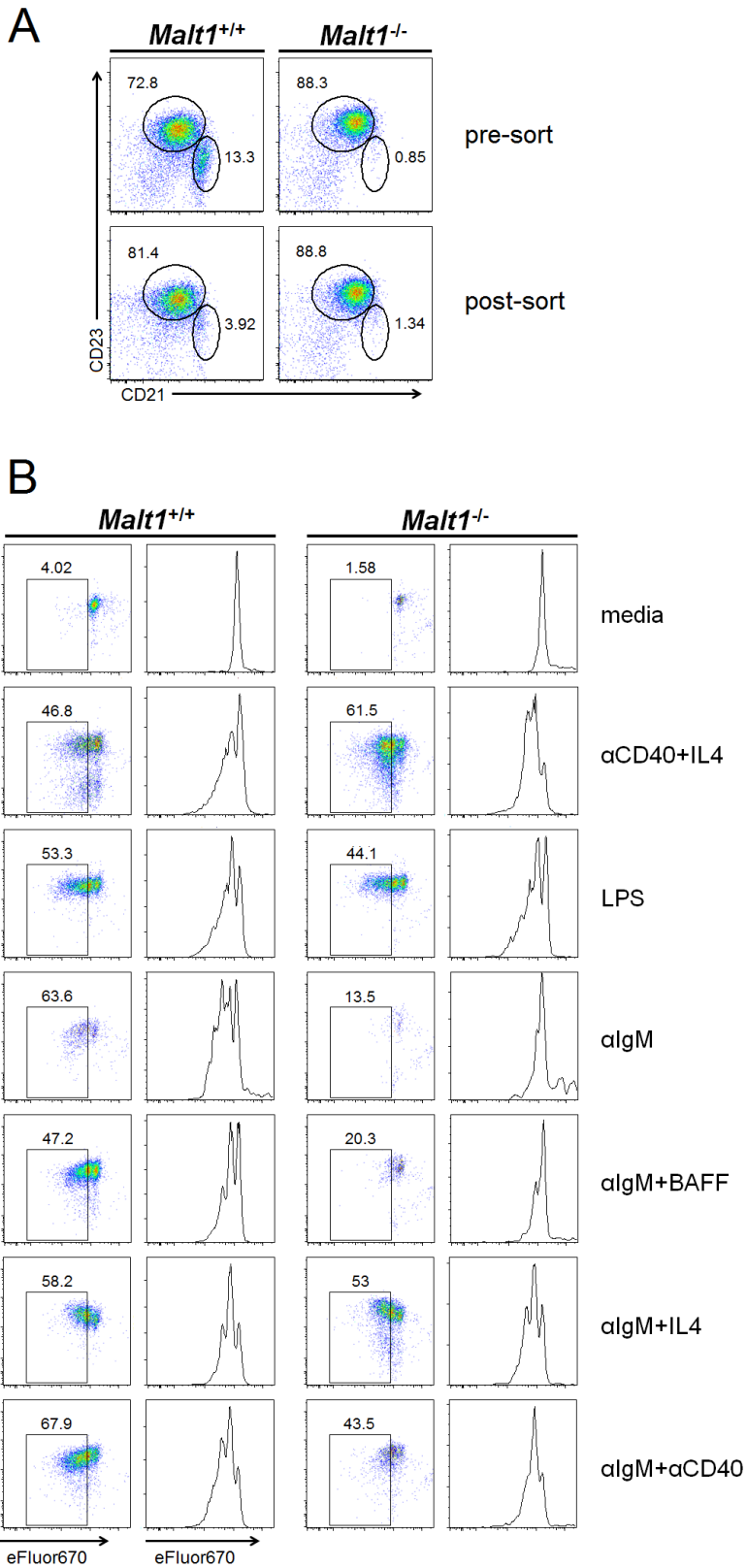


Figure 2-6. MALT1 is dispensable for *in vitro* GC differentiation under strong cytokine stimulation. WT and *Malt1*$^{-/-}$ splenic B cells were cultured with IL4 on 40LB feeder cells for 5 days to allow for induction of phenotypically GC B cells. (A) Characterization of the iGBs by flow cytometric analysis of surface markers. Numbers indicate the percentages of cells in the gate. The data shown here is representative of 5 experiments (n = 5-6 per genotype). (B) Immunoblotting analysis of WT and *Malt1*$^{-/-}$ iGBs. (C) WT and *Malt1*$^{-/-}$ iGBs were removed on day 5 from culture, washed with PBS, labeled with eFluor670, and stimulated with αIgM F(ab')$_2$ or αIgG F(ab')$_2$ for 24 hours. Proliferation was assessed by dilution of eFluor670.

Figure 2-7. MALT1 is specifically required for BCR-induced proliferation. (A) Splenic FO B cells were enriched from WT and *Malt1*$^{-/-}$ mice by depletion of non-B cells (CD43^{+}) and MZ B cells (CD9^{+}). Purity was determined by flow cytometry. (B) Sorted splenic FO B cells were cultured with the indicated stimuli for 3 days and cell proliferation was measured by dilution of the eFluor670 dye. The percentage of divided cells is shown in each dot plot. Representative plots from 2 independent experiments (n = 2 per genotype) are shown.


A
Malt1+/+
Malt1-/-
72.8
13.3
88.3
0.85
pre-sort
81.4
3.92
88.8
1.34
post-sort
CD23
CD21
B
Malt1+/+
Malt1-/-
4.02
1.58
media
46.8
61.5
αCD40+IL4
53.3
44.1
LPS
63.6
13.5
αIgM
47.2
20.3
αIgM+BAFF
58.2
53
αIgM+IL4
67.9
43.5
αIgM+αCD40
B220
eFluor670
eFluor670

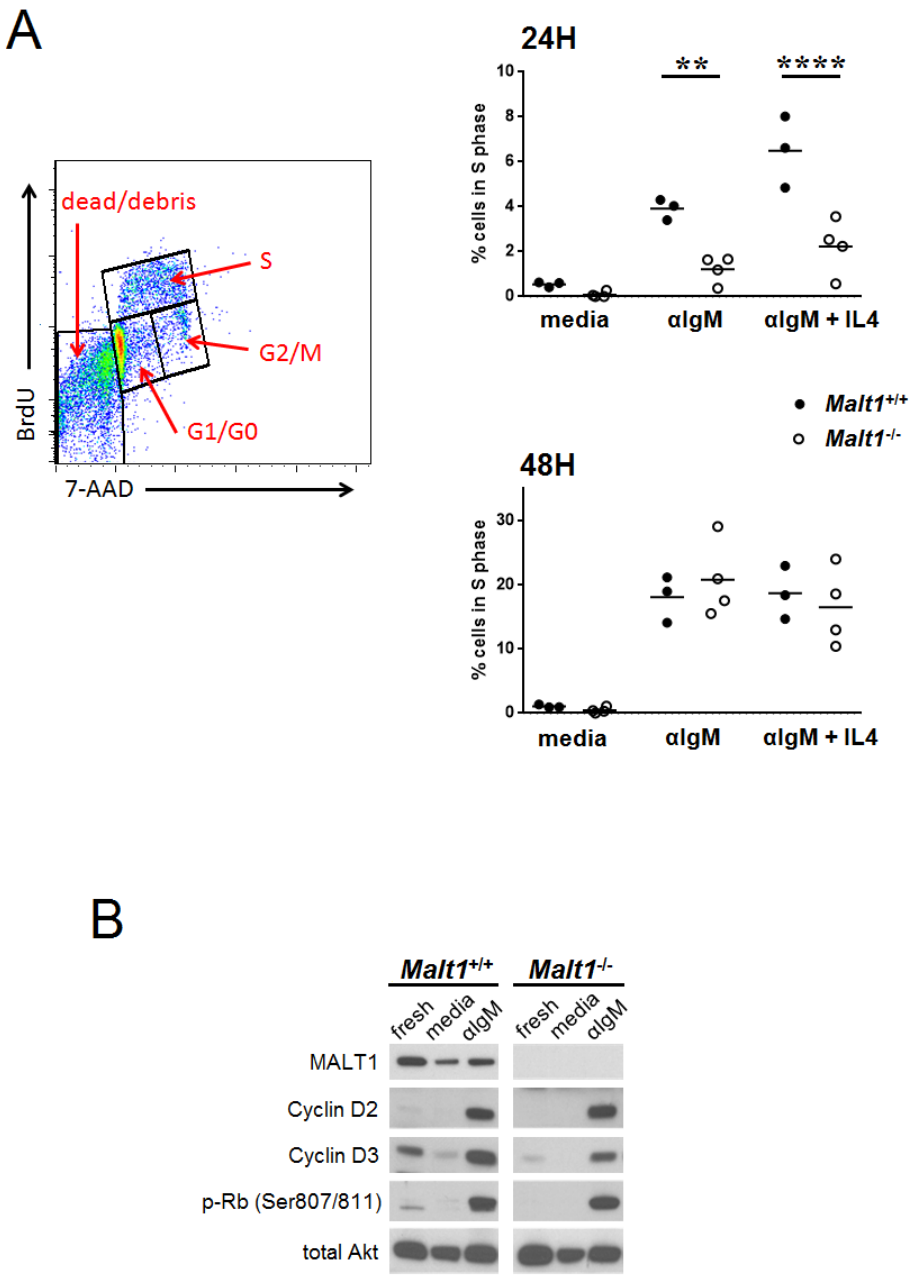


Figure 2-8. MALT1 is required for efficient BCR-induced cell cycle entry. (A) WT and *Malt1*$^{-/-}$ splenic B cells were stimulated as indicated and for 24 or 48 hours. The cells were pulsed with BrdU for 1 hour prior to harvest, fixed and permeabilized, and followed by staining with αBrdU and 7-AAD. The left panel depicts the gating strategy. The percentage of S phase cells is plotted in the right panels. (B) Immunoblot analysis of WT and *Malt1*$^{-/-}$ splenic B cells that were freshly isolated or cultured with or without αIgM F(ab')$_2$ for 24 hours. Blots are representative of at 3 independent experiments (n = 3-4 per genotype).

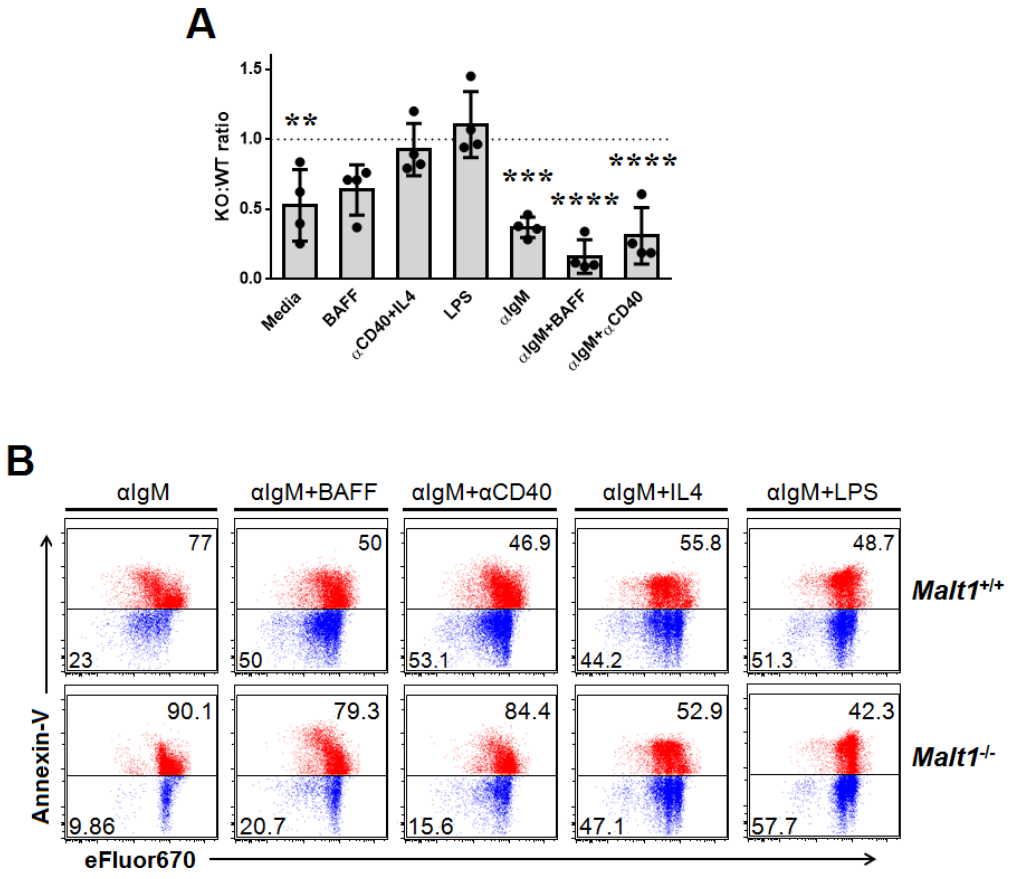


Figure 2-9. MALT1 is required for protection from BCR-induced apoptosis. (A) Viability of WT and *Malt1*$^{-/-}$ B cells cultured with indicated stimuli for 3 days was assessed by flow cytometry looking at the FSC versus SSC profiles. Results from 4 experiments were pooled together (n = 4 per genotype). To account for variation across experiments, the viability of *Malt1*$^{-/-}$ B cells was normalized to that of WT littermate in individual experiments and the mean KO:WT ratios were plotted. Error bars represent SD. Significant deviation from the value 1 (ie. no difference between *Malt1*$^{-/-}$ and WT) was marked with asterisks. (B) WT and *Malt1*$^{-/-}$ splenic B cells were cultured in the presence of αIgM alone or in combination with other stimuli for 3 days. Cell proliferation and apoptosis were evaluated by combined eFluor670 dilution and Annexin V staining. The cells were gated based on their Annexin V status and the percentage of cells within each gate is indicated. The result is representative of 3 independent experiments (n = 3 per genotype).

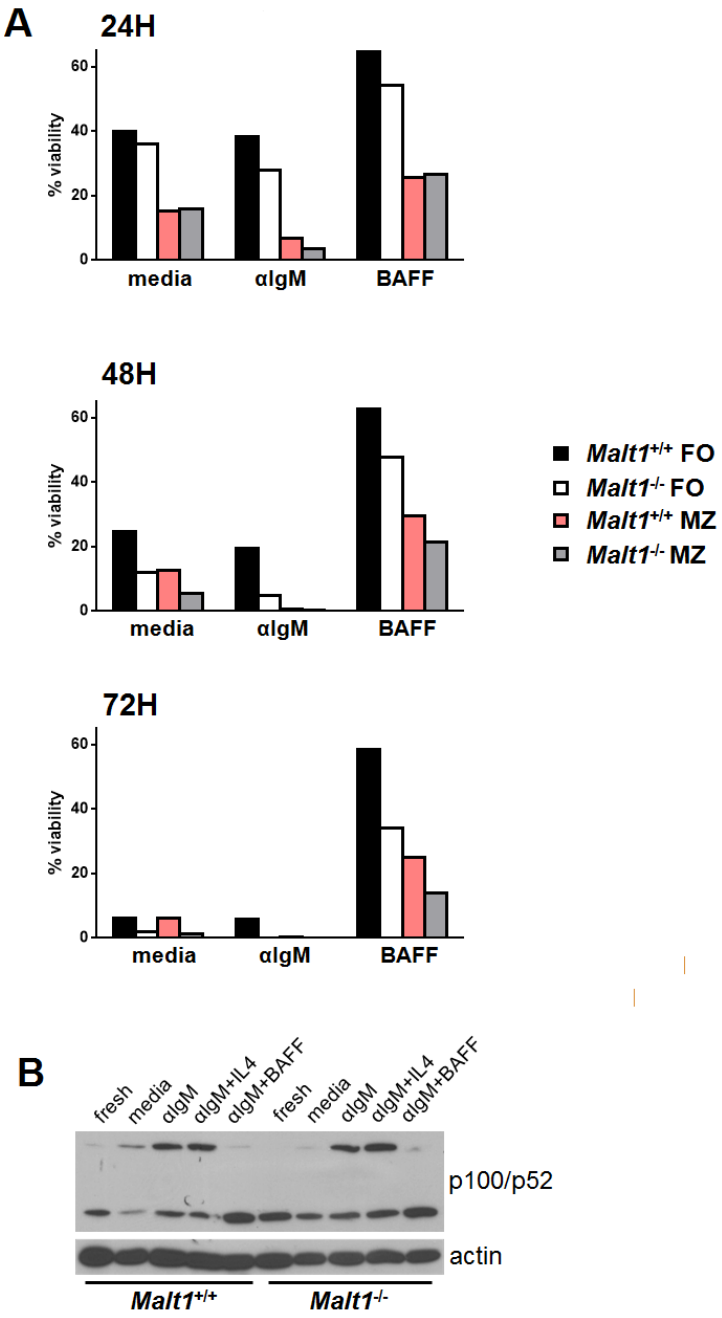


Figure 2-10. MALT1 is required for survival of both FO and MZ B cells. (A) FACS-sorted FO ($B220^{+}CD21^{lo}CD23^{hi}$) and MZ ($B220^{+}CD21^{hi}CD23^{lo}$) B cells from WT and *Malt1*$^{-/-}$ spleens were cultured for up to 72 hours with the indicated stimuli. Splenocytes from two animals from each genotype were pooled for FACS-sorting. Viability was assessed every 24 hours by flow cytometry looking at the FSC versus SSC profile. (B) Immunoblot analysis of WT and *Malt1*$^{-/-}$ splenic B cells, either freshly isolated or cultured for 24 hours with the indicated stimuli.

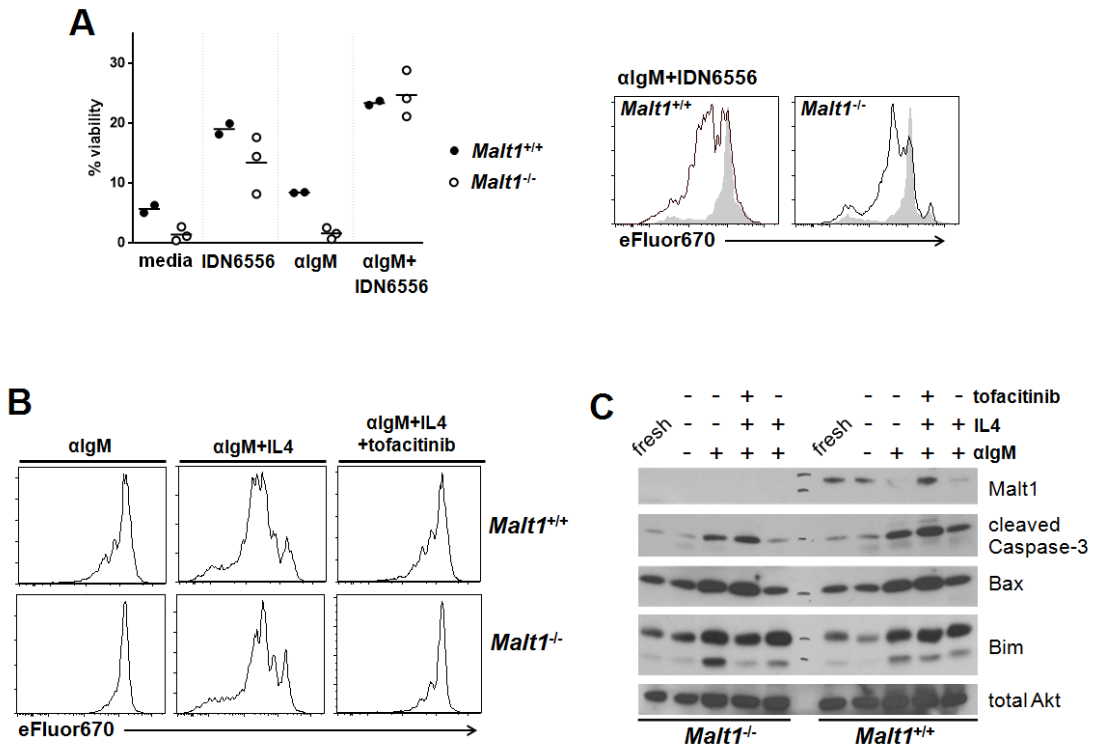


Figure 2-11. MALT1 protects B cells from caspase-dependent cell death. (A) WT and *Malt1*$^{-/-}$ splenic B cells were cultured as indicated for 3 days and assessed for viability (FSC vs SSC profile) and cell proliferation (eFluor670 dilution) by flow cytometry. Results from 2 experiments (n = 2-3 per genotype) were pooled. (B) WT and *Malt1*$^{-/-}$ splenic B cells were stimulated with αIgM in the presence of IL4 or tofacitinib for 3 days and proliferation was determined by flow cytometric measure of eFluor670 dilution The histograms are representative of 2 independent experiments (n = 2 per genotype). (C) Immunoblot analysis of WT and *Malt1*$^{-/-}$ splenic B cells, either freshly isolated or cultured for 24 hours with the indicated stimuli. Total Akt was used as loading control. The blots are representative of 2 independent experiments (n = 2-3 per genotype).

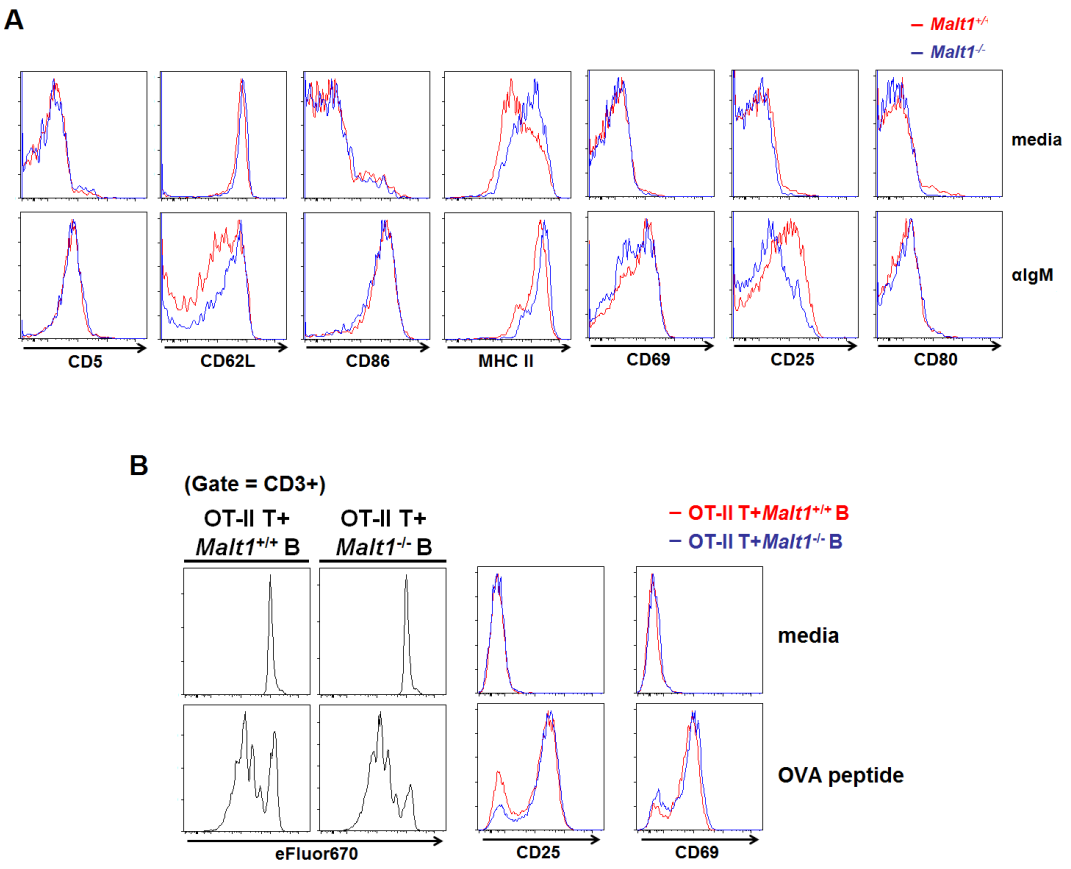


Figure 2-12. MALT1-deficient B cells retain the capacity to prime T cells. (A) WT and *Malt1*$^{-/-}$ splenic B cells were stimulated with αIgM for 24 hours and activation was assessed by flow cytometry. Overlaid histograms of a panel of surface markers in WT (red) and *Malt1*$^{-/-}$ (blue) B cells are shown. The data is representative of 3 independent experiments (n = 3 per genotype). (B) T cells purified from OT-II mice were labeled with eFluor670 and cultured with CD43-depleted splenic B cells from WT or *Malt1*$^{-/-}$ animals, with or without OVA peptide. Proliferation and activation of T cells were assessed 3 days later by flow cytometry. Representative plots from 3 independent experiments are shown (n = 3 per genotype).

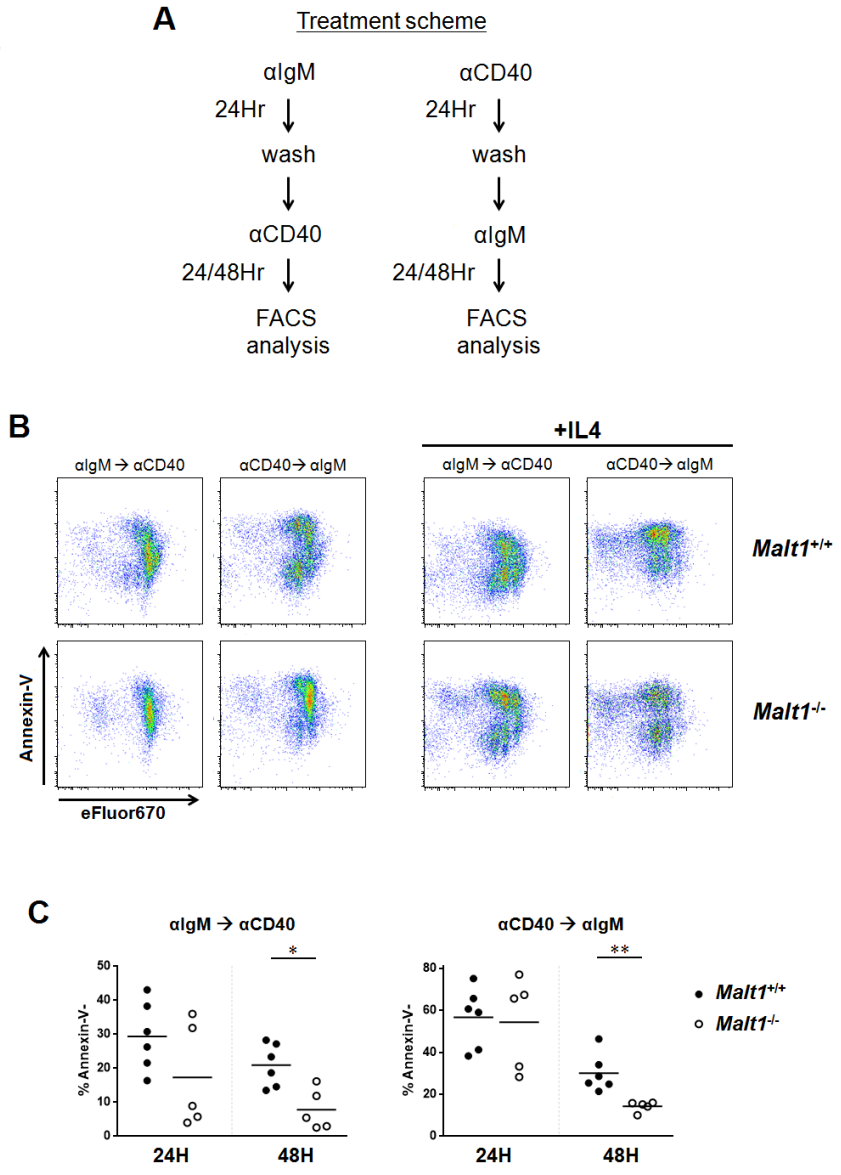
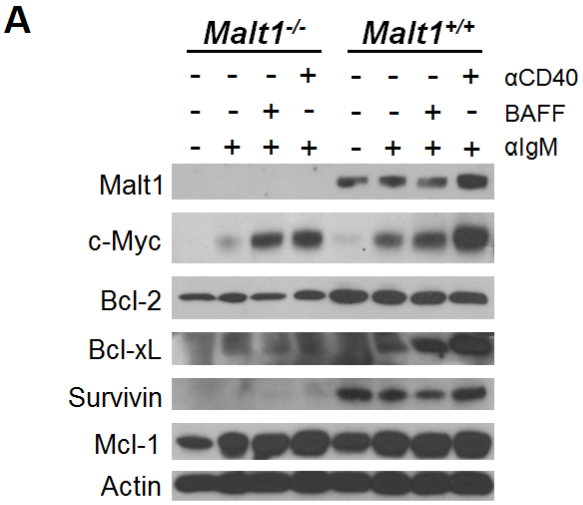
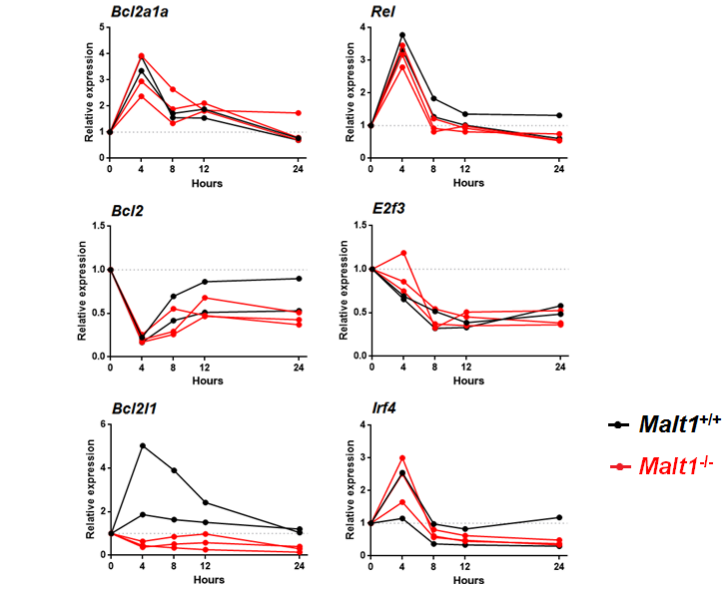
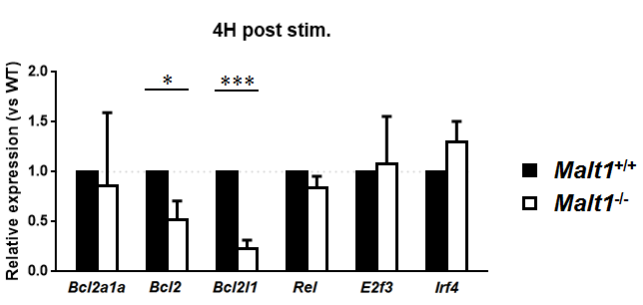


Figure 2-13. Help from T cells cannot rescue the BCR-induced survival and proliferative defects in *Malt1*$^{-/-}$ B cells. (A) Schematic diagram outlining the sequential treatment of αIgM and αCD40 (top panel). (B) Cells were harvested at 24 or 48 hours post-second stimulus and assessed by flow cytometry analysis of combined Annexin V staining and eFluor670 dilution (bottom panel). FACS plots are representative of 5 experiments (n = 5-6 per genotype). (C) Viability was determined by gating on the Annexin V^{-} cells and the results from 5 experiments were pooled and plotted.

Figure 2-14. MALT1 is required for sustained Bcl-2 expression and Bcl-xL upregulation in BCR-activated B cells. (A) Immunoblot analysis of WT and *Malt1*$^{-/-}$ splenic B cells stimulated as indicated for 24 hours. Blots are representative of 3 independent experiments (n = 3 per genotype). (B) Time-course induction of several c-Rel target genes in response to αIgM treatment was monitored by real-time PCR. WT (n = 2, black) and *Malt1*$^{-/-}$ (n = 3, red) splenic B cells were stimulated with 1 µg of αIgM F(ab')2 for 4, 8, 12, and 24 hours. At each time point, expression of each gene was first corrected for expression of the reference gene *Gapdh*, then the fold-changes, relative to the zero-time point of the same animal, are plotted. (C) Expression of same set of c-Rel target genes at 4 hours post-stimulation in *Malt1*$^{-/-}$ B cells was normalized to that of WT.


A
Malt1-/-
Malt1+/+
- - - + - - - + αCD40
- - + - - - + - BAFF
- + + + - + + + αIgM
Malt1
c-Myc
Bcl-2
Bcl-xL
Survivin
Mcl-1
Actin


B
Bcl2a1a
Rel
Bcl2
E2f3
Bcl2l1
Irf4
Relative expression
Hours
Malt1+/+
Malt1-/-


C
4H post stim.
Relative expression (vs WT)
*
***
Bcl2a1a
Bcl2
Bcl2l1
Rel
E2f3
Irf4
Malt1+/+
Malt1-/-

Chapter 3. Partial rescue of germinal center defect in *Malt1*$^{-/-}$ mice with Bcl-2 overexpression

Introduction

Apoptosis plays an essential role in the development and maintenance of cellular homeostasis of the immune system. Since either insufficient or excessive apoptosis has deleterious effects, the survival and death of hematopoietic cells, including B lymphocytes, must be precisely regulated. Apoptosis in B cells is mediated by two pathways, both converge on the activation of effector caspases (158). The extrinsic pathway is intiated by binding of the death receptor by its ligand, such as the Fas receptor-ligand system, and leads to activation of the initiator caspase-8 via formation of the death-inducing signaling complex. The intrinsic pathway can be activated by a multitude of stimuli and regulated by the interactions between the pro- and anti-apoptotic members of the Bcl-2 family. This results in permeabilization of the mitochondrial membrane and the release of cytochrome C, and subsequent activation of caspase-9.

During the later stages of B cell maturation, the transcription factor NF-kB protects against apoptosis via induction of gene transcripts encoding pro-survival Bcl-2 homologs (132). NF-κB inactivation in mature B cells, achieved by the administration of fungal metabolic gliotoxin, is sufficient to cause drastic B cell death by downregulating Bcl-2 (159). The Bcl-2 family of pro-survival proteins are differentially regulated during B cell development; while NF-κB induces both Bcl-2 and A1 in immature B cells and only Bcl-2 in quiescent mature B cells (132),

additional stmulation is required for the upregulation of Bcl-xL and A1 in mature B cells (160, 161).

The BCR is essential for provision of survival signals for maintaining the mature B cells pool, as deletion of the *Igh* gene resulted in complete loss of B cells (43, 162). On the other hand, BCR can also initiates apoptosis, most evidently in immature B cells after binding of self-antigens to surface IgM, thereby enabling negative selection (163). While BCR crosslinking also induces death in mature B cells, the effect is less pronounced than in immature B cells, due to the protective function of c-Rel by inducing *Bcl2l1* and *Bcl2a1* gene transcription (154, 160). In contrast, RelA plays a less of a role in the survival of BCR-activated mature B cells, presumably because of difference in the kinetics of RelA versus c-Rel induction (164).

Morever, viability can be further enhanced during BCR crosslinking with the inclusion of other stimuli including LPS, BAFF, and CD40L (165). Notably, signaling via CD40 is the basis of T cell help and activates both canonical and non-canonical NF-κB pathways, mediating B cell survival via induction of pro-survival genes such as Bcl-xL independent of c-Rel (166). BAFF signal attenuates apoptosis by several mechanisms, including directly targeting of Bim (167) and activation of Pim-2 expression (168).

As introduced in Chapter 1, GC is a unique site where clonal expansion and selection of antigen-specific B cells take place. To ensure the elimination of low-affinity and autoreactive cells generated by somatic mutations, GC B cells are rendered sensitive to apoptosis by a specific downregulation of the Bcl-2 protein (169), as well as increase in Fas expression (170). However, Fas-induced death of B cells can

be antagonized by signals transduced by the engagement of BCR and also treatment with IL4 (171, 172). On the other hand, survival of GC B cells is equally important, given that it directly influences the attributes of the effectors cells, and the key mediators are other members of the Bcl-2 family, including Bcl-xL and Mcl-1 (156). A1 expression is also found in the GC, but its role in GC B cell survival has not yet been fully established (158). While all of the pro-survival proteins in the Bcl-2 family functions by maintaintaing mitochondrial integrity, they differ from each other in their ability to inhibit the pro-apoptotic memers of the family and therefore, are uniquely involved during B cell development and GC reactions.

Ectopic expression of Bcl-2 in the B lineage has been demonstrated to result in an expansion of resting mature B cells that showed enhanced survival *in vitro* (173, 174). Furthermore, Bcl-2 trasgenic (Tg) animals also displayed an accumulation of plasma cells and exhibit amplified and prolonged response upon immunization (175). In Chapter 2, the defective GC phenotype in MALT1-deficient animals was linked to impaired survival and delayed cell cycle entry of B cells in response to BCR stimulation. Based on these findings, I hypothesize that providing *Malt1*$^{-/-}$ B cells with a survival signal would be sufficient to overcome the block in GC formation. Therefore, in the present chapter, the effect of B-cell specific enforced expression of Bcl-2 in the MALT1-deficient background is examined. While I find that Bcl-2 transgene alone does not rectify the GC phenotype, in the presence of WT T cells, ectopic expression of Bcl-2 in *Malt1*$^{-/-}$ B cells leads to a partial rescue in GC formation. However, plasma cell differentiation and production of antigen-specific antibodies remain abrogated in the absence of MALT1.

Materials and Methods

Mice

Bcl-2 Tg x *Malt1*$^{-/-}$ mice were generated by intercrossing Eμ-Bcl-2-22 mice (The Jackson Laboratory) with *Malt1*$^{-/-}$ mice. Littermates without the Bcl-2 transgene or of the genotype *Bcl-2 Tg* x *Malt1*$^{+/-}$ were used as controls. For generation of μMT chimeras, bone marrow cells from *Bcl-2 Tg* x *Malt1*$^{+/-}$ or *Bcl-2 Tg* x *Malt1*$^{-/-}$ animals (n = 2 each) were pooled and injected intravenously into four sublethally irradiated (5 Gy) μMT recipients.

sRBC immunization and antibody detection

Mice were injected intraperitoneally with 100 μL of packed sRBCs and sacrificed after 7 days for analysis by flow cytometry and histology. Sera were collected on day 0 and day 7 for measuring the levels of sRBC-specific IgM and IgG1 levels using the flow cytometry-based approach described in Chapter 2.

Flow cytometry

Single cell suspensions were prepared from the spleens and Peyer's patches and stained according to the same procedures outlined in Chapter 2.

Histology

Spleens from the immunized animals were frozen, sectioned, fixed, and stained following the same steps as described in Chapter 2.

In vitro proliferation and apoptosis assay

CD43-depleted splenic B cells were labeled with eFluor670 and cultured in complete RPMI with or without the indicated stimuli. Proliferation was determined by dilution of the eFluor670 dye and apoptosis was detected by Annexin-V staining. Alternatively, cell viability was assessed by the forward/side scatter profile of the cells.

In vitro antibody-secreting cell (ASC) differentiation assay

CD43-depleted splenic B cells were cultured in complete RPMI with LPS or αCD40 plus IL4 for 4 days. The presence of ASCs was assessed by flow cytometry.

Results

Ectopic expression of Bcl-2 does not rescue GC defect in MALT1-deficient mice

To determine whether ectopic expression of Bcl-2 can rescue the GC defect caused by MALT1 deficiency, *Malt1*$^{-/-}$ mice were interbred with Eμ-Bcl-2-22 transgenic mice (175), which specifically express the human Bcl-2 transgene in the B lineage. Enforced Bcl-2 expression on the *Malt1*$^{+/-}$ background (*Bcl-2 Tg* x *Malt1*$^{+/-}$) resulted in expanded GC B population in the Peyer's patches, while the frequency of Tfh cells remained comparable with that detected in WT animals (Figure 3-1A). In contrast, *Bcl-2 Tg* x *Malt1*$^{-/-}$ mice were almost completely devoid of GC B cells in the Peyer's patches, which correlated with a severe reduction in Tfh cells. Further characterization of the spleens revealed a similar expansion of B220^{+} B cells in both *Malt1*$^{+/-}$ and *Malt1*$^{-/-}$ animals expressing Bcl-2 transgene. (Figure 3-1B). However, Bcl-2 overexpression did not rescue the MZ B compartment in *Malt1*$^{-/-}$ mice since only a few CD21^{hi}CD23^{lo} cells were detected in the spleen (Figure 3-1C). In comparison to WT animals, *Malt1*$^{+/-}$ mice expressing the Bcl-2 transgene also exhibited a reduced MZ and increased FO B subsets, in line with what has been previously described (176).

Immune response of *Bcl-2 Tg* x *Malt1*$^{-/-}$ mice to TD antigens was also examined. In line with the findings in the Peyer's patches, there was a drastic redction (~50-fold) in GC B cells in *Bcl-2 Tg* x *Malt1*$^{-/-}$ mice upon sRBC immunization (Figure 3-2A). The GC defect further translates into a significant decrease (~15-fold) in plasma cell differentiation (Figure 3-2B). Consistently, the production of sRBC-

specific IgM and IgG1 was abrogated in *Bcl-2 Tg* x *Malt1*$^{-/-}$ animals (Figure 3-2 C). Collectively, these results indicate that ecoptic expression of Bcl-2 in B cells is not sufficient to rectify the GC response in *Malt1*$^{-/-}$ animals.

In the presence of WT T cells, Bcl-2 overexpression partially recues GC formation but not plasma cell differentiation in Malt1-deficient B cells

To rule out the possibility that the impairment in GC formation observed in the *Bcl-2 Tg* x *Malt1*$^{-/-}$ mice was due to defective T cell response, bone marrow cells from *Bcl-2 Tg* x *Malt1*$^{+/-}$ or *Bcl-2 Tg* x *Malt1*$^{-/-}$ littermates were transferred into sublethally irraidiated B cell-deficient µMT mice, generating µMT/*Bcl-2 Tg* x *Malt1*$^{+/-}$ and µMT/*Bcl-2 Tg* x *Malt1*$^{+/-}$ chimeras, respectively. Similar to the system described in Chapter 2, the B cells in the recipeient mice would be completely donor-derived, while the rest of the cells of the hematopoeitic lineage would be of a mix of both donor and recipient origins. Remarkably, immunization with sRBCs elicited GC response in µMT mice reconstituted with *Bcl-2 Tg* x *Malt1*$^{-/-}$ bone marrow, albeit not as robust (~2-fold reduction) as in µMT/*Bcl-2 Tg* x *Malt1*$^{+/-}$ animals (Figure 3-3A, middle panels). A similar proportion of the GC B cells in either set of chimeras stained positive for IgG1, confirming that MALT1 is not essential for class-switch recombination (Figure 3-4A, bottom panels). However, tthe frequencies of plasma cells were substantially decreased in µMT/*Bcl-2 Tg* x *Malt1*$^{-/-}$ animals (Figure 3-3A, top panels). While the two sets of chimerice mice showed comparable frequencies of

splenic CD4+ T cells, µMT/*Bcl-2 Tg* x *Malt1*$^{-/-}$ animals also showed a 2-fold reduction in Tfh population (Figure 3-3B), which correlated with the reduction in GC B cells.

Unlike the spleens, the Peyer's patches from each chimera set showed similar frequencies of total B cells, GC B, and CD4+ T cells (Figure 3-3C). Interestingly, there was a modest but significant increase in the Tfh population in µMT/*Bcl-2 Tg* x *Malt1*$^{-/-}$ mice (Figure 3-3C). The effect of Bcl-2 overpression on FO and MZ B cell subsets were also examined. Recapitulating the phenotypes of intact *Bcl-2 Tg* x *Malt1*$^{-/-}$ animals, µMT/*Bcl-2 Tg* x *Malt1*$^{-/-}$ mice showed a slight increase in FO B cells and a significant reduction in MZ B compartment (Figure 3-3D). Also similar to intact *Malt1*$^{-/-}$ and *Bcl-2 Tg* x *Malt1*$^{-/-}$ animals, there was an expansion of the IgM^{hi}IgD^{hi} population in the µMT/*Bcl-2 Tg* x *Malt1*$^{-/-}$ spleens (Figure 3-3E).

Histology of the spleen sections from the immunized chimeras was performed to understand the nature of the reduction in GC B cells. In the µMT/*Bcl-2 Tg* x *Malt1*$^{-/-}$ chimeras, well-defined PNA^{+} clusters were detected in almost all the follicles examined, but the size of each cluster was smaller compared to the ones observed in µMT/*Bcl-2 Tg* x *Malt1*$^{+/-}$ mice (Figure 3-4A). Proliferating cells, indicated by Ki67 positivity, were detected in both groups. Despite of the partial rescue in GC formation, the defect in antibody response prevailed in µMT/*Bcl-2 Tg* x *Malt1*$^{-/-}$ mice, since the levels of sRBC-specific IgM and IgG1 remained significantely reduced compared to the levels measured in µMT/*Bcl-2 Tg* x *Malt1*$^{+/-}$ mice (Figure 3-4B).

To investigate whether the reduction in GC B cells observed at the peak of GC response was due to impaired maturation of early GC founder cells, PNA, GL7, and IgD staining was incorporated to resolve subsets of GC B cells in the immunized µMT

chimeras. Total GC cohorts were first identified by staining with the pan-GC marker PNA and the frequencies of PNA^+ B cells were consistently reduced by 2-fold in µMT/*Bcl-2 Tg* x *Malt1*$^{-/-}$ mice (Figue 3-5A). However, there was no appreciable difference in the proportions of mature IgD^-GL7^+ and early IgD^+GL7^- GC B cells in µMT/*Bcl-2 Tg* x *Malt1*$^{-/-}$ animals relative to µMT/*Bcl-2 Tg* x *Malt1*$^{+/-}$ controls (Figure 3-5B). Collectively, these data indicate that *Bcl2* transgene partially restored the ability of MALT1-deficient animals to initate GC response in the presence of WT T cells. While MALT1 is dispenable for the maturation of GC B cells, it is required for differentiation into plasma cells and also for the production of immunogen-specific antibodies.

Enforced Bcl-2 expression prolongs the lifespan of MALT1-defcient B cells but does not restore proliferation in response to BCR stimulation

To investigate whether enforced expression of Bcl-2 rescues the responses MALT1-deficient B cells to BCR stimulation, proliferation and apoptosis of splenic B cells, in response to αIgM alone or in combination with other stimuli, were monitored by combined Annexin-V and eFluor670 labeling. Similar to WT B cells, *Bcl-2 Tg* x *Malt1*$^{+/-}$ B cells underwent several rounds of division after 72 hours in culture in response to IgM, and proliferation was further enhanced by the addition of BAFF or αCD40 (Figure 3-6A). Notably, *Bcl-2 Tg* x *Malt1*$^{-/-}$ B cells cycled at most two rounds to BCR triggering. While the addition of BAFF or αCD40 had minimal effect on αIgM-induced proliferation in *Bcl-2 Tg* x *Malt1*$^{-/-}$ B cells, the inclusion of either IL4 or

LPS triggered a more robust proliferative profile that was more similar to what *Bcl-2 Tg* x *Malt1*$^{+/-}$ B cells exhibited (Figure 3-6A). B cells from intact *Malt1*$^{+/-}$ and *Malt1*$^{-/-}$ animals were included as controls and enforced Bcl-2 expression clearly blocked apoptosis in both resting and BCR-activated MALT1-sufficient and MALT1-deficient B cells, compared to their respective controls without the transgene (Figure 3-6B). Interestingly, there was no difference in viability between *Bcl-2 Tg* x *Malt1*$^{+/-}$ and *Bcl-2 Tg* x *Malt1*$^{-/-}$ B cells that were cultured in media alone, but viability of *Bcl-2 Tg* x *Malt1*$^{-/-}$ B cells was reduced in all BCR-stimulating conditions, suggesting that the protective mechanism of MALT1 following BCR activation also includes a Bcl-2-independent component.

The *in vitro* survival responses of *Bcl-2 Tg* x *Malt1*$^{+/-}$ and *Bcl-2 Tg* x *Malt1*$^{-/-}$ B cells to other stimuli were also explored and the incubation period was extended from 72 to 96 hours to see if more differences can be detected. However, viabilities were similar between the two when cultured under resting condition or in the presence of stimuli excluding αIgM F(ab')$_2$ (Figure 3-7). Even in the presence of the *Bcl2* transgene, MALT1-deificent B cells exhibited reduced viability when stimulated with αIgM and the inclusion of BAFF or αCD40 failed to enhance viability. Taken together, these results suggest that MALT1 is not only required for the survival of resting and BCR-activated B cells, it also drives proper proliferative response to BCR crosslinking.

MALT1 is essential for ASC differentiation in vitro

Previously published reports have established that MALT1 is required for TI humoral response (91, 92). However, I have consistenly observed that LPS-induced proliferation remains intact in MALT1-deficient B cells. Therefore, the effect of MALT1 on the differentiation of antibody-secreting cells (ASC) was further examined. Due to the fact that the analysis of immune responses *in vivo* can be influenced by B cell-extrinsic factors including T cell priming and activation, *in vitro* assay under defined conditions were utilized instead. When splenic B cells from *Malt1*$^{+/-}$ mice were stimulated with LPS, a portion of the cells differentiated into the ASC phenotype, defined by B220^{lo}CD138^{+}, after four days in culture (Figure 3-8A, top panel). A smaller frequency was detected when stimulated with αCD40+IL4, but the level of CD138 was higher, leading to a more distinct separation of the ASC population (Figure 3-8A, bottom panel). In comparison, there was a 3-fold reduction in ASC formation in MALT1-deficient B cells in response to LPS stimulation, and a 30-fold decrease in response to αCD40+IL4. Furthermore, enforced Bcl-2 expression failed to reverse the block in ASC differentiation in *Malt1*$^{-/-}$ B cells, in reponse to either LPS or αCD40+IL4 (Figure 3-8B). Consistent with the *in vivo* findings, these results suggest that MALT1 is necessary for the generation of ASCs.

Discussion

The outcome of B cell responses to antigenic or mitogenic activation reflects a balance between the relative rates of cell division, death, and differentiation. The findings presented thus far are in favor of the model that, in the absence of MALT1, the balance is tipping toward apoptosis. Enforced expression of Bcl-2 clearly expanded the B cell compartment in *Malt1*$^{-/-}$ animals and enhanced the survival of resting MALT1-deficient B cells *in vitro*. However, viability of *Bcl-2 Tg* x *Malt1*$^{-/-}$ B cells was reduced in all stimulating conditions involving αIgM stimulation, suggesting that the protective mechanism of MALT1 following BCR activation also includes a Bcl-2-independent component. Based on the immunoblotting and gene expression analyses shown in Chapter 2, a likely candidate is Bcl-xL. Although overexpression of either Bcl-2 or Bcl-xL leads to accumulation of mature B cells, intriguingly, Bcl-xL but not Bcl-2 becomes rapidly upregulated upon activation via surface immunoglobulin, LPS, or CD40 signaling (173). Furthermore, within the GC, CD40-CD40L interaction rescues centrocytes from apoptosis primarily via the induction of Bcl-xL (177). More definitive experiments are required to verify whether combined enforced expression of Bcl-xL and Bcl-2 in B cells can completely reverse the defects associated with MALT1 deficiency. How MALT1 regulates the expression of both Bcl-2 and Bcl-xL also awaits further analysis. Previous studies have identified c-Rel as the key transcription factor in protecting B cells from BCR-mediated apoptosis, partly via induction of Bcl-xL and A1 (99, 154). In contrast, the link between Bcl-2 and NF-κB in B cells is less firmly established and the regulation of Bcl-2 may

requires additional mechanisms. Interestingly, the downregulation of Bcl-2 in GC B cells occurs not at the transcription level but rather at the translation step (178), thus highlighting the importance of post-transcriptional control. One possible mechanism involves the 3'-untranslated region of the *Bcl2* mRNA, which contains binding sites for the RNA binding protein nucleolin, as well as miR-15a and miR-16-1 (179). Binding of nucleolin has been shown to enhance stability of *Bcl2* mRNA, whereas high levels of miR-15a and miR-16-1 suppress *Bcl2* expression (180, 181). Post-translational modification of Bcl-2 also exits, including phosphorylation of serine 70 residue, which entails stabilization of the interaction between Bcl-2 and Bax and inhibits degradation of Bcl-2 (182, 183).

The evidence presented in this chapter indicate that the *Bcl2* transgene, in the presence of WT T cells, lead to a partial rescue of GC formation by prolonging the survival of MALT1-deficient B cells. Strikingly, GC reactions and antibody response remained defective in intact *Bcl-2 Tg* x *Malt1*$^{-/-}$ animals, which may be explained by the intrinsic block in BCR-induced proliferation associated with MALT1 deficiency, as well as the lack of T cell activation. Similar to the findings made in an early publication on Bcl-2 transgenic mice (174), the *Bcl-2 Tg* x *Malt1*$^{+/-}$ B cells did not spontaneously proliferate but were responsive to mitogens. In contrast, despite that the *Bcl2* transgene drastically increased the viability of αIgM-stimulated MALT1-deficient B cells, cell cycle did not progress beyond the second division. The inclusion of αCD40 or BAFF signal allowed the cells to undergo for more rounds of division, but never to the same degree as in *Bcl-2 Tg* x *Malt1*$^{+/-}$ B cells. The *in vitro* observations also reflect the *in vivo* outcomes in μMT/*Bcl-2 Tg* x *Malt1*$^{-/-}$ chimeras;

although T cell-derived help allowed for the initiation of GC reactions, the magnitude of the GC was reduced due to limited cell cycle progression in MALT1-deficient B cells. Intriguingly, both µMT/*Bcl-2 Tg* x *Malt1*$^{+/-}$ and µMT/*Bcl-2 Tg* x *Malt1*$^{-/-}$ chimeric animals showed similar frequencies of GC B cells in the Peyer's patches, which may be explained by the differential requirements for GC formation in the Peyer's patches in response to microbial antigenss than in the spleen upon antigenic stimulation (184). B cells in gut-associated lymphoid tissue can be driven into GCs through the interaction of innate immune receptors with intestinal microbial antigens, in the absence of BCR engagement (184).

The µMT/mixed chimera model in Chapter 2 showed that even in the same context as WT T cells, MALT1-deficient T cells less readily differentiate into the Tfh phenotype. Tfh cells have been acknowledged as the key cell type required for GC formation and generation of memory B cells (185). While Tfh cells provide help to antigen-activated B cells, reciprocal signals from B cells are also important for the maintenance of Tfh differentiation, leading to the induction of the master regulator transcription factor Bcl-6 in either cell type (186, 187). Consequently, the reduction in Tfh phenotype in *Malt1*$^{-/-}$ mice may be attributed to the inability of MALT1-deficient B cell to successfully prime Tfh differentiation. It has been reported that sustained antigen presentation by dendritic cells facilitates early Tfh formation in the absence of B cells (188). Therefore, the block in Tfh differentiation may also arise from a cell-intrinsic defect in *Malt1*$^{-/-}$ T cells, but additional studies are needed to validate and decipher the molecular mechanism. The MALT1 substrate, Roquin, is a likely

candidate since it inhibits ICOS and the loss of both Roquin1 and Roquin2 has been reported to result in spontaneous Tfh differentiation and GC development (189).

Nevertheless, there remains a B cell-intrinsic requirement of MALT1 for optimal TD GC response, plasma cell differentiation, and Ig production. Similar to intact *Malt1*$^{-/-}$ animals, the MZ B compartment is largely compromised in *Bcl-2* x *Malt1*$^{-/-}$ mice. This suggests that in the absence of MALT1, not only is the reduction in MZ B cells a B cell autonomous defect, it is unlikely due to impaired survival since it cannot be rescued by enforced Bcl-2 expression. The partial decline in GC B cells observed in μMT/*Bcl-2* x *Malt1*$^{-/-}$ animals can be explained by the intrinsic block in BCR-induced proliferation associated with MALT1 deficiency, thereby restricting the expansion of cetroblasts during early GC development. Despite that defective c-Rel translocation has been implicated in *Malt1*$^{-/-}$ B cells (94), the involvement of MALT1 in regulating activation of RelA cannot be completely ruled out, given that RelA is essential for the generation of GC-derived plasma cells (126). The findings shown in this chapter also indicate that MALT1 is indispensable for plasmablast differentiation induced by BCR-indepenent stimuli. The fact that, in Chapter 2, LPS-induced proliferation was shown to be intact in *Malt1*$^{-/-}$ B cells seems paradoxical at first, but analysis of *in vitro* B cell culture revealed an impairment in plasmablast generation. Given that differentiation into plasma cells is a stochastic event that is tightly associated with cell divisions and is governed by a distinct gene regulatory network (190, 191), MALT1 may promote plasma cell differentiation by either regulating cell cycle progression or the induction of transcription factors including Blimp-1, XBP-1

and IRF4. However, these functions of MALT1 are likely independent of its scaffolding role as part of the CBM signalosome during BCR stimulation.

Chapter 3 contains work modified from the submitted manuscript titled “Differing requirements for MALT1 function in the survival and differentiation of peripheral B cell subsets”. The co-authors are Zilu Zhu, Janna Hachmann, Takuya Nojima, Daisuke Kitamura, Guy Salvesen, and Robert Rickert. The author of this dissertation is the primary investigator and author of the manuscript.

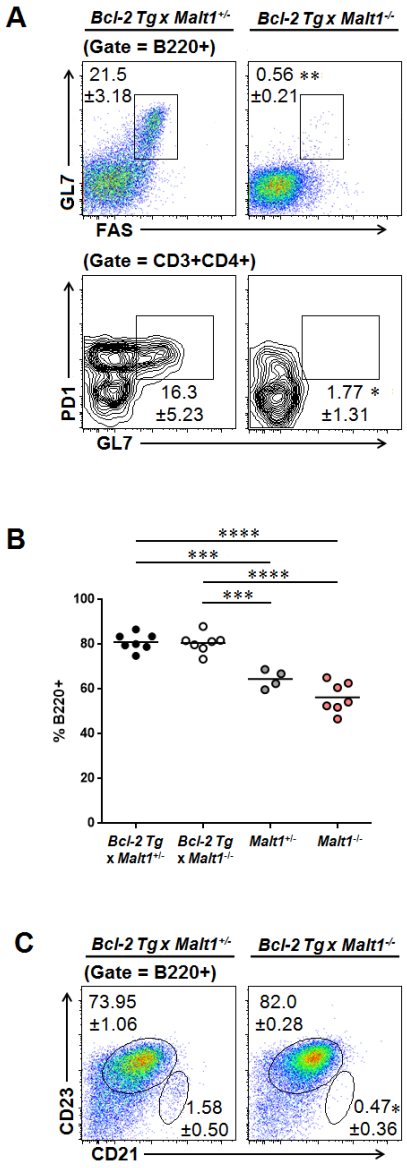


Figure 3-1 Constitutive Bcl-2 overexpression fails to rescue the GC defect and MZ B differentiation in MALT1-deficient animals. (A). Representative FACS plots showing the frequencies of GC B cells ($B220^+GL7^+Fas^+$) and Tfh cells ($CD3^+CD4^+PD1^+GL7^+$) in the Peyer's patches of *Bcl-2 Tg* x *Malt1*+/- (n=2) and *Bcl-2 Tg* x *Malt1*-/- animals (n=3). The values indicated are mean ± SD. (B) The percentage of total $B220^+$ B cells in the spleens was assessed by flow cytometry. (C) Representative FACS plots showing the frequencies of FO and MZ B cells in the spleens of *Bcl-2 Tg* x *Malt1*$^{+/-}$ (n = 2) and *Bcl-2 Tg* x *Malt1*$^{-/-}$ (n = 3) animals. The values indicated are mean ± SD.

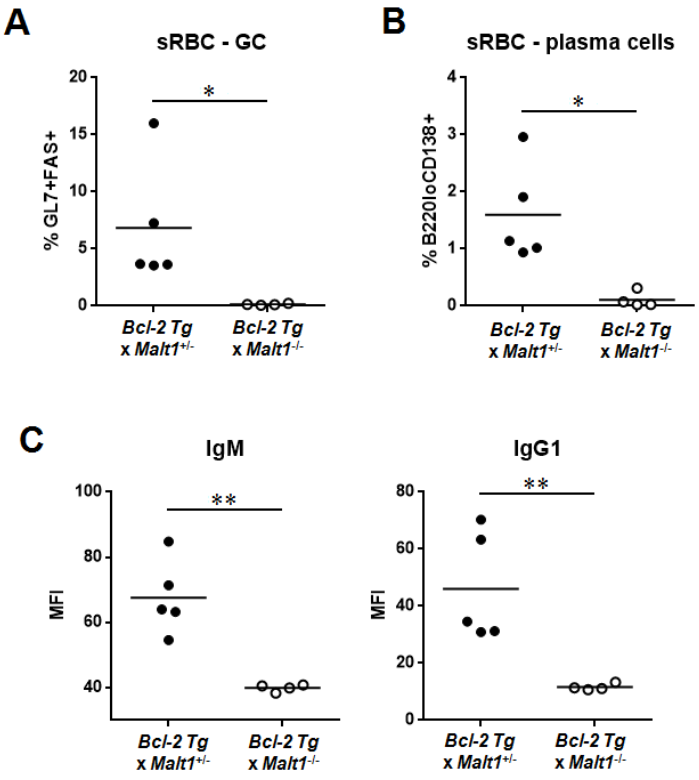
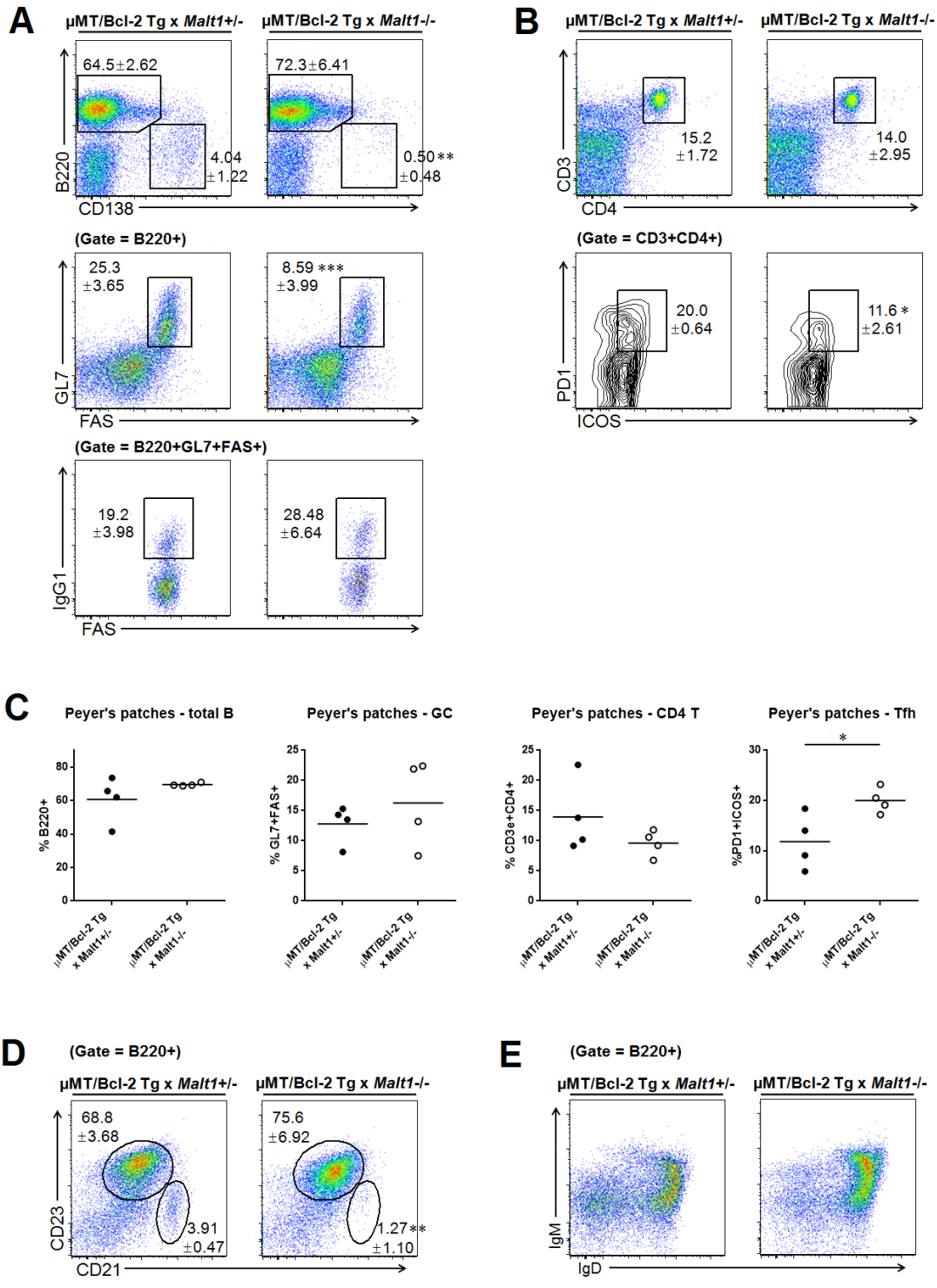


Figure 3-2. Constitutve Bcl-2 expression does not rectify the production of antigen-specific antibodies in MALT1-deificent animals. *Bcl-2 Tg* x *Malt1*$^{+/-}$ (n = 5) and *Bcl-2 Tg* x *Malt1*$^{-/-}$ (n = 4) mice were immunized with sRBCs. (A) On day 7 post-immunization, GC formation and plasma cell differentiation were assessed by flow cytometry. (B) Sera were collected on day 7 and the levels of sRBC-specific IgM and IgG1 were measured by low cytometry. The values shown represent mean fluorescent intensities.

Figure 3-3. Bcl-2 overexpression and the presence of WT T cells partially rescue the GC phenotype but not plasma differentiation in *Malt1*$^{-/-}$ B cells. Sublethally irradiated μMT mice were reconstituted with bone marrow from *Bcl-2 Tg* x *Malt1*$^{+/-}$ or *Bcl-2 Tg* x *Malt1*$^{-/-}$ donors and immunized with sRBCs 8 weeks afterward. (A) Representative FACS plots showing the frequencies of total B cells (B220^{+}), GC B cells (GL7^{+}Fas^{+}), class-switched GC B cells (GL7^{+}Fas^{+}IgG1^{+}), plasma cells (B220^{lo}CD138^{+}), CD4^{+} T cells (CD3^{+}CD4^{+}), as well as Tfh cells (PD1^{+}ICOS^{+}) in the spleens. (B) Total B cells, GC B cells, CD4^{+} T cells, and Tfh cells in the Peyer's patches were determined using the same staining strategies as in (A) by flow cytometry. (C) Representative FACS plots showing the frequencies of FO (CD21^{lo}CD23^{hi}) and MZ (CD21^{hi}CD23^{lo}) B cells in the spleen. (D) Representative FACS plots showing the IgM vs IgD prolife of splenic B cells in the chimeric animals.


A
μMT/Bcl-2 Tg x Malt1+/-
μMT/Bcl-2 Tg x Malt1-/-
64.5±2.62
4.04 ±1.22
72.3±6.41
0.50** ±0.48
B220
CD138
(Gate = B220+)
25.3 ±3.65
8.59 *** ±3.99
GL7
FAS
(Gate = B220+GL7+FAS+)
19.2 ±3.98
28.48 ±6.64
IgG1
FAS
B
μMT/Bcl-2 Tg x Malt1+/-
μMT/Bcl-2 Tg x Malt1-/-
15.2 ±1.72
14.0 ±2.95
CD3
CD4
(Gate = CD3+CD4+)
20.0 ±0.64
11.6 * ±2.61
PD1
ICOS
C
Peyer's patches - total B
Peyer's patches - GC
Peyer's patches - CD4 T
Peyer's patches - Tfh
% B220+
% GL7+FAS+
% CD3e+CD4+
%PD1+ICOS+
*
μMT/Bcl-2 Tg x Malt1+/-
μMT/Bcl-2 Tg x Malt1-/-
D
(Gate = B220+)
μMT/Bcl-2 Tg x Malt1+/-
μMT/Bcl-2 Tg x Malt1-/-
68.8 ±3.68
3.91 ±0.47
75.6 ±6.92
1.27** ±1.10
CD23
CD21
E
(Gate = B220+)
μMT/Bcl-2 Tg x Malt1+/-
μMT/Bcl-2 Tg x Malt1-/-
IgM
IgD

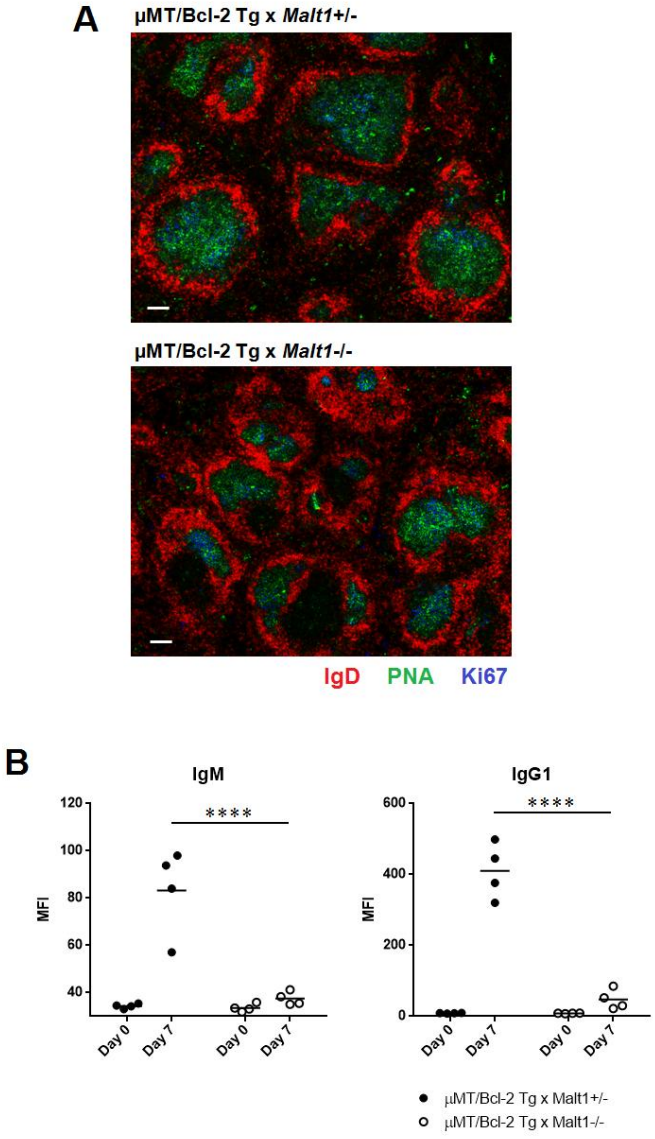


Figure 3-4 sRBC-immunized µMT/*Bcl-2 Tg* x *Malt1*$^{-/-}$ animals display smaller PNA^{+} clusters and fail to produce antigen-specific antibodies. (A) Representative images of spleen sections from sRBC-immunized µMT/*Bcl-2 Tg* x *Malt1*$^{+/-}$ and µMT/*Bcl-2 Tg* x *Malt1*$^{+/-}$ chimeras stained with antibodies against IgD (PE) and Ki67 (APC), as well as PNA (FITC). The images were taken at 5x magnification. Scale bar reprents 50 µM. (B) Levels of sRBC-specific IgM and IgG1 antibodies in the sera collected on day 0 and day 7 post-immunization were measured by flow cytometry. The graphs show mean fluorescent intensities.

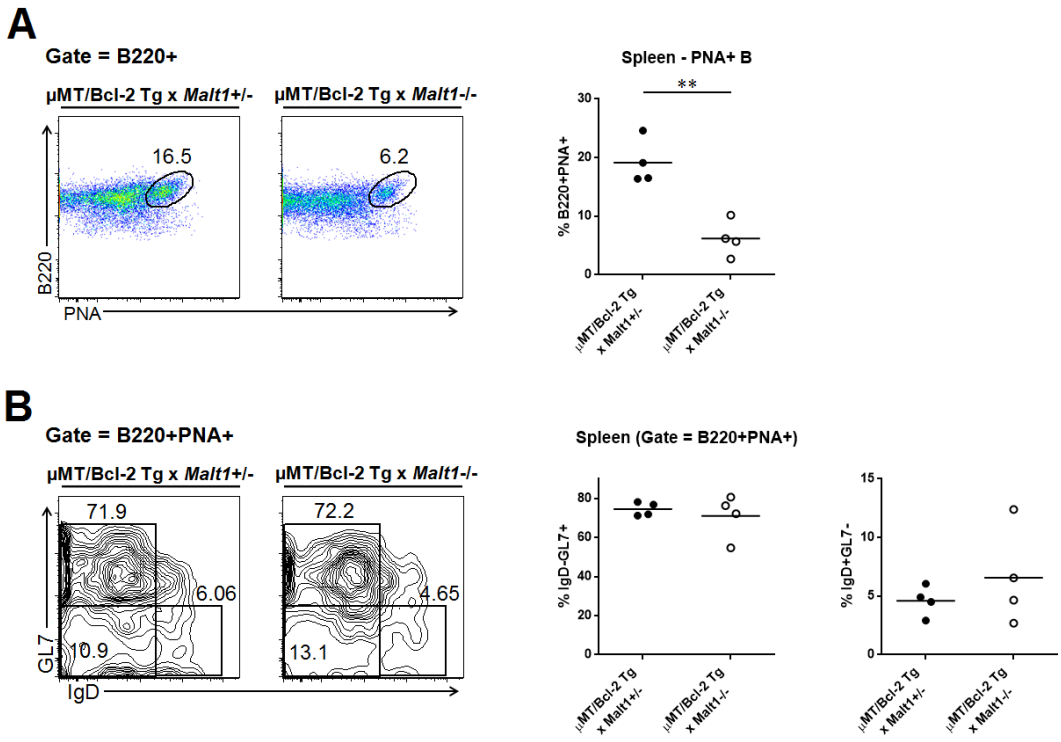


Figure 3-5. MALT1 is dispensable for maturation of GC B cells. Total GC pool in sRBC-immunized μMT chimeras reconstituted with either *Bcl-2 Tg* x *Malt1*$^{+/-}$ or *Bcl-2 Tg* x *Malt1*$^{-/-}$ (n = 4 per group) were first identified by B220^{+}PNA^{+} staining as shown in (A) and further characterized by surface IgD and GL7 staining to distinguish between early (IgD^{+}GL7^{-}) and mature (IgD^{-}GL7^{+}) stages of GC B cells in (B). The representative FACS plots are shown on the left and the frequencies were plotted on the right.

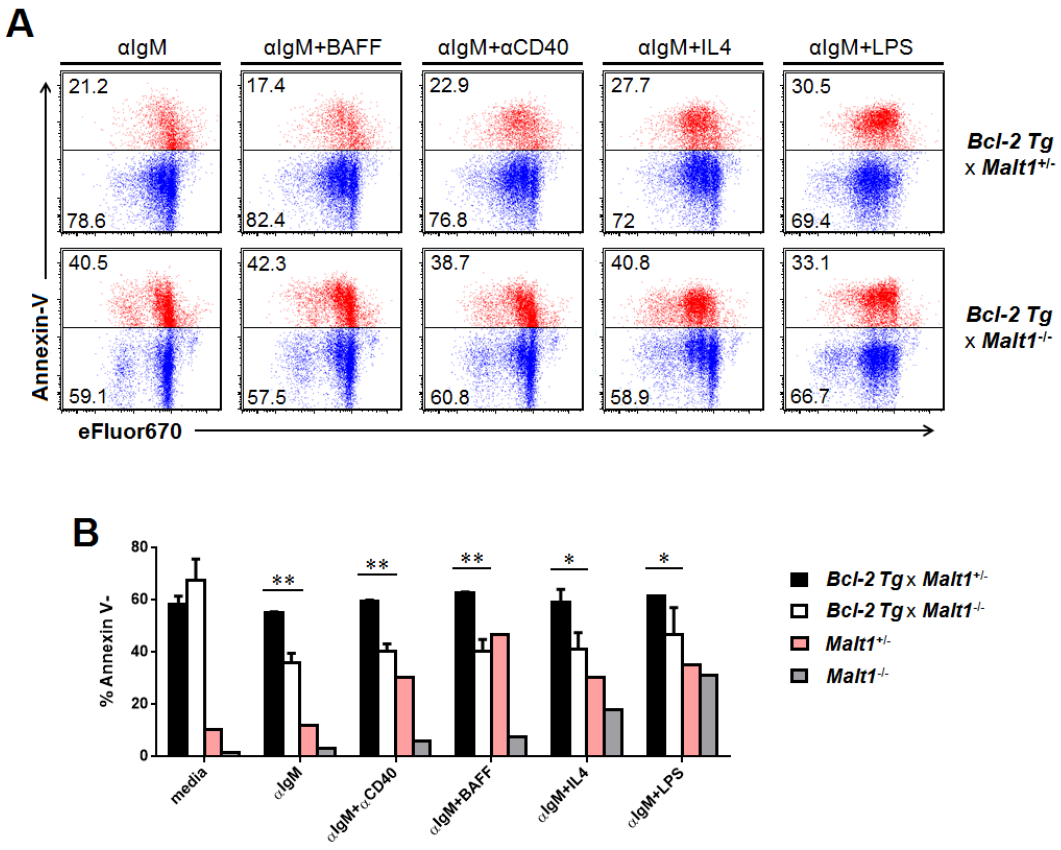


Figure 3-6. Constitutive Bcl-2 expression prolongs the survival of MALT1-deficient B cells but only partially rescues BCR-induced proliferation. (A) CD43-depleted splenic B cells from *Bcl-2 Tg* x *Malt1*$^{+/-}$ or *Bcl-2 Tg* x *Malt1*$^{-/-}$ mice were cultured with the indicated stimuli for 3 days. Cell proliferation and apoptosis were evaluated by combined eFluor670 dilution and Annexin-V staining. Representative FACS plots from n = 2-3 animals per genotype are shown. (B) Histogram showing the viability of *Bcl-2 Tg* x *Malt1*$^{+/-}$ (n = 2), *Bcl-2 Tg* x *Malt1*$^{-/-}$ (n = 3), *Malt1*$^{+/-}$ (n = 1), and *Malt1*$^{-/-}$ (n = 1) B cells in the same assay as in (A), determined by negative Annexin-V staining in the gated region specified in each FACS plot. Error bars represent SD.

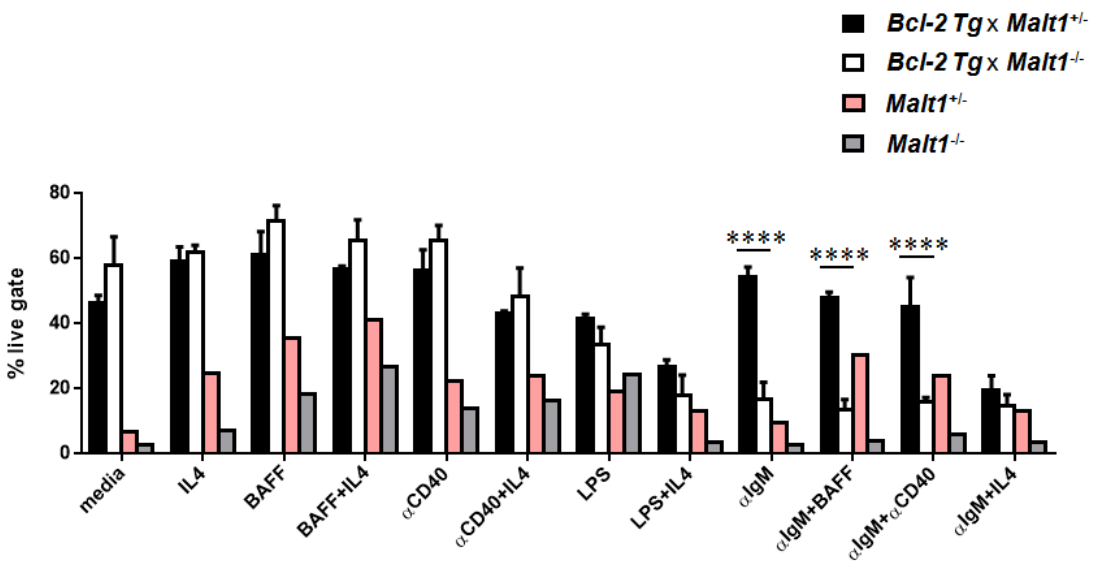


Figure 3-7. Constitutive Bcl-2 expression fails to completely rescue BCR-induced apoptosis in MALT1-deficient B cells. CD43-depleted splenic B cells from *Bcl-2 Tg* x *Malt1*$^{+/-}$ (n = 2), *Bcl-2 Tg* x *Malt1*$^{-/-}$ (n = 3), *Malt1*$^{+/-}$ (n = 1), and *Malt1*$^{-/-}$ (n = 1) mice were cultured as indicated for 4 days. The percentage of cells in the live gate according to the FSC versus SSC profile was determined by flow cytometry. Error bars indicate SD.

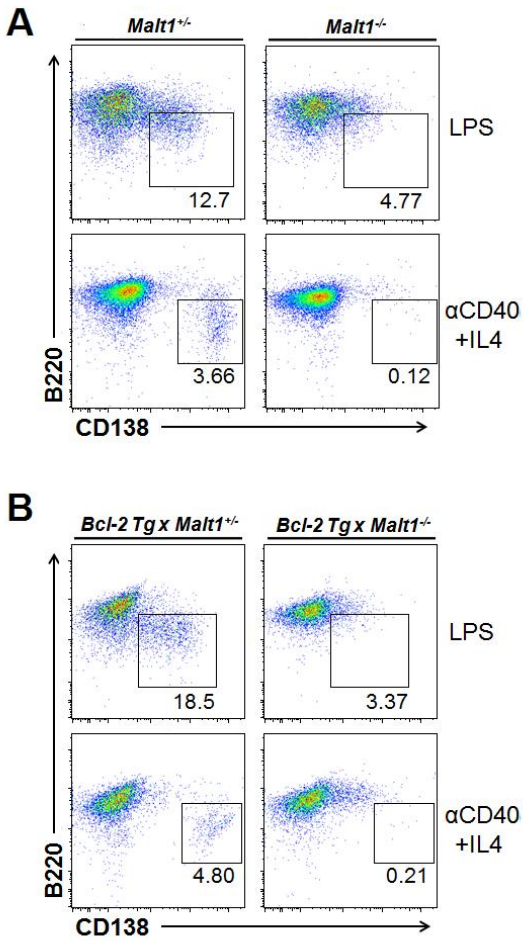


Figure 3-8. MALT1 is required for *in vitro* ASC differentiation. Purified B cells from (A) *Malt1*$^{+/-}$ and Malt1$^{-/-}$ littermates and (B) *Bcl-2 Tg* x *Malt1*$^{+/-}$ and *Bcl-2 Tg* x *Malt1*$^{-/-}$ littermates were cultured with the indicated mitogens for 4 days and stained with B220 and CD138 to assess the formation of ASCs. Representative FACS plots from 3 independent experiments (n = 3 per genotypes) are shown.

Chapter 4. Regulation of MALT1 proteolytic activity in B cells

Introduction

MALT1 was classified as the first human paracaspase in the clan CD cysteine protease based on modeling of its C-terminal caspase-like domain (192). However, early attempt using an activity-based protease active probe in conjunction with combinatorial peptide substrate library approach failed to detect proteolytic activity of MALT1 on synthetic substrates (193). It was not until almost a decade later that first two substrates of MALT1 were independently discovered and since then, the list expanded to encompass six proteins, including BCL10, RelB, cylindromatosis tumor suppressor protein (CYLD), tumor necrosis factor, alpha-induced protein 3 (A20), regulatory RNase 1 (Regnase-1) and roquin (Figure 4-1). At the same time, it became clear that in addition to its scaffolding function, MALT1 also regulates antigen receptor signaling through proteolytic cleavage of several substrates. MALT1-mediated processing of A20 and CYLD, two deubiquitinases that act as negative regulators of canonical NF-κB activation, dampen their inhibition and therefore sustain antigen receptor signaling (194, 195). MALT1 also cleaves RelB and induces its proteasomal degradation, which results in enhanced expression of canonical NF-κB target genes (196).

The link between MALT1 protease activity and NF-κB signaling pathway has been established in an earlier study, which reported that NF-κB activation becomes diminished after deletion of the caspase domain of MALT1 or mutation of its active

site cysteine residue (197). On the other hand, dysregulated MALT1 proteolytic activity contributes to the growth and survival of the activated-B cell subtype of diffuse large B cell lymphoma (ABC-DLBCL) that is characterized by constitutive NF-κB activation (198, 199). Inhibition of constitutive MALT1 activity with the use of small molecule inhibitor or peptide has proven effective in inducing apoptosis via inhibition of NF-κB pathway in ABC-DLBCL cells (199-201).

MALT1-mediated substrate processing has other biological consequences. While cleavage of BCL10 had minimal effect on NF-κB signaling, it was shown to be a requirement for TCR-induced cell adhesion to β1 fibronectin, possibly modulating integrin-dependent T cell priming and extravasation (202). Proteolytic inactivation of CYLD by MALT1 was found to be required for JNK activation and helps to induce expression of specific gene sets in activated T cells (195). Cleavage of the RNase Regnase-1 in $CD4^+$ T helper cells lifts Regnase-1-mediated suppression of specific mRNAs including *Rel* and *Il2*, thereby facilitating T cell activation (203). Most recently, it was demonstrated that TCR-induced MALT1 proteolytic activity results in the cleavage of roquin and regnase-1, thereby releasing their cooperatively repressed target mRNAs to promote Th17 differentiation (204).

To understand the physiological consequences of MALT1-mediated proteolysis, three independent groups have recently generated knock-in mice expressing the catalytically inactive version of MALT1 by substituting the catalytic cysteine residue with alanine (100, 205, 206). Despite of some subtle discrepancies, the phenotypes described by each of the groups were largely consistent. Similar to MALT1-deficient mice, the protease-inactive mice showed reduced MZ and B1 B cell

compartment, lower basal serum Ig levels, as well as defective humoral responses to both TI and TD antigen immunization. However, inactivation of MALT1 protease does not affect BCR signaling, since stimulation-induced IKK, ERK, and JNK activation were normal in both T and B cells from the knock-in mice. Intriguingly, one group reported modest reductions in spontaneous GC formation and Tfh population in the Peyer's patches of the protease inactive mice, but the GCs contain increased proportional of $IgG1^+$ B cells (100). Moreover, all three groups noted that the protease-deficient mice develop spontaneous systemic autoimmunity that can attributed to regulatory T cells (Treg) deficiency and Th1- and Th2-skwed responses.

A comparison of all known MALT1 substrates indicates that the paracaspase preferentially cleaves after an arginine residue. Recent biochemical work revealed that the enzymatic activity of MALT11 is dependent on its dimerization and using positional-scanning peptidyl substrate libraries, the optimal substrate preference for MALT1 was determined: leucine-valine-serine-arginine (LVSR) (207). This insight led to the development of novel activity-based probes that are designed to specifically bind and inhibit MALT1, allowing for detection and monitoring of its activity in cell lysates (208, 209).

In this chapter, the regulation of MALT1 protease activity in B cells is further explored. I show that MALT1-mediated cleavage events occur only after αIgM treatment but not with other stimuli. Also, the use of the MALT1 activity probe allows for monitoring of MALT1 proteolytic activity *in situ* in the spleens of immunized animals. Furthermore, the connection between upstream regulators, including Btk and PDK1, with MALT1 activity in B cells is examined.

Materials and methods

B cell activation

Freshly splenic B cells purifed by MACS depletion and were plated at 4 x 10^6 cells per 1 mL of complete RPMI in each well in a 12-well plate format. Cells were stimulated with 10 µg/mL αIgM $F(ab')_2$, 25 ng/mL BAFF, 5 µg/mL αCD40 supplemented with 10 ng/mL recombinant murin IL-4, or 10 µg/mL LPS for the indicated amount time. Thioridazine hydrochloride (Sigma) was dissolved in PBS and was added to cell culture at the indicated concentrations to inhibit MALT1 protease activity. Cells were collected by centrifugation and subject to immunoblotting.

Immunoblotting

Cells were lysed in RIPA buffer plus complete protease inhibitor. Lysates were cleared by centrifugation and protein content was determined by BCA assay. Equal amount of proteins were separated on 4-12% Bis-Tris SDS gel (Life Technologies) and transferred onto PVDF membrane, and blotted with the indicated primary antibodies. The following antibodies were purchased from Cell Signaling: A20 (D13H3), actin, MEK1/2, BCL10 (C78F1), cleaved caspase-3, MALT, and RelB (C1E4). Antibodies against CYLD (E-10) was purchased from Santa Cruz. The primary antibodies were detected with HRP-conjudated donkey anti-rabbit IgG (H+L) or goat anti-mouse IgG (H+L) secondary antibodies.

sRBCs immunization

For the induction of GC response, animals were injected intraperitoneally with 200 µL of 10% (vol/vol) sRBCs in PBS. Intraperitoneal administration of thioridazine (or equal volume of solvent) was initiated on day 3 post-immunization and given daily for 4 days at a dose of 100 µg per animal.

Flow cytometry

On day 7 post-immunization, the mice were sacrificed and single cell suspensions were prepared from the spleen. After removal of RBCs by hypotonic lysis, the cells were blocked and stained with the indicated antibodies and analyzed on a FACSCanto flow cytometer.

Histology

The Cy5-conjugated MALT1-specific probe LVSR-AOMK (208) was a gerenous gift from the laboratory of Dr. Guy Salvesen. Spleens from sRBC-immunized animals were embedded in OCT and 5 µM cryosections were prepared. Spleen sections were fixed in acetone, air-dried, blocked in PBS with 5% BSA containing 10 µM E64 and incubated with Cy5-LVSR-AOMK and various other antibodies including B220, IgD, and CD21/35, as well as PNA.

Results

MALT1 protease activity is specifically induced by stimuation through BCR

MALT1 has been reported to play an essential role in both BCR and BAFF-induced signal transduction in B cells (93). To explore whether MALT1 enzymatic activity is induced in response to these stimuli, splenic B cells were treated with αIgM or BAFF and the the cleavage of MALT1 substrates was assessed by immunoblotting analysis. In line with the previously published reports on MALT1 substrates, proteolysis of CYLD and A20 was observed in WT splenic B cells after stimulation with αIgM $F(ab')_2$ for 4 hours and the cleavage events were even more prononuced by 24 hours post-stimulation (Figure 4-2). In contrast, treatment with BAFF did not lead to accumulation of A20 or CYLD cleaved products. Processing of A20 and CYLD is dependent on MALT1 since the cleavage events were not detected in αIgM-stimulated *Malt1*$^{-/-}$ B cells. Interestingly, BCR crosslinking also did not lead to upregulation of A20 proteins in *Malt1*$^{-/-}$ B cells, as it did in WT B cells.

Next, the induction of MALT1-mediated proteolysis in response to other B cell stimuli were assessed. The incubation period of 24 hours was chosen since it resulted in greater accumulation of cleavage products. Unlike αIgM treatment, which led to processing of both A20 and CYLD, neither LPS or αCD40 plus IL-4 induced cleavage of either substrates (Figure 4-3). Other substrates of MALT1, RelB and BCL10, were also included for the analysis. While the cleaved products of RelB and BCL10 were unable to be detected with the antibodies that were avaiable in my hand, αIgM but not LPS or αCD40 plus IL-4 stimulation did lead to a disappearance of the full-length

protein (Figure 4-3). Thus, the induction of MALT1 protease activity is specifically mediated through the BCR.

In situ detection of MALT1 protease activity during GC response

To investigate how MALT1 protease activity is regulated during immune response, I employed the recently developed MALT1 activity probe (208) and evaluated its potential for use in cryo-preserved tissues. Frozen spleen sections from sRBC-immunized mice were incubated with the Cy5-conjugated LVSR-AOMK probe, along with antibodies against B220 and the lectin PNA. Intriguingly, MALT1 protease activity, indicated by Cy5 positivity, was confined to the PNA^+ GC region and not detected in the rest of the follicle (Figure 4-4). A few cells exhibiting MALT1 activity but stained negatively with either αB220 or PNA were also observed, suggesting that active MALT1 may become induced in other cell types in the GC. Spleen sections from *Malt1*$^{-/-}$ mice were used as a control. Consistent with the findings presented in Chapter 2, *Malt1*$^{-/-}$ mice displayed a lack of well-defined PNA^+ cluster in each follicle, indicative of impairment in GC response upon sRBC immunization. In addition, Cy5 staining was not detected in the spleens of *Malt1*$^{-/-}$ mice, thus demonstrating the specificity of the MALT1 activity probe (Figure 4-4).

Next, the spatial distribution of cells showing MALT1 activity within the GC microenvironment was explored. As discussed in Chapter 1, the GC region is largely compartmentalized; rapidly proliferating centroblasts constitute the dark zone, whereas centrocytes undergoing selection and affinity maturation are localized to the light zone, which encompasses well-developed FDC network and $CD4^+$ T cells.

Therefore, IgD and CD35 staining were incorporated with the MALT1 activity probe to visualize the follicular mantle and FDCs, respectively. Consistent with the previous finding, Cy5 staining was only detected in IgD^- region that is surrounded by the follicular mantle (Figure 4-5). However, a portion of the cells possessing MALT1 activity were found in proximity with the $CD35^+$ FDCs, while the others situated outside the light zone. Collectively, these results suggest that in response to antigen stimulation, MALT1 protease activity becomes induced in the GC and is not confined to either the dark zone or light zone.

MALT1 protease activity is dispensable for GC formation and BCR-induced response

Recent study characterizting the MALT1 protease inactive mice revealed an partially impaired GC response to TD antigen (100). However, the approach using the knock-in mice cannot distinguish whether MALT1-mediated proteolysis is required for the inifiation or maintenance of GC reactions. Thus, an alternative approach utilizing small molecule inhibitor was opted instead. The effect of thioridazine on selectively inhibiting MALT1 cleavage activity and subsequent NF-κB signaling has been demonstrated in ABC-DLBCL lines (200). Following immuniziation with TD antigen, GC B cells become apparent by day 4 postimmunization and reach a peak frequency by days 6-8 (210). Therefore, after sRBC immunization, WT mice were given 2 days to allow for antigen-specific B cells to become activated and cognate interactions between B and T cells to occur (211). Then the animals were administered daily with thioridazine starting on day 3 for 4 consecutive days and GC response was evaluated on day 7. Similar to what was observed in MALT1 protease inactive mice,

treatment with thiodazine only led to a modest reduction in the frequency of $GL7^+Fas^+$ GC B cells, whereas GC formation was abrogated in *Malt1*$^{-/-}$ mice (Figure 4-6A). Interestingly, administration of thioridazine led to a decrease in total B cells to similar level as in *Malt1*$^{-/-}$ B cells, as well as a partial reduction in MZ B compartment, while the FO B cell pool remained intact (Figure 4-6B). Thioridazine appears to target only B cells since the frequencies of both the $CD4^+$ T and Tfh populations were comparable between DMSO- and thioridazine-treated *Malt1*$^{+/+}$ animals. In contrast, the Tfh pool was drastically reduced in *Malt1*$^{-/-}$ mouse, similar to what was described in Chapters 2.

To investigate how thioridazine acts on B cells, the effect of thioridazine *in vitro* was examined. While thioridazine efficiently blocked MALT1-mediated cleavage of A20 in αIgM-activated splenic B cells (Figure 4-7A), it did not affect the prolferative response to BCR stimulation (Figure 4-7C). Furthermore, thioridazine reduced the viability of unstimulated B cells only, whereas B cells under all the stimulating conditions tested showed comparable survival as in DMSO-treated group (Figure 4-7B). It should be noted that at the highest dose (2 μg/mL), thioridazine appears to be toxic to splenic B cells, evident by the accumulation of cleaved Caspase-3 (Figure 4-7A) and few viable cells in all the conditions tested (results not shown), and was therefore excluded from the analysis in panels B and C. Taken together, these data suggest that MALT1 protease activity is not essential for proper BCR-induced proliferative and survival response.

PDK1 does not regulate MALT1 protease acitivity in B cells

PI3K and PDK1 have been implicated in the regulation of MALT1 protease activity and the evidence came from a report that showed treatment of ABC DLBCL cells with PI3K or PDK1 inhibitor attenuates MALT1 cleavage activity and NF-κB activation (212). PDK1-deficient mice haved been described (213) and they exhibit overlapping phenotypes with *Malt1*$^{-/-}$ animals, including drastic reductions in both MZ and B1 compartments, impaired GC B cell differentiation upon sRBC immunization, as well as defective proliferation and survival response to αIgM stimulation that can only be rescued with IL4 costimulation. Thus, the possibility of PDK1 being an upstream regulator of MALT1 protease activity in B cells was explored. Due to a pronounced reduction in MZ B cells in the *CD21Cre* x *PDK1*$^{L/L}$ mice, splenic FO B cells were purified by combined depletion of CD43^{+} and CD9^{+} cells to avoid skewing the result. After stimulation with αIgM F(ab')$_2$, both *CD21Cre* x *PDK1*$^{L/L}$ and control FO B cells displayed comparable cleavage of A20, CYLD, and BCL10 (Figure 4-7). Also, PMA plus ionomycin (PMA/iono) bypasses the antigen requirement and stimulates PKC directly, resulting in accumulation of A20 in both groups. Consistent with the result shown in Figure 4-2, BAFF stimulation did not lead to indction of MALT1 activity. Thus, contrary to what was observed in ABC DLBCL cells, PDK1 is not required for induction of MALT1 cleavage activity in splenic B cells.

BCR-induced MALT1 activity is dependent on Btk activity

The finding that MALT1-mediated proteolysis is induced specifically by BCR stimulation supports a role for BCR signaling in regulating MALT1 activity. As reviewed in Chapter 1, BCR signaling results in the activation of Btk, which in turn

phosphorylates PLCγ2 and subsequently leads to activation of PKCβ. It has been established that silencing of Btk or blocking of Btk activity with the irreversible inhibitor, ibrutinib, kills ABC DLBCL cells, which are known to depend on chronic active BCR signaling for survival (214). Since ABC DLBCL lines have also been shown to rely on MALT1 proteolytic activity (198, 199), the connection between BTK activity and MALT1-mediated cleavage was investigated in the context of splenic B cells. In support of the hypothesis, pre-treatment of WT splenic B cells with ibrutinib was found to inhibit αIgM-induced cleavage of A20 and CYLD (Figure 4-9). Interestingly, ibrutinib appears to suppress Bcl-2 in both unstimulated and BCR-activated B cells, while it prevented the induction of Bcl-xL in BCR-activated B cells. Furthermore, ibrutinib blocked the upregulation of A20 in response to BCR stimulation, possibly via inhibition of protein synthesis, but did not alter CYLD level (Figure 4-9).

Lastly, the effect of ibrutinib on the proliferative and survival response to BCR-stimulation was examined. WT splenic B cells were pre-treated with varying concentrations of ibrutinib prior to stimulation with mitogens. When cultured in media alone or with αIgM or αIgM plus αCD40 stimulation, inhibition of BTK activity efficiently reduced viability of the WT B cells while sparing the MALT1-deficient B cells (Figure 4-10A). In contrast, ibrutinib pre-treatment had no selective impact on viability when B cells were stimulated with the TLR9 agonist CpG oligodeoxynucleotides (CpG), which occurs independently of BCR signaling. Similarly, ibrutinib also blocked BCR-induced proliferation in WT B cells, but had minimal effect on cell divisions induced by CpG stimulation. (Figure 4-10B). These

results confirm that Btk locates upstream of MALT1 and regulates its proteolytic acitivity in B cells upon BCR triggering.

Discussion

As described in Chapter 1, triggering of BCR induces formation of the ternary CBM complex, leading to activation of the IKK complex and subsequentlyly, nuclear translocation NF-κB. While expression of either BCL10 or CARMA1 resulted in potent NF-κB activation (215), MALT1 cannot independently activate NF-κB but rather associates and synergizes with BCL10 to enhance NF-κB activation (192, 197). Further analysis revealed that the caspase-like domain of MALT1 is critical for the synergistic enhancement and strikingly, lignad-induced oligomeriztation of the MALT1 protease domain was sufficient for activating NF-κB (197). The theme of activation by oligomerization also applies to the reguation of MALT1 proteolytic activity, with evidence coming from both structural and biochemical studies (207, 216). The findings presented in this chapter stress the importance of the CBM signalosome in enabling the protease activity of MALT1 in splenic B cells. MALT1-mediated cleavage events were not detected when B cells were stimulated through CD40, BAFFR, or TLR4, since none of which normally involves formation of the CBM complex. Furthermore, all the studies on mice expressing the catalytically inactive version of MALT1 (MALT1-C464A) consistently reported that antigen receptor-induced IKK activation can still take place in the absence of MALT1 protease function (100, 205, 206), suggesting that MALT1 proteolytic activity is a consequence rather than the cause of antigen receptor signaling. The specfic induction of MALT1-mediated cleavage events upon BCR engagement supports the emerging

view that the protease function of MALT1 further fine-tuness NF-κB signaling inititated by its scaffolding function (95).

Ubiquitination adds another layer of complexity to the regulation of MALT1 unctions. It has been demonstrated in T cells that stimulation-induced polyubibuitination of MALT1 by TRAF6 controls its scaffolding function by enabling recruitment of NEMO and NF-κB activation (217). A recent study also identified monoubiqutination on lysine 644 residue, which interestingly, peaks at a later time point than the polyubiquitination event, as necessary to activate the protease function of MALT1 (218). Further studies are needed to determine if these regulatory mechanisms also exist in B cells upon BCR activation. Whether the interplay of dimerization and monoubiquitination influences the conformation of the MALT1 protease domain also awaits additional investigation.

Intriguingly, *in situ* detection of MALT1 activity with the probe revealed that MALT1 proteolysis is restricted to the GC region after TD immunization. Since BCR signaling is reduced in GC B cells (125), the cells exhibiting active MALT1 may have been simply "recent recruits" that just entered GC and still retained high BCR signaling. However, a recent study utilizing in vivo reporter detected induction of BCR signaling among a subset of GC B cells in the LZ, where they encounter antigen and Tfh cells (219). Conseqeuntly, MALT1 catalytic actiiy may also become induced in the LZ and since frequent shuttling of GC B cells between the DZ and LB have been documented (220), the staining pattern is not necessarily restricted to the LZ. To distinguish between these possibilities, the LVSR-labeled cells need to be sorted and carefully scrutinized by gene expression analysis.

Results from the study on MALT1 protease-inactive mice (100) and the thioridazine inhibition experiment presented in this chapter are in agreement, showing that MALT1-mediated proteolysis is not essential for GC formation and maintenance. Inhibition of MALT1 activity also had minimal effect on the proliferative and survival responses of B cells to αIgM stimulation. The only noticeable effect of thioridazine *in vivo* is the reduction of total B cell cohort to the level seen in MALT1-deficient animals, possibily as a result of apoptosis. The specificity of thioridazine has been evaluated and the small molecule selectively targets ABC DLBCL but not GCB DLBCL cells in a mouse xenograft model (200). It is unclear, at this point, why it is selectively toxic to resting but not stimulated B cells. Neverthelss, it would be interesting to evaluate the *in vivo* consequences of MALT1 substrate cleavage during the progression of GC response. This would entail generation of knock-in mice expressing cleavage-resistant version, by mutating the amino acid sequences of the cleavage site, of each of the reported MALT1 substrates and assess their response to TD immunizations.

MALT1 was initially discovered as part of the oncogenic fusion gene product that occurs as a result of t(11;18)(q21;q21) chromosmal translocation in a subset of MALT lymphoma patients (221). The resulting cIAP2-MALT1 fusion protein is a potent activator of NF-κB, due to spontaneous oligomerization through its N-terminal cIAP2-derived sequence with the MALT1 C-terminus (222). cIAP2-MALT1 also retains a functional paracaspase domain derived from the MALT1 portion of the gene product and it was shown that substitution of the catalytic cysteine residue in the caspase-like domain with alanine is sufficient to abrogate NF-κB activation induced

by cIAP2-MALT1 (192). While cIAP2-MALT1 shares most of the proteolytic substrates as WT MALT1, it became clear that cIAP-2MALT1 possesses its own unique set of substrates, including NIK (223) and LIM domain and actin binding 1 (LIMA1) (224) identfied thus far. It would be imperative to understand how the protease domain of the fusion protein is regulated and if it is distinct from that of WT MALT1, since the cleavage events mediated by cIAP2-MALT1 were found to enhance ongenic properties (223, 224). The cIAP2 moieity appears to contributte to the interaction with these unnatural substrates that normally cannot be recruited to WT MALT1 protein. Thus, inhibition of such interaction could potentially be uilized as a treatment option for MALT lymphoma patients harboring the cIAP2-MALT1 fusion, since complete inhibition of MALT1 protease activity risks undesirable autoimmunity (100, 205, 206).

Although it has been described in T cells that PDK1 associates with PKCθ and CARMA1, possibly facilitating CARMA1 phosphorylation by function as a molecular bridge (225), in the context of splenic B cells, the contribution of PDK1 to the CBM signalosome is questionable. The role of PDK1 in regulating MALT1 protease funcion is also ruled out by the findings presented in this chapter. In contrast, inhibition of Btk, which has been recognized as upstream regulator of the CBM complex, abrogated cleavage of A20 and CYLD and blocked αIgM-induced signaling, as indciated by the lack of A20 and Bcl-xL induction. These data are, again, in line with the notion of oligomerization-induced activation. The fact that, in response to αIgM stimulation, ibrutinib-treated B cells display reduced viability and cell dvision

similar to MALT1-deficient B cells also illustrates the importance of this pathway in regulating BCR-induced proliferative and survival responses.

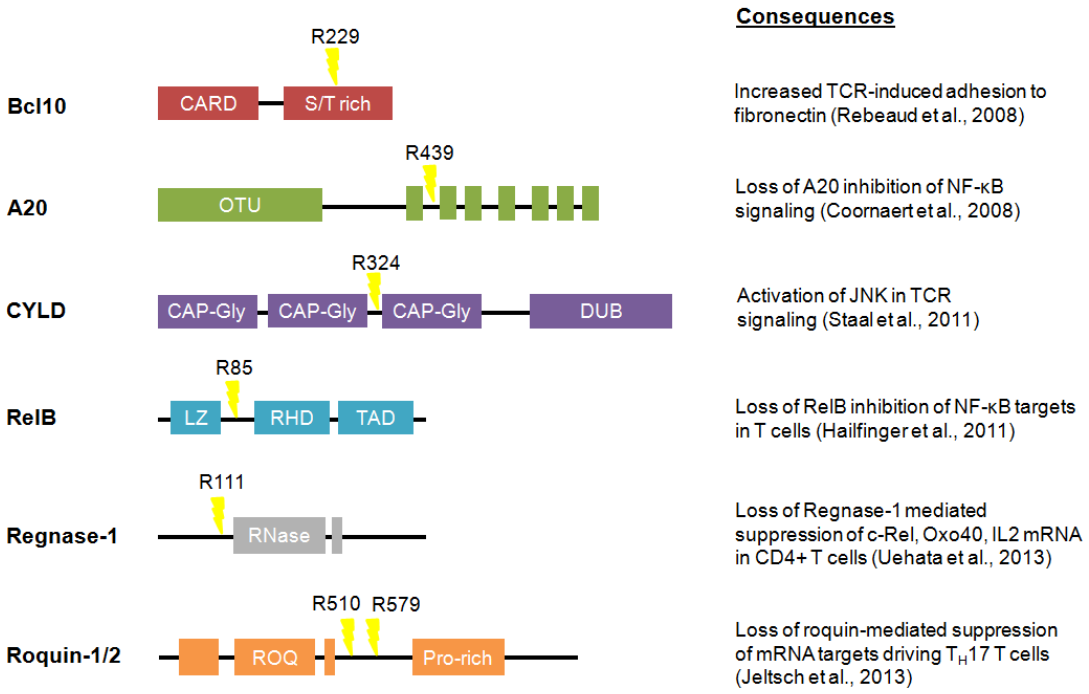


Figure 4-1. List of MALT1 substrates that have been identified to date. and the consequences of their cleavage. The cleavage sites were marked in yellow at the indicated arginine residue.

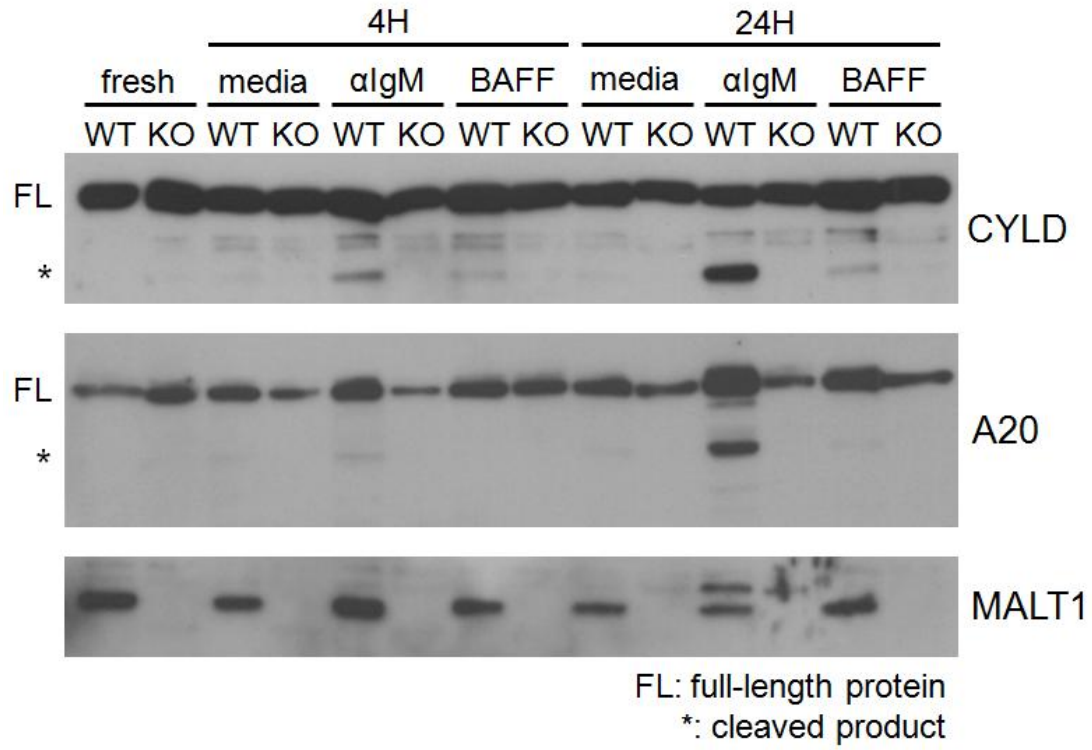


Figure 4-2. BAFF does not induce MALT1-mediated cleavage events. Immunoblot analysis of WT and *Malt1*$^{-/-}$ (KO) splenic B cells, freshly isolated or stimulated as indicated for 4 hours or 24 hours. Blots are representative of at least 3 independent experiments.

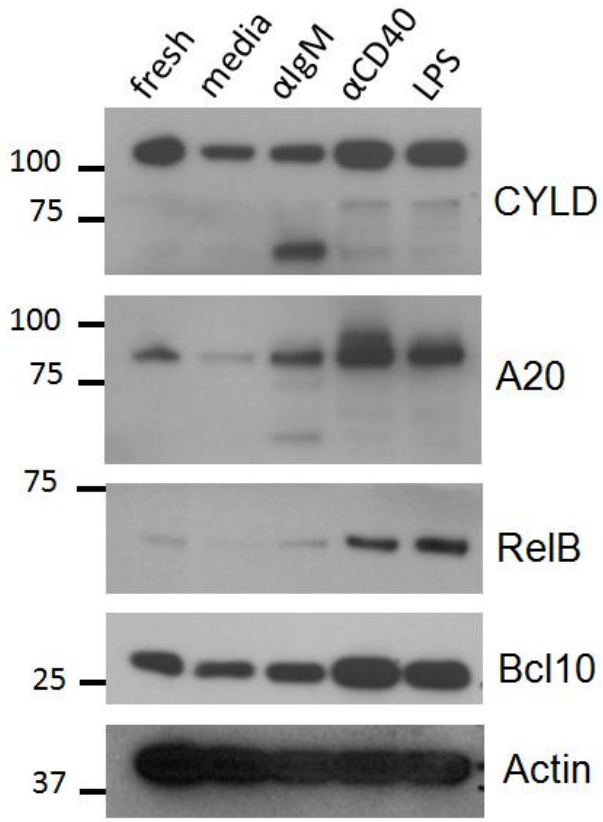


Figure 4-3. MALT1 proteolytic activity is induced specifically via BCR stimulation. Immunoblot analysis of WT splenic B cells, freshly isolated or stimulated for 24 hours with the indicated stimuli. Blots are representative of at least 3 independent experiments.

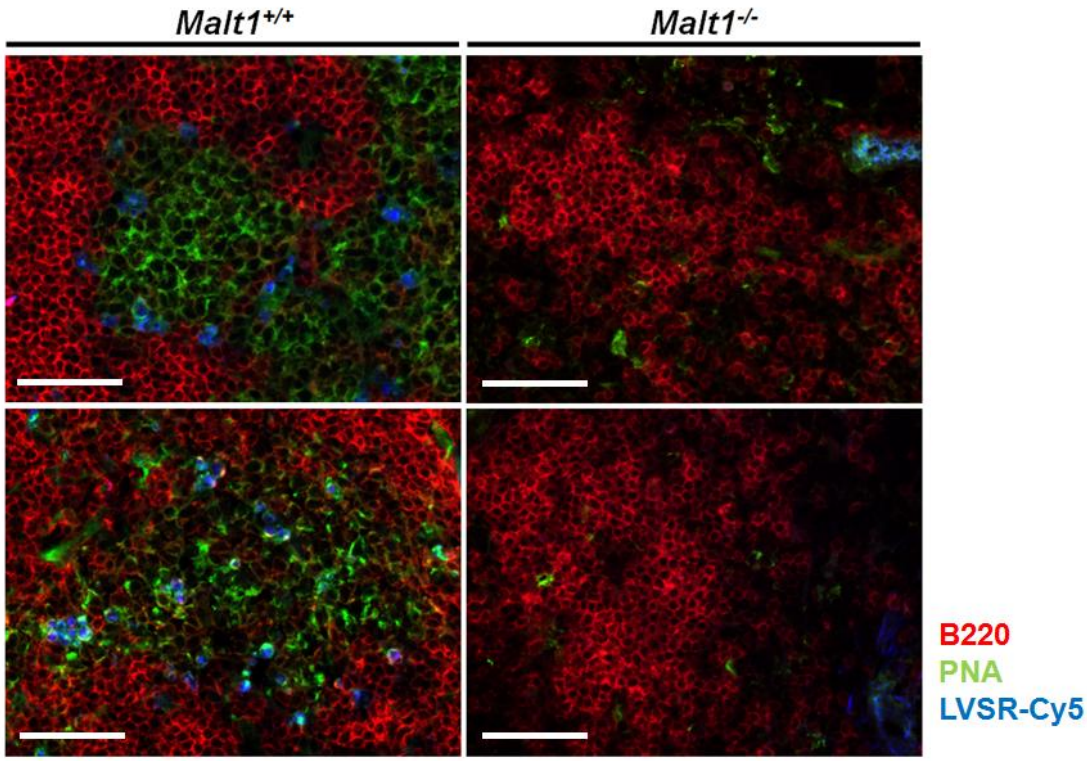


Figure 4-4. *In situ* detection of MALT1 proteolytic activity in the GC. Spleen sections from sRBC-immunized WT and *Malt1*-/- mice were stained with PNA (green), α-B220 Ab (red), and the MALT1 activity probe LVSR-Cy5 (blue). The images were taken at 40X magnification. Scale bar represents 50 µM.

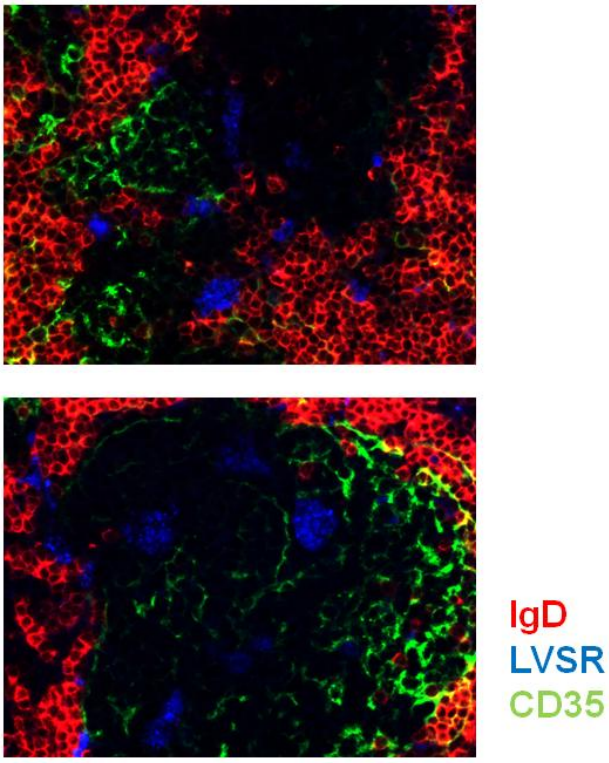


Figure 4-5. MALT1 proteolytic activity can be detected in both LZ and DZ of GC. Spleen sections from sRBC-immunized WT and *Malt1*$^{-/-}$ mice were stained with antibodies specific for CD35 (green) and IgD (red), as well as LVSR-Cy5 (blue). The images were taken at 40x magnification.

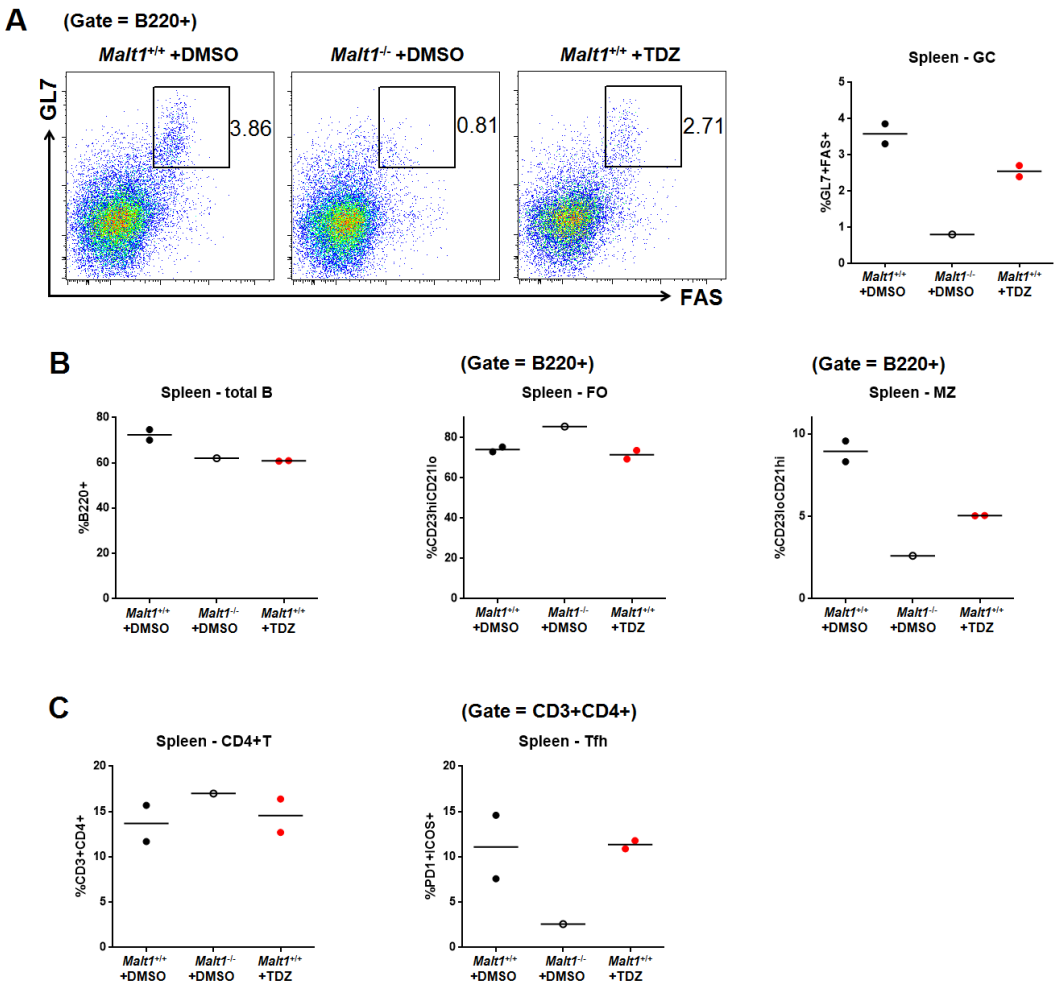


Figure 4-6. Inhibition of MALT1 protease activity does not affect GC formation. sRBC-immunized WT animals were treated daily with DMSO or thioridazine (TDZ) (n = 2 for each group) starting on day 3 post-immunization for 4 days. *Malt1*$^{-/-}$ animal (n = 1) was included as a control and was administered with DMSO post-sRBC immunization. (A) GC formation was assessed on day 7 by flow cytometry. (B) The frequencies of total B220^{+} B cells, as well as CD21^{lo}CD23^{hi} FO B cells and CD21^{hi}CD23^{lo} MZ B cells in the spleens were measured by flow cytometry. (C) Total CD3^{+}CD4^{+} T cells and PD1^{+}ICOS^{+} Tfh population were enumerated by flow cytometry.

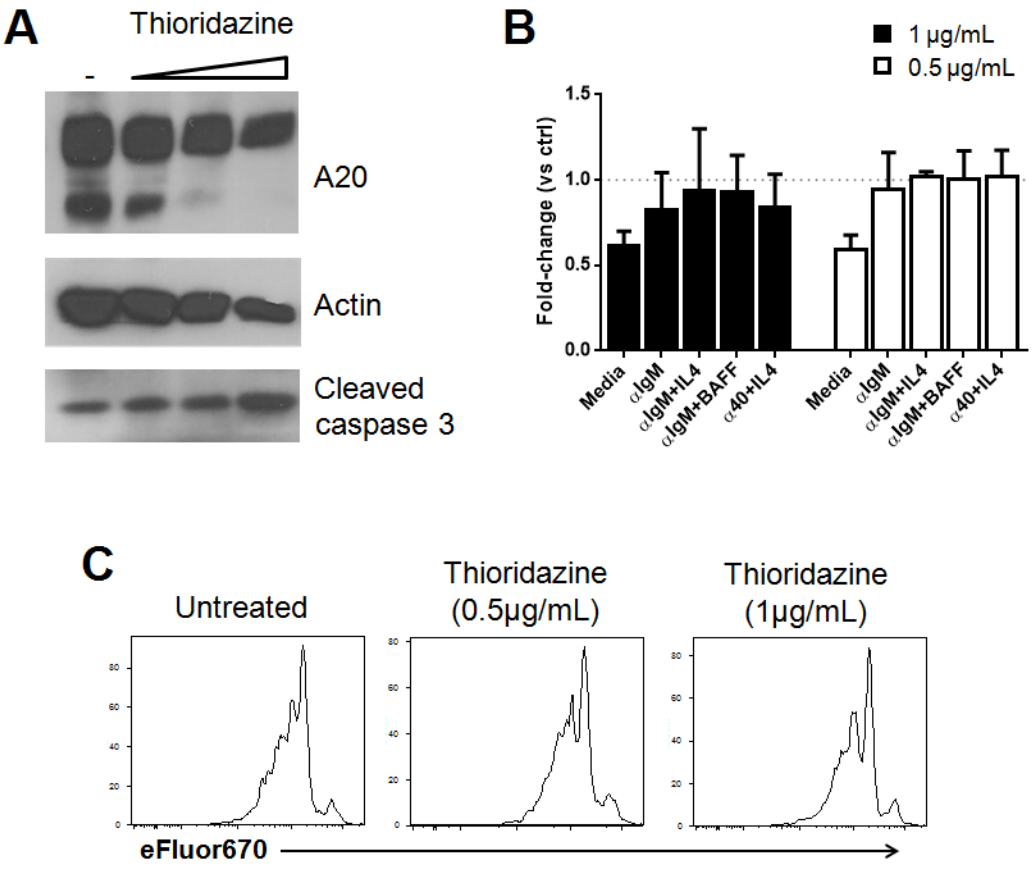


Figure 4-7. Inhibition of MALT1 activity does not affect BCR-induced proliferative and survival response. (A) Immunoblot analysis of αIgM-treated WT splenic B cells with or without increasing concentrations of thioridazine from 0.5 µg/mL to 2 µg/mL. Blots are representative of 2 independent experiments. (B) WT splenic B cells were cultured as indicated for 3 days in the presence DMSO (control), or thioridazine at 0.5 µg/mL or 1 µg/mL. Viability was assessed by flow cytometry and normalized to the corresponding conditions in the control group. (C) WT splenic B cells were treated with αIgM for 3 days alone or in the presence of thioridazine. Proliferation was determined by eFluor670 dilution. Results are representative of 3 independent experiments.

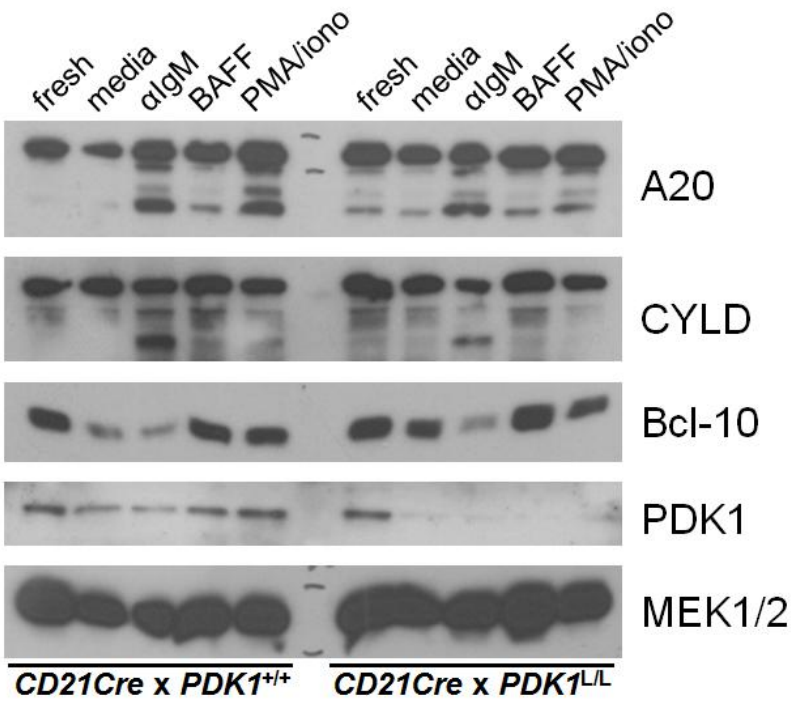


Figure 4-8. PDK1 is dispensable for MALT1-mediated proteolysis in B cells. Immunoblot analyses of splenic $CD43^{-}CD9^{-}$ FO B cells from *CD21Cre* x $PDK1^{+/+}$ and *CD21Cre* x $PDK1^{L/L}$ freshly isolated or cultured with the indicated stimuli for 24 hours. Blots are representative of 2 independent experiments (n = 2 per genotype).

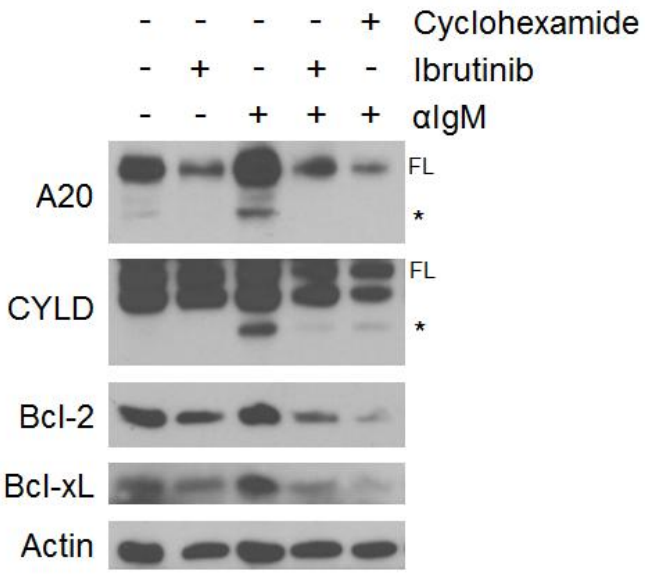


Figure 4-9. BTK activity is required for MALT1-mediated proteolysis. Immunoblot analysis of WT splenic B cells cultured as indicated for 4 hours. Blots are representative of 3 independent experiments. FL: full-length protein, *: cleaved product.

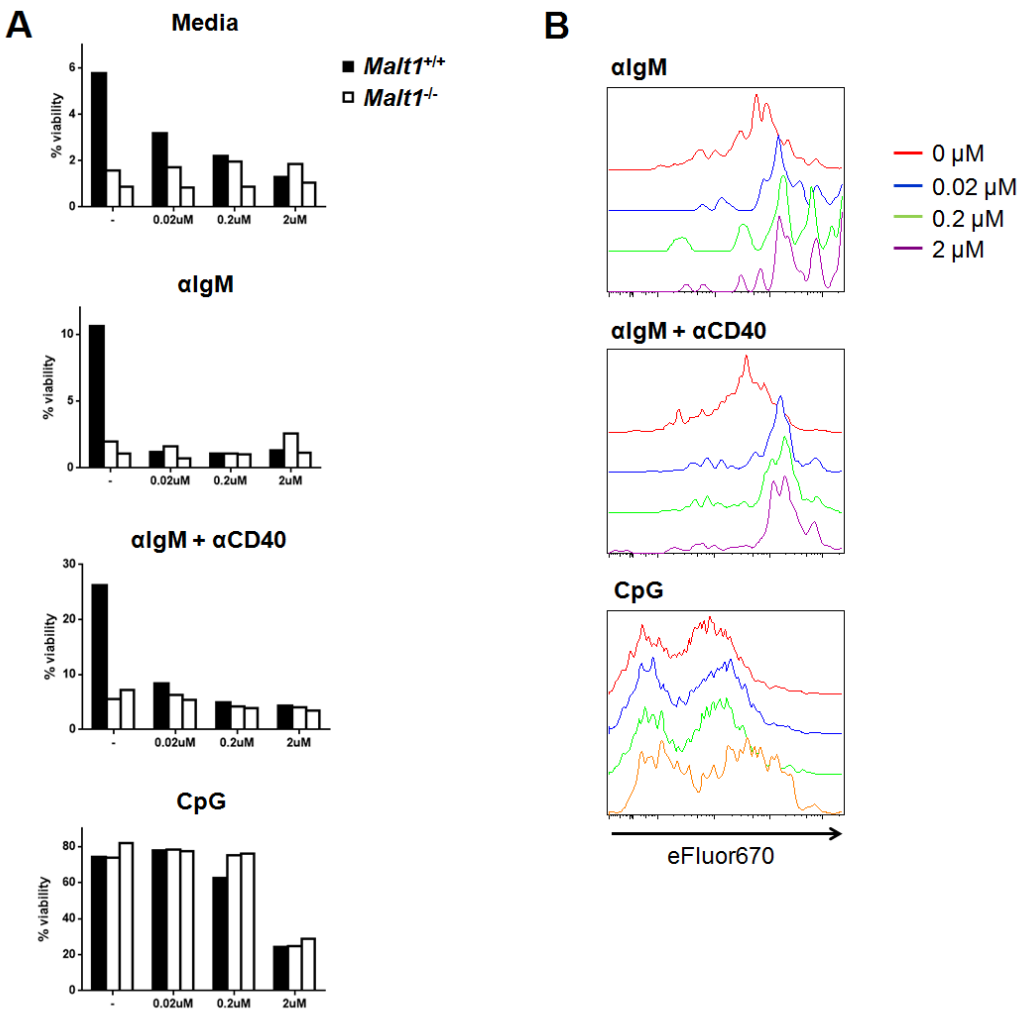


Figure 4-10. BTK activity is required for BCR-induced proliferation and survival. CD43-depleted WT (n = 1) and Malt1-/- (n = 2) B cells were cultured for 3 days with the indicated stimuli alone or in the the presence of increasing concentrations of Ibrutinib. (A) Viability was assessed by flow cytometry based on the FSC vs SSC profiles. (B) Proliferation was monitored by eFluor670 dilution.

Chapter 5. Conclusion

The hallmark of TD immune response is the formation of GC, which depends on an intact CBM complex for canonical NF-κB signaling induced by the antigen receptor. However, whether MALT1 is differentitally required in B and T cells for proper GC response has been elusive. In this dissertation, I utilized various chimeric mouse models to explore the role of MALT1 in regulating B cell functions. The evidence presented in Chapter 2 and 3 clearly indicate that B cells require MALT1 to mount an effective GC response. Furthermore, the defect associated with MALT1 deficiency has been further pinpointed to the initiation of GC reactions. I clarified the discrepancies about the role of MALT1 in B cells that were shown in previously published reports (91, 92, 94) and found that MALT1 is essential for the regulation of proliferation and survival specifically in response to BCR stimulation. Based on my data, there appears to be a dominant death signal when the BCR-Btk-CBM pathway becomes disrupted, which can be partially attributed to reduced Bcl-2 level. The same pathway is also critical for the induction of MALT1 proteolytic activity, as the results alluded to in Chapter 4. The findings described in this work are not inconsistent with the results from prior studies but rather emphasize a crucial role of MALT1 in B cells that has been previously overlooked.

The work presented here also uncovers additional cell-intrinsic roles of MALT1 in both B and T cells. Firstly, I show that MALT1 is requird in T cells for Tfh differentiation. Two models of Tfh differentiation have been proposed according to current literature. While several groups demonstrated that interactions with antigen-

specific B cells are necessary for Tfh differentiation and maintenance (101, 226, 227), others held an opposing theory that strong and persistent TCR signal is sufficient to drive Tfh differentiation (188, 228). Further investigation is necessary to distinguish between these possibilities and to delinate the mechanism of how MALT1 contributes to Tfh differentiation. Secondly, there is a B-cell instrinsic requirement for MALT1 in MZ B development. As dicussed in Chapter 2, the defective MZ pheotype is associated specifically with ablation of the CBM complex members but not the upstream kinases. It woud be of interest to see how signaling through CBM regulates differentiation into MZ B cells. Lastly, by prolonging the lifespan of MALT1-deficient B cells with forced Bcl-2 expression to allow for the initiation of GC reactions, I find that MALT1 is also required for the generation of antibody-secreting plasma cells, possibly via regulation of the transcriptional circuitry governing plasma cell differentiation.

While BCR crosslinking by soluble antibodies is a useful way to mimic antigen binding to the BCR, a more physiological revelent mouse model would be one that expresses a transgenic immunoglobulin that is specific to one type of antigen on the MALT1-deficient background. This would provide an opportunity to carefully trace the fate of *Malt1*$^{-/-}$ B cells upon encounter with their cognate antigens. Furthermore, a new mouse strain carrying a loxP-flanked *Malt1* allele has recently been made available (European Conditional Mouse Mutagenesis Program), which would enable dissection of the role of MALT1 in specific cell types to address the unanswered issues and to follow up on the new findings arising from this work.

As discussed in Chapter 4, MALT1 protease activity has been implicated in both ABC DLBCLs and MALT lymphomas that involvie the t(11;18)(q21;q21) translocation. Therapeutic inhibition of MALT1 proteolytic activity has emerged as a promising approach for the treatment of B cell lymphomas with deregulated NF-κB activation. Furthermore, MALT1 inhibition has advantages over targeting of upstream protein kinases in ABC-DLBCL tumors with lesions that are located downstream or in parallel pathways, such as CARMA1 or MYD88, respectively, since they are unlikely to respond to inhibition of Syk, Btk or PKCβ. The results from this dissertation also indicate that deletion of MALT1 induces a dominant pro-apoptotic signal in B cells that cannot be fully rescued by other pro-survival factors, such as Bcl-2 overexpression, which is a hallmark of follicular lymphoma (229) and one-third of germinal center B cells (GCB)-DLBCLs (230) Thus, even in B cell malignancies which do not depend on MALT1 proteolytic activity for survival, inhibition of MALT1 scaffolding function, by disrupting the interaction between BCL-10 and MALT1, can also generate a pro-death response.

References

1. Burnet, F. M. 1959. *The Clonal Selection Theory of Acquired Immunity* Cambridge University Press, London, and Vanderbilt University Press, Nashville.

2. Sitnicka, E., D. Bryder, K. Theilgaard-Monch, N. Buza-Vidas, J. Adolfsson, and S. E. Jacobsen. 2002. Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. *Immunity* 17: 463-472.

3. Peschon, J. J., P. J. Morrissey, K. H. Grabstein, F. J. Ramsdell, E. Maraskovsky, B. C. Gliniak, L. S. Park, S. F. Ziegler, D. E. Williams, C. B. Ware, J. D. Meyer, and B. L. Davison. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* 180: 1955-1960.

4. Rich, B. E., J. Campos-Torres, R. I. Tepper, R. W. Moreadith, and P. Leder. 1993. Cutaneous lymphoproliferation and lymphomas in interleukin 7 transgenic mice. *J Exp Med* 177: 305-316.

5. Schlissel, M. S. 2003. Regulating antigen-receptor gene assembly. *Nat Rev Immunol* 3: 890-899.

6. Nishimoto, N., H. Kubagawa, T. Ohno, G. L. Gartland, A. K. Stankovic, and M. D. Cooper. 1991. Normal pre-B cells express a receptor complex of mu heavy chains and surrogate light-chain proteins. *Proc Natl Acad Sci U S A* 88: 6284-6288.

7. Herzog, S., M. Reth, and H. Jumaa. 2009. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. *Nat Rev Immunol* 9: 195-205.

8. Mostoslavsky, R., F. W. Alt, and K. Rajewsky. 2004. The lingering enigma of the allelic exclusion mechanism. *Cell* 118: 539-544.

9. Donjerkovic, D., and D. W. Scott. 2000. Activation-induced cell death in B lymphocytes. *Cell Res* 10: 179-192.

10. Halverson, R., R. M. Torres, and R. Pelanda. 2004. Receptor editing is the main mechanism of B cell tolerance toward membrane antigens. *Nat Immunol* 5: 645-650.

11. Goodnow, C. C., J. Sprent, B. Fazekas de St Groth, and C. G. Vinuesa. 2005. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature* 435: 590-597.

12. Loder, F., B. Mutschler, R. J. Ray, C. J. Paige, P. Sideras, R. Torres, M. C. Lamers, and R. Carsetti. 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: 75-89.

13. Allman, D., R. C. Lindsley, W. DeMuth, K. Rudd, S. A. Shinton, and R. R. Hardy. 2001. Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. *J Immunol* 167: 6834-6840.

14. Mackay, F., and J. L. Browning. 2002. BAFF: a fundamental survival factor for B cells. *Nat Rev Immunol* 2: 465-475.

15. Pillai, S., and A. Cariappa. 2009. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev Immunol* 9: 767-777.

16. Cerutti, A., M. Cols, and I. Puga. 2013. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nat Rev Immunol* 13: 118-132.

17. Baumgarth, N. 2011. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* 11: 34-46.

18. Rajewsky, K. 1996. Clonal selection and learning in the antibody system. *Nature* 381: 751-758.

19. Carrasco, Y. R., and F. D. Batista. 2006. B cell recognition of membrane-bound antigen: an exquisite way of sensing ligands. *Curr Opin Immunol* 18: 286-291.

20. Batista, F. D., and N. E. Harwood. 2009. The who, how and where of antigen presentation to B cells. *Nat Rev Immunol* 9: 15-27.

21. Lanzavecchia, A. 1985. Antigen-specific interaction between T and B cells. *Nature* 314: 537-539.

22. Reif, K., E. H. Ekland, L. Ohl, H. Nakano, M. Lipp, R. Forster, and J. G. Cyster. 2002. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature* 416: 94-99.

23. Quezada, S. A., L. Z. Jarvinen, E. F. Lind, and R. J. Noelle. 2004. CD40/CD154 interactions at the interface of tolerance and immunity. *Annu Rev Immunol* 22: 307-328.

24. Kawabe, T., T. Naka, K. Yoshida, T. Tanaka, H. Fujiwara, S. Suematsu, N. Yoshida, T. Kishimoto, and H. Kikutani. 1994. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity* 1: 167-178.

25. Liu, Y. J., D. E. Joshua, G. T. Williams, C. A. Smith, J. Gordon, and I. C. MacLennan. 1989. Mechanism of antigen-driven selection in germinal centres. *Nature* 342: 929-931.

26. Tsubata, T., J. Wu, and T. Honjo. 1993. B-cell apoptosis induced by antigen receptor crosslinking is blocked by a T-cell signal through CD40. *Nature* 364: 645-648.

27. Grewal, I. S., J. Xu, and R. A. Flavell. 1995. Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. *Nature* 378: 617-620.

28. Gatto, D., D. Paus, A. Basten, C. R. Mackay, and R. Brink. 2009. Guidance of B cells by the orphan G protein-coupled receptor EBI2 shapes humoral immune responses. *Immunity* 31: 259-269.

29. Pereira, J. P., L. M. Kelly, Y. Xu, and J. G. Cyster. 2009. EBI2 mediates B cell segregation between the outer and centre follicle. *Nature* 460: 1122-1126.

30. Jacob, J., and G. Kelsoe. 1992. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers. *J Exp Med* 176: 679-687.

31. Revy, P., T. Muto, Y. Levy, F. Geissmann, A. Plebani, O. Sanal, N. Catalan, M. Forveille, R. Dufourcq-Labelouse, A. Gennery, I. Tezcan, F. Ersoy, H. Kayserili, A. G. Ugazio, N. Brousse, M. Muramatsu, L. D. Notarangelo, K. Kinoshita, T. Honjo, A. Fischer, and A. Durandy. 2000. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* 102: 565-575.

32. Muramatsu, M., K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 102: 553-563.

33. Liu, Y. J., J. Zhang, P. J. Lane, E. Y. Chan, and I. C. MacLennan. 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. *Eur J Immunol* 21: 2951-2962.

34. Allen, C. D., K. M. Ansel, C. Low, R. Lesley, H. Tamamura, N. Fujii, and J. G. Cyster. 2004. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol* 5: 943-952.

35. Shih, T. A., E. Meffre, M. Roederer, and M. C. Nussenzweig. 2002. Role of BCR affinity in T cell dependent antibody responses in vivo. *Nat Immunol* 3: 570-575.

36. Anderson, S. M., A. Khalil, M. Uduman, U. Hershberg, Y. Louzoun, A. M. Haberman, S. H. Kleinstein, and M. J. Shlomchik. 2009. Taking advantage: high-affinity B cells in the germinal center have lower death rates, but similar rates of division, compared to low-affinity cells. *J Immunol* 183: 7314-7325.

37. Allen, C. D., T. Okada, H. L. Tang, and J. G. Cyster. 2007. Imaging of germinal center selection events during affinity maturation. *Science* 315: 528-531.

38. Nutt, S. L., P. D. Hodgkin, D. M. Tarlinton, and L. M. Corcoran. 2015. The generation of antibody-secreting plasma cells. *Nat Rev Immunol* 15: 160-171.

39. Yefenof, E., V. M. Sanders, J. W. Uhr, and E. S. Vitetta. 1986. In vitro activation of murine antigen-specific memory B cells by a T-dependent antigen. *J Immunol* 137: 85-90.

40. Good, K. L., D. T. Avery, and S. G. Tangye. 2009. Resting human memory B cells are intrinsically programmed for enhanced survival and responsiveness to diverse stimuli compared to naive B cells. *J Immunol* 182: 890-901.

41. Stavnezer, J., J. E. Guikema, and C. E. Schrader. 2008. Mechanism and regulation of class switch recombination. *Annu Rev Immunol* 26: 261-292.

42. Norvell, A., L. Mandik, and J. G. Monroe. 1995. Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. *J Immunol* 154: 4404-4413.

43. Lam, K. P., R. Kuhn, and K. Rajewsky. 1997. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* 90: 1073-1083.

44. Niiro, H., and E. A. Clark. 2002. Regulation of B-cell fate by antigen-receptor signals. *Nat Rev Immunol* 2: 945-956.

45. Rolli, V., M. Gallwitz, T. Wossning, A. Flemming, W. W. Schamel, C. Zurn, and M. Reth. 2002. Amplification of B cell antigen receptor signaling by a Syk/ITAM positive feedback loop. *Mol Cell* 10: 1057-1069.

46. Fu, C., C. W. Turck, T. Kurosaki, and A. C. Chan. 1998. BLNK: a central linker protein in B cell activation. *Immunity* 9: 93-103.

47. Takata, M., and T. Kurosaki. 1996. A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-gamma 2. *J Exp Med* 184: 31-40.

48. Berridge, M. J. 1993. Inositol trisphosphate and calcium signalling. *Nature* 361: 315-325.

49. Johnson, G. L., and R. Lapadat. 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298: 1911-1912.

50. Oh-hora, M., S. Johmura, A. Hashimoto, M. Hikida, and T. Kurosaki. 2003. Requirement for Ras guanine nucleotide releasing protein 3 in coupling phospholipase C-gamma2 to Ras in B cell receptor signaling. *J Exp Med* 198: 1841-1851.

51. Hashimoto, A., H. Okada, A. Jiang, M. Kurosaki, S. Greenberg, E. A. Clark, and T. Kurosaki. 1998. Involvement of guanosine triphosphatases and phospholipase C-gamma2 in extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, and p38 mitogen-activated protein kinase activation by the B cell antigen receptor. *J Exp Med* 188: 1287-1295.

52. Bustelo, X. R., and M. Barbacid. 1992. Tyrosine phosphorylation of the vav proto-oncogene product in activated B cells. *Science* 256: 1196-1199.

53. Johmura, S., M. Oh-hora, K. Inabe, Y. Nishikawa, K. Hayashi, E. Vigorito, D. Kitamura, M. Turner, K. Shingu, M. Hikida, and T. Kurosaki. 2003. Regulation of Vav localization in membrane rafts by adaptor molecules Grb2 and BLNK. *Immunity* 18: 777-787.

54. Dal Porto, J. M., S. B. Gauld, K. T. Merrell, D. Mills, A. E. Pugh-Bernard, and J. Cambier. 2004. B cell antigen receptor signaling 101. *Mol Immunol* 41: 599-613.

55. Rao, A., C. Luo, and P. G. Hogan. 1997. Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* 15: 707-747.

56. Fearon, D. T., and M. C. Carroll. 2000. Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. *Annu Rev Immunol* 18: 393-422.

57. Tuveson, D. A., R. H. Carter, S. P. Soltoff, and D. T. Fearon. 1993. CD19 of B cells as a surrogate kinase insert region to bind phosphatidylinositol 3-kinase. *Science* 260: 986-989.

58. Inabe, K., M. Ishiai, A. M. Scharenberg, N. Freshney, J. Downward, and T. Kurosaki. 2002. Vav3 modulates B cell receptor responses by regulating phosphoinositide 3-kinase activation. *J Exp Med* 195: 189-200.

59. Fruman, D. A. 2004. Phosphoinositide 3-kinase and its targets in B-cell and T-cell signaling. *Curr Opin Immunol* 16: 314-320.

60. Varnai, P., T. Bondeva, P. Tamas, B. Toth, L. Buday, L. Hunyady, and T. Balla. 2005. Selective cellular effects of overexpressed pleckstrin-homology domains that recognize PtdIns(3,4,5)P3 suggest their interaction with protein binding partners. *J Cell Sci* 118: 4879-4888.

61. Stephens, L., K. Anderson, D. Stokoe, H. Erdjument-Bromage, G. F. Painter, A. B. Holmes, P. R. Gaffney, C. B. Reese, F. McCormick, P. Tempst, J. Coadwell, and P. T. Hawkins. 1998. Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science* 279: 710-714.

62. Sarbassov, D. D., D. A. Guertin, S. M. Ali, and D. M. Sabatini. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307: 1098-1101.

63. Calnan, D. R., and A. Brunet. 2008. The FoxO code. *Oncogene* 27: 2276-2288.

64. Limon, J. J., and D. A. Fruman. 2012. Akt and mTOR in B Cell Activation and Differentiation. *Front Immunol* 3: 228.

65. Baldwin, A. S., Jr. 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 14: 649-683.

66. Liou, H. C., W. C. Sha, M. L. Scott, and D. Baltimore. 1994. Sequential induction of NF-kappa B/Rel family proteins during B-cell terminal differentiation. *Mol Cell Biol* 14: 5349-5359.

67. Baeuerle, P. A., and D. Baltimore. 1988. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* 242: 540-546.

68. Sommer, K., B. Guo, J. L. Pomerantz, A. D. Bandaranayake, M. E. Moreno-Garcia, Y. L. Ovechkina, and D. J. Rawlings. 2005. Phosphorylation of the CARMA1 linker controls NF-kappaB activation. *Immunity* 23: 561-574.

69. Shinohara, H., S. Maeda, H. Watarai, and T. Kurosaki. 2007. IkappaB kinase beta-induced phosphorylation of CARMA1 contributes to CARMA1 Bcl10 MALT1 complex formation in B cells. *J Exp Med* 204: 3285-3293.

70. Qiao, Q., C. Yang, C. Zheng, L. Fontan, L. David, X. Yu, C. Bracken, M. Rosen, A. Melnick, E. H. Egelman, and H. Wu. 2013. Structural architecture of

the CARMA1/Bcl10/MALT1 signalosome: nucleation-induced filamentous assembly. *Mol Cell* 51: 766-779.

71. Sun, L., L. Deng, C. K. Ea, Z. P. Xia, and Z. J. Chen. 2004. The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. *Mol Cell* 14: 289-301.

72. Shinohara, H., T. Yasuda, Y. Aiba, H. Sanjo, M. Hamadate, H. Watarai, H. Sakurai, and T. Kurosaki. 2005. PKC beta regulates BCR-mediated IKK activation by facilitating the interaction between TAK1 and CARMA1. *J Exp Med* 202: 1423-1431.

73. Liao, G., M. Zhang, E. W. Harhaj, and S. C. Sun. 2004. Regulation of the NF-kappaB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation. *J Biol Chem* 279: 26243-26250.

74. Claudio, E., K. Brown, S. Park, H. Wang, and U. Siebenlist. 2002. BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells. *Nat Immunol* 3: 958-965.

75. Kayagaki, N., M. Yan, D. Seshasayee, H. Wang, W. Lee, D. M. French, I. S. Grewal, A. G. Cochran, N. C. Gordon, J. Yin, M. A. Starovasnik, and V. M. Dixit. 2002. BAFF/BLyS receptor 3 binds the B cell survival factor BAFF ligand through a discrete surface loop and promotes processing of NF-kappaB2. *Immunity* 17: 515-524.

76. Gardam, S., and R. Brink. 2014. Non-Canonical NF-kappaB Signaling Initiated by BAFF Influences B Cell Biology at Multiple Junctures. *Front Immunol* 4: 509.

77. Schiemann, B., J. L. Gommerman, K. Vora, T. G. Cachero, S. Shulga-Morskaya, M. Dobles, E. Frew, and M. L. Scott. 2001. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* 293: 2111-2114.

78. Gross, J. A., S. R. Dillon, S. Mudri, J. Johnston, A. Littau, R. Roque, M. Rixon, O. Schou, K. P. Foley, H. Haugen, S. McMillen, K. Waggie, R. W. Schreckhise, K. Shoemaker, T. Vu, M. Moore, A. Grossman, and C. H. Clegg. 2001. TACI-Ig neutralizes molecules critical for B cell development and autoimmune disease. impaired B cell maturation in mice lacking BLyS. *Immunity* 15: 289-302.

79. Rolink, A. G., J. Tschopp, P. Schneider, and F. Melchers. 2002. BAFF is a survival and maturation factor for mouse B cells. *Eur J Immunol* 32: 2004-2010.

80. Patke, A., I. Mecklenbrauker, and A. Tarakhovsky. 2004. Survival signaling in resting B cells. *Curr Opin Immunol* 16: 251-255.

81. Hu, H. M., K. O'Rourke, M. S. Boguski, and V. M. Dixit. 1994. A novel RING finger protein interacts with the cytoplasmic domain of CD40. *J Biol Chem* 269: 30069-30072.

82. Cheng, G., A. M. Cleary, Z. S. Ye, D. I. Hong, S. Lederman, and D. Baltimore. 1995. Involvement of CRAF1, a relative of TRAF, in CD40 signaling. *Science* 267: 1494-1498.

83. Rothe, M., V. Sarma, V. M. Dixit, and D. V. Goeddel. 1995. TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. *Science* 269: 1424-1427.

84. Ishida, T., S. Mizushima, S. Azuma, N. Kobayashi, T. Tojo, K. Suzuki, S. Aizawa, T. Watanabe, G. Mosialos, E. Kieff, T. Yamamoto, and J. Inoue. 1996. Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region. *J Biol Chem* 271: 28745-28748.

85. Shih, V. F., R. Tsui, A. Caldwell, and A. Hoffmann. 2011. A single NFkappaB system for both canonical and non-canonical signaling. *Cell Res* 21: 86-102.

86. Coope, H. J., P. G. Atkinson, B. Huhse, M. Belich, J. Janzen, M. J. Holman, G. G. Klaus, L. H. Johnston, and S. C. Ley. 2002. CD40 regulates the processing of NF-kappaB2 p100 to p52. *EMBO J* 21: 5375-5385.

87. Pasare, C., and R. Medzhitov. 2005. Control of B-cell responses by Toll-like receptors. *Nature* 438: 364-368.

88. Hayden, M. S., A. P. West, and S. Ghosh. 2006. NF-kappaB and the immune response. *Oncogene* 25: 6758-6780.

89. Hara, H., T. Wada, C. Bakal, I. Kozieradzki, S. Suzuki, N. Suzuki, M. Nghiem, E. K. Griffiths, C. Krawczyk, B. Bauer, F. D'Acquisto, S. Ghosh, W. C. Yeh, G. Baier, R. Rottapel, and J. M. Penninger. 2003. The MAGUK family protein CARD11 is essential for lymphocyte activation. *Immunity* 18: 763-775.

90. Ruland, J., G. S. Duncan, A. Elia, I. del Barco Barrantes, L. Nguyen, S. Plyte, D. G. Millar, D. Bouchard, A. Wakeham, P. S. Ohashi, and T. W. Mak. 2001. Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. *Cell* 104: 33-42.

91. Ruefli-Brasse, A. A., D. M. French, and V. M. Dixit. 2003. Regulation of NF-kappaB-dependent lymphocyte activation and development by paracaspase. *Science* 302: 1581-1584.

92. Ruland, J., G. S. Duncan, A. Wakeham, and T. W. Mak. 2003. Differential requirement for Malt1 in T and B cell antigen receptor signaling. *Immunity* 19: 749-758.

93. Tusche, M. W., L. A. Ward, F. Vu, D. McCarthy, M. Quintela-Fandino, J. Ruland, J. L. Gommerman, and T. W. Mak. 2009. Differential requirement of MALT1 for BAFF-induced outcomes in B cell subsets. *J Exp Med* 206: 2671-2683.

94. Ferch, U., C. M. zum Buschenfelde, A. Gewies, E. Wegener, S. Rauser, C. Peschel, D. Krappmann, and J. Ruland. 2007. MALT1 directs B cell receptor-induced canonical nuclear factor-kappaB signaling selectively to the c-Rel subunit. *Nat Immunol* 8: 984-991.

95. Afonina, I. S., L. Elton, I. Carpentier, and R. Beyaert. 2015. MALT1 - a universal soldier: multiple strategies to ensure NF-kappaB activation and target gene expression. *FEBS J* 282: 3286-3297.

96. Allen, C. D., T. Okada, and J. G. Cyster. 2007. Germinal-center organization and cellular dynamics. *Immunity* 27: 190-202.

97. Goetz, C. A., and A. S. Baldwin. 2008. NF-kappaB pathways in the immune system: control of the germinal center reaction. *Immunol Res* 41: 233-247.

98. Carrasco, D., J. Cheng, A. Lewin, G. Warr, H. Yang, C. Rizzo, F. Rosas, C. Snapper, and R. Bravo. 1998. Multiple hemopoietic defects and lymphoid hyperplasia in mice lacking the transcriptional activation domain of the c-Rel protein. *J Exp Med* 187: 973-984.

99. Tumang, J. R., A. Owyang, S. Andjelic, Z. Jin, R. R. Hardy, M. L. Liou, and H. C. Liou. 1998. c-Rel is essential for B lymphocyte survival and cell cycle progression. *Eur J Immunol* 28: 4299-4312.

100. Bornancin, F., F. Renner, R. Touil, H. Sic, Y. Kolb, I. Touil-Allaoui, J. S. Rush, P. A. Smith, M. Bigaud, U. Junker-Walker, C. Burkhart, J. Dawson, S. Niwa, A. Katopodis, B. Nuesslein-Hildesheim, G. Weckbecker, G. Zenke, B. Kinzel, E. Traggiai, D. Brenner, A. Brustle, M. St Paul, N. Zamurovic, K. D. McCoy, A. Rolink, C. H. Regnier, T. W. Mak, P. S. Ohashi, D. D. Patel, and T. Calzascia. 2015. Deficiency of MALT1 paracaspase activity results in unbalanced regulatory and effector T and B cell responses leading to multiorgan inflammation. *J Immunol* 194: 3723-3734.

101. Baumjohann, D., S. Preite, A. Reboldi, F. Ronchi, K. M. Ansel, A. Lanzavecchia, and F. Sallusto. 2013. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* 38: 596-605.

102. Han, S., K. Hathcock, B. Zheng, T. B. Kepler, R. Hodes, and G. Kelsoe. 1995. Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *J Immunol* 155: 556-567.

103. Reiter, R., and K. Pfeffer. 2002. Impaired germinal centre formation and humoral immune response in the absence of CD28 and interleukin-4. *Immunology* 106: 222-228.

104. Che, T., Y. You, D. Wang, M. J. Tanner, V. M. Dixit, and X. Lin. 2004. MALT1/paracaspase is a signaling component downstream of CARMA1 and mediates T cell receptor-induced NF-kappaB activation. *J Biol Chem* 279: 15870-15876.

105. Jabara, H. H., T. Ohsumi, J. Chou, M. J. Massaad, H. Benson, A. Megarbane, E. Chouery, R. Mikhael, O. Gorka, A. Gewies, P. Portales, T. Nakayama, H. Hosokawa, P. Revy, H. Herrod, F. Le Deist, G. Lefranc, J. Ruland, and R. S. Geha. 2013. A homozygous mucosa-associated lymphoid tissue 1 (MALT1) mutation in a family with combined immunodeficiency. *J Allergy Clin Immunol* 132: 151-158.

106. McKinnon, M. L., J. Rozmus, S. Y. Fung, A. F. Hirschfeld, K. L. Del Bel, L. Thomas, N. Marr, S. D. Martin, A. K. Marwaha, J. J. Priatel, R. Tan, C. Senger, A. Tsang, J. Prendiville, A. K. Junker, M. Seear, K. R. Schultz, L. M. Sly, R. A. Holt, M. S. Patel, J. M. Friedman, and S. E. Turvey. 2014. Combined immunodeficiency associated with homozygous MALT1 mutations. *J Allergy Clin Immunol* 133: 1458-1462, 1462 e1451-1457.

107. Vicente-Duenas, C., L. Fontan, I. Gonzalez-Herrero, I. Romero-Camarero, V. Segura, M. A. Aznar, E. Alonso-Escudero, E. Campos-Sanchez, L. Ruiz-Roca, M. Barajas-Diego, A. Sagardoy, J. I. Martinez-Ferrandis, F. Abollo-Jimenez, C. Bertolo, I. Penuelas, F. J. Garcia-Criado, M. B. Garcia-Cenador, T. Tousseyn, X. Agirre, F. Prosper, F. Garcia-Bragado, E. D. McPhail, I. S. Lossos, M. Q. Du, T. Flores, J. M. Hernandez-Rivas, M. Gonzalez, A. Salar, B. Bellosillo, E. Conde, R. Siebert, X. Sagaert, C. Cobaleda, I. Sanchez-Garcia, and J. A. Martinez-Climent. 2012. Expression of MALT1 oncogene in hematopoietic stem/progenitor cells recapitulates the pathogenesis of human lymphoma in mice. *Proc Natl Acad Sci U S A* 109: 10534-10539.

108. Nojima, T., K. Haniuda, T. Moutai, M. Matsudaira, S. Mizokawa, I. Shiratori, T. Azuma, and D. Kitamura. 2011. In-vitro derived germinal centre B cells differentially generate memory B or plasma cells in vivo. *Nat Commun* 2: 465.

109. Spandidos, A., X. Wang, H. Wang, and B. Seed. 2010. PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. *Nucleic Acids Res* 38: D792-799.

110. Cato, M. H., S. K. Chintalapati, I. W. Yau, S. A. Omori, and R. C. Rickert. 2011. Cyclin D3 is selectively required for proliferative expansion of germinal center B cells. *Mol Cell Biol* 31: 127-137.

111. Dufner, A., and W. W. Schamel. 2011. B cell antigen receptor-induced activation of an IRAK4-dependent signaling pathway revealed by a MALT1-IRAK4 double knockout mouse model. *Cell Commun Signal* 9: 6.

112. Solvason, N., W. W. Wu, N. Kabra, X. Wu, E. Lees, and M. C. Howard. 1996. Induction of cell cycle regulatory proteins in anti-immunoglobulin-stimulated mature B lymphocytes. *J Exp Med* 184: 407-417.

113. Natori, S., H. Higuchi, P. Contreras, and G. J. Gores. 2003. The caspase inhibitor IDN-6556 prevents caspase activation and apoptosis in sinusoidal endothelial cells during liver preservation injury. *Liver Transpl* 9: 278-284.

114. Changelian, P. S., M. E. Flanagan, D. J. Ball, C. R. Kent, K. S. Magnuson, W. H. Martin, B. J. Rizzuti, P. S. Sawyer, B. D. Perry, W. H. Brissette, S. P. McCurdy, E. M. Kudlacz, M. J. Conklyn, E. A. Elliott, E. R. Koslov, M. B. Fisher, T. J. Strelevitz, K. Yoon, D. A. Whipple, J. Sun, M. J. Munchhof, J. L. Doty, J. M. Casavant, T. A. Blumenkopf, M. Hines, M. F. Brown, B. M. Lillie, C. Subramanyam, C. Shang-Poa, A. J. Milici, G. E. Beckius, J. D. Moyer, C. Su, T. G. Woodworth, A. S. Gaweco, C. R. Beals, B. H. Littman, D. A. Fisher, J. F. Smith, P. Zagouras, H. A. Magna, M. J. Saltarelli, K. S. Johnson, L. F. Nelms, S. G. Des Etages, L. S. Hayes, T. T. Kawabata, D. Finco-Kent, D. L. Baker, M. Larson, M. S. Si, R. Paniagua, J. Higgins, B. Holm, B. Reitz, Y. J. Zhou, R. E. Morris, J. J. O'Shea, and D. C. Borie. 2003. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. *Science* 302: 875-878.

115. Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol* 76: 34-40.

116. Donahue, A. C., and D. A. Fruman. 2003. Proliferation and survival of activated B cells requires sustained antigen receptor engagement and phosphoinositide 3-kinase activation. *J Immunol* 170: 5851-5860.

117. Leitges, M., C. Schmedt, R. Guinamard, J. Davoust, S. Schaal, S. Stabel, and A. Tarakhovsky. 1996. Immunodeficiency in protein kinase cbeta-deficient mice. *Science* 273: 788-791.

118. Saijo, K., I. Mecklenbrauker, C. Schmedt, and A. Tarakhovsky. 2003. B cell immunity regulated by the protein kinase C family. *Ann N Y Acad Sci* 987: 125-134.

119. Gilmore, T. D., and S. Gerondakis. 2011. The c-Rel Transcription Factor in Development and Disease. *Genes Cancer* 2: 695-711.

120. Ridderstad, A., G. J. Nossal, and D. M. Tarlinton. 1996. The xid mutation diminishes memory B cell generation but does not affect somatic hypermutation and selection. *J Immunol* 157: 3357-3365.

121. Benson, M. J., V. Rodriguez, D. von Schack, S. Keegan, T. A. Cook, J. Edmonds, S. Benoit, N. Seth, S. Du, D. Messing, C. L. Nickerson-Nutter, K. Dunussi-Joannopoulos, A. L. Rankin, M. Ruzek, M. E. Schnute, and J. Douhan, 3rd. 2014. Modeling the clinical phenotype of BTK inhibition in the mature murine immune system. *J Immunol* 193: 185-197.

122. Hikida, M., S. Casola, N. Takahashi, T. Kaji, T. Takemori, K. Rajewsky, and T. Kurosaki. 2009. PLC-gamma2 is essential for formation and maintenance of memory B cells. *J Exp Med* 206: 681-689.

123. Xue, L., S. W. Morris, C. Orihuela, E. Tuomanen, X. Cui, R. Wen, and D. Wang. 2003. Defective development and function of Bcl10-deficient follicular, marginal zone and B1 B cells. *Nat Immunol* 4: 857-865.

124. Shaffer, A. L., A. Rosenwald, E. M. Hurt, J. M. Giltnane, L. T. Lam, O. K. Pickeral, and L. M. Staudt. 2001. Signatures of the immune response. *Immunity* 15: 375-385.

125. Khalil, A. M., J. C. Cambier, and M. J. Shlomchik. 2012. B cell receptor signal transduction in the GC is short-circuited by high phosphatase activity. *Science* 336: 1178-1181.

126. Heise, N., N. S. De Silva, K. Silva, A. Carette, G. Simonetti, M. Pasparakis, and U. Klein. 2014. Germinal center B cell maintenance and differentiation are controlled by distinct NF-kappaB transcription factor subunits. *J Exp Med* 211: 2103-2118.

127. Weih, F., D. Carrasco, and R. Bravo. 1994. Constitutive and inducible Rel/NF-kappa B activities in mouse thymus and spleen. *Oncogene* 9: 3289-3297.

128. Beg, A. A., W. C. Sha, R. T. Bronson, S. Ghosh, and D. Baltimore. 1995. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 376: 167-170.

129. Doi, T. S., T. Takahashi, O. Taguchi, T. Azuma, and Y. Obata. 1997. NF-kappa B RelA-deficient lymphocytes: normal development of T cells and B cells, impaired production of IgA and IgG1 and reduced proliferative responses. *J Exp Med* 185: 953-961.

130. Pohl, T., R. Gugasyan, R. J. Grumont, A. Strasser, D. Metcalf, D. Tarlinton, W. Sha, D. Baltimore, and S. Gerondakis. 2002. The combined absence of NF-kappa B1 and c-Rel reveals that overlapping roles for these transcription factors in the B cell lineage are restricted to the activation and function of mature cells. *Proc Natl Acad Sci U S A* 99: 4514-4519.

131. Grumont, R. J., I. J. Rourke, L. A. O'Reilly, A. Strasser, K. Miyake, W. Sha, and S. Gerondakis. 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: 663-674.

132. Grossmann, M., L. A. O'Reilly, R. Gugasyan, A. Strasser, J. M. Adams, and S. Gerondakis. 2000. The anti-apoptotic activities of Rel and RelA required during B-cell maturation involve the regulation of Bcl-2 expression. *EMBO J* 19: 6351-6360.

133. Gerondakis, S., and U. Siebenlist. 2010. Roles of the NF-kappaB pathway in lymphocyte development and function. *Cold Spring Harb Perspect Biol* 2: a000182.

134. Thome, M. 2004. CARMA1, BCL-10 and MALT1 in lymphocyte development and activation. *Nat Rev Immunol* 4: 348-359.

135. Martin, F., A. M. Oliver, and J. F. Kearney. 2001. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* 14: 617-629.

136. Oliver, A. M., F. Martin, G. L. Gartland, R. H. Carter, and J. F. Kearney. 1997. Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. *Eur J Immunol* 27: 2366-2374.

137. Balazs, M., F. Martin, T. Zhou, and J. Kearney. 2002. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. *Immunity* 17: 341-352.

138. Hardy, R. R., K. Hayakawa, D. R. Parks, and L. A. Herzenberg. 1983. Demonstration of B-cell maturation in X-linked immunodeficient mice by simultaneous three-colour immunofluorescence. *Nature* 306: 270-272.

139. Hikida, M., S. Johmura, A. Hashimoto, M. Takezaki, and T. Kurosaki. 2003. Coupling between B cell receptor and phospholipase C-gamma2 is essential for mature B cell development. *J Exp Med* 198: 581-589.

140. Pappu, B. P., and X. Lin. 2006. Potential role of CARMA1 in CD40-induced splenic B cell proliferation and marginal zone B cell maturation. *Eur J Immunol* 36: 3033-3043.

141. Cariappa, A., H. C. Liou, B. H. Horwitz, and S. Pillai. 2000. Nuclear factor kappa B is required for the development of marginal zone B lymphocytes. *J Exp Med* 192: 1175-1182.

142. Sasaki, Y., E. Derudder, E. Hobeika, R. Pelanda, M. Reth, K. Rajewsky, and M. Schmidt-Supprian. 2006. Canonical NF-kappaB activity, dispensable for B cell development, replaces BAFF-receptor signals and promotes B cell proliferation upon activation. *Immunity* 24: 729-739.

143. Feng, B., S. Cheng, C. Y. Hsia, L. B. King, J. G. Monroe, and H. C. Liou. 2004. NF-kappaB inducible genes BCL-X and cyclin E promote immature B-cell proliferation and survival. *Cell Immunol* 232: 9-20.

144. Khan, W. N., F. W. Alt, R. M. Gerstein, B. A. Malynn, I. Larsson, G. Rathbun, L. Davidson, S. Muller, A. B. Kantor, L. A. Herzenberg, and et al. 1995. Defective B cell development and function in Btk-deficient mice. *Immunity* 3: 283-299.

145. Su, T. T., B. Guo, Y. Kawakami, K. Sommer, K. Chae, L. A. Humphries, R. M. Kato, S. Kang, L. Patrone, R. Wall, M. Teitell, M. Leitges, T. Kawakami, and D. J. Rawlings. 2002. PKC-beta controls I kappa B kinase lipid raft recruitment and activation in response to BCR signaling. *Nat Immunol* 3: 780-786.

146. Moore, P. A., O. Belvedere, A. Orr, K. Pieri, D. W. LaFleur, P. Feng, D. Soppet, M. Charters, R. Gentz, D. Parmelee, Y. Li, O. Galperina, J. Giri, V. Roschke, B. Nardelli, J. Carrell, S. Sosnovtseva, W. Greenfield, S. M. Ruben, H. S. Olsen, J. Fikes, and D. M. Hilbert. 1999. BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science* 285: 260-263.

147. Patke, A., I. Mecklenbrauker, H. Erdjument-Bromage, P. Tempst, and A. Tarakhovsky. 2006. BAFF controls B cell metabolic fitness through a PKC beta- and Akt-dependent mechanism. *J Exp Med* 203: 2551-2562.

148. Craxton, A., D. Magaletti, E. J. Ryan, and E. A. Clark. 2003. Macrophage- and dendritic cell--dependent regulation of human B-cell proliferation requires the TNF family ligand BAFF. *Blood* 101: 4464-4471.

149. Castro, I., J. A. Wright, B. Damdinsuren, K. L. Hoek, G. Carlesso, N. P. Shinners, R. M. Gerstein, R. T. Woodland, R. Sen, and W. N. Khan. 2009. B cell receptor-mediated sustained c-Rel activation facilitates late transitional B

cell survival through control of B cell activating factor receptor and NF-kappaB2. *J Immunol* 182: 7729-7737.

150. Guo, B., and T. L. Rothstein. 2005. B cell receptor (BCR) cross-talk: IL-4 creates an alternate pathway for BCR-induced ERK activation that is phosphatidylinositol 3-kinase independent. *J Immunol* 174: 5375-5381.

151. Dufort, F. J., B. F. Bleiman, M. R. Gumina, D. Blair, D. J. Wagner, M. F. Roberts, Y. Abu-Amer, and T. C. Chiles. 2007. Cutting edge: IL-4-mediated protection of primary B lymphocytes from apoptosis via Stat6-dependent regulation of glycolytic metabolism. *J Immunol* 179: 4953-4957.

152. Hasbold, J., A. B. Lyons, M. R. Kehry, and P. D. Hodgkin. 1998. Cell division number regulates IgG1 and IgE switching of B cells following stimulation by CD40 ligand and IL-4. *Eur J Immunol* 28: 1040-1051.

153. Dedeoglu, F., B. Horwitz, J. Chaudhuri, F. W. Alt, and R. S. Geha. 2004. Induction of activation-induced cytidine deaminase gene expression by IL-4 and CD40 ligation is dependent on STAT6 and NFkappaB. *Int Immunol* 16: 395-404.

154. Owyang, A. M., J. R. Tumang, B. R. Schram, C. Y. Hsia, T. W. Behrens, T. L. Rothstein, and H. C. Liou. 2001. c-Rel is required for the protection of B cells from antigen receptor-mediated, but not Fas-mediated, apoptosis. *J Immunol* 167: 4948-4956.

155. Sochalska, M., E. Ottina, S. Tuzlak, S. Herzog, M. Herold, and A. Villunger. 2015. Conditional knockdown of BCL2A1 reveals rate-limiting roles in BCR-dependent B-cell survival. *Cell Death Differ*.

156. Vikstrom, I., S. Carotta, K. Luthje, V. Peperzak, P. J. Jost, S. Glaser, M. Busslinger, P. Bouillet, A. Strasser, S. L. Nutt, and D. M. Tarlinton. 2010. Mcl-1 is essential for germinal center formation and B cell memory. *Science* 330: 1095-1099.

157. Wen, R., Y. Chen, L. Xue, J. Schuman, S. Yang, S. W. Morris, and D. Wang. 2003. Phospholipase Cgamma2 provides survival signals via Bcl2 and A1 in different subpopulations of B cells. *J Biol Chem* 278: 43654-43662.

158. Peperzak, V., I. B. Vikstrom, and D. M. Tarlinton. 2012. Through a glass less darkly: apoptosis and the germinal center response to antigen. *Immunol Rev* 247: 93-106.

159. Bureau, F., A. Vanderplasschen, F. Jaspar, F. Minner, P. P. Pastoret, M. P. Merville, V. Bours, and P. Lekeux. 2002. Constitutive nuclear factor-kappaB activity preserves homeostasis of quiescent mature lymphocytes and

granulocytes by controlling the expression of distinct Bcl-2 family proteins. *Blood* 99: 3683-3691.

160. Grumont, R. J., I. J. Rourke, and S. Gerondakis. 1999. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. *Genes Dev* 13: 400-411.

161. Lee, H. H., H. Dadgostar, Q. Cheng, J. Shu, and G. Cheng. 1999. NF-kappaB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. *Proc Natl Acad Sci U S A* 96: 9136-9141.

162. Kraus, M., M. B. Alimzhanov, N. Rajewsky, and K. Rajewsky. 2004. Survival of resting mature B lymphocytes depends on BCR signaling via the Igalpha/beta heterodimer. *Cell* 117: 787-800.

163. Hartley, S. B., J. Crosbie, R. Brink, A. B. Kantor, A. Basten, and C. C. Goodnow. 1991. Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. *Nature* 353: 765-769.

164. Sen, R. 2006. Control of B lymphocyte apoptosis by the transcription factor NF-kappaB. *Immunity* 25: 871-883.

165. Smith, S. H., and M. P. Cancro. 2003. Cutting edge: B cell receptor signals regulate BLyS receptor levels in mature B cells and their immediate progenitors. *J Immunol* 170: 5820-5823.

166. Zarnegar, B., J. Q. He, G. Oganesyan, A. Hoffmann, D. Baltimore, and G. Cheng. 2004. Unique CD40-mediated biological program in B cell activation requires both type 1 and type 2 NF-kappaB activation pathways. *Proc Natl Acad Sci U S A* 101: 8108-8113.

167. Craxton, A., K. E. Draves, A. Gruppi, and E. A. Clark. 2005. BAFF regulates B cell survival by downregulating the BH3-only family member Bim via the ERK pathway. *J Exp Med* 202: 1363-1374.

168. Enzler, T., G. Bonizzi, G. J. Silverman, D. C. Otero, G. F. Widhopf, A. Anzelon-Mills, R. C. Rickert, and M. Karin. 2006. Alternative and classical NF-kappa B signaling retain autoreactive B cells in the splenic marginal zone and result in lupus-like disease. *Immunity* 25: 403-415.

169. Merino, R., L. Ding, D. J. Veis, S. J. Korsmeyer, and G. Nunez. 1994. Developmental regulation of the Bcl-2 protein and susceptibility to cell death in B lymphocytes. *EMBO J* 13: 683-691.

170. Hao, Z., G. S. Duncan, J. Seagal, Y. W. Su, C. Hong, J. Haight, N. J. Chen, A. Elia, A. Wakeham, W. Y. Li, J. Liepa, G. A. Wood, S. Casola, K. Rajewsky,

and T. W. Mak. 2008. Fas receptor expression in germinal-center B cells is essential for T and B lymphocyte homeostasis. *Immunity* 29: 615-627.

171. Rothstein, T. L., J. K. Wang, D. J. Panka, L. C. Foote, Z. Wang, B. Stanger, H. Cui, S. T. Ju, and A. Marshak-Rothstein. 1995. Protection against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B cells. *Nature* 374: 163-165.

172. Foote, L. C., A. Marshak-Rothstein, and T. L. Rothstein. 1998. Tolerant B lymphocytes acquire resistance to Fas-mediated apoptosis after treatment with interleukin 4 but not after treatment with specific antigen unless a surface immunoglobulin threshold is exceeded. *J Exp Med* 187: 847-853.

173. McDonnell, T. J., N. Deane, F. M. Platt, G. Nunez, U. Jaeger, J. P. McKearn, and S. J. Korsmeyer. 1989. bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* 57: 79-88.

174. McDonnell, T. J., G. Nunez, F. M. Platt, D. Hockenberry, L. London, J. P. McKearn, and S. J. Korsmeyer. 1990. Deregulated Bcl-2-immunoglobulin transgene expands a resting but responsive immunoglobulin M and D-expressing B-cell population. *Mol Cell Biol* 10: 1901-1907.

175. Strasser, A., S. Whittingham, D. L. Vaux, M. L. Bath, J. M. Adams, S. Cory, and A. W. Harris. 1991. Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. *Proc Natl Acad Sci U S A* 88: 8661-8665.

176. Sakai, T., M. Nishikori, M. Tashima, R. Yamamoto, T. Kitawaki, A. Takaori-Kondo, T. Suzuki, S. Tsuzuki, and T. Uchiyama. 2009. Distinctive cell properties of B cells carrying the BCL2 translocation and their potential roles in the development of lymphoma of germinal center type. *Cancer Sci* 100: 2361-2367.

177. Tuscano, J. M., K. M. Druey, A. Riva, J. Pena, C. B. Thompson, and J. H. Kehrl. 1996. Bcl-x rather than Bcl-2 mediates CD40-dependent centrocyte survival in the germinal center. *Blood* 88: 1359-1364.

178. Kondo, E., S. Nakamura, H. Onoue, Y. Matsuo, T. Yoshino, H. Aoki, K. Hayashi, K. Takahashi, J. Minowada, S. Nomura, and et al. 1992. Detection of bcl-2 protein and bcl-2 messenger RNA in normal and neoplastic lymphoid tissues by immunohistochemistry and in situ hybridization. *Blood* 80: 2044-2051.

179. Willimott, S., and S. D. Wagner. 2010. Post-transcriptional and post-translational regulation of Bcl2. *Biochem Soc Trans* 38: 1571-1575.

180. Otake, Y., S. Soundararajan, T. K. Sengupta, E. A. Kio, J. C. Smith, M. Pineda-Roman, R. K. Stuart, E. K. Spicer, and D. J. Fernandes. 2007. Overexpression of nucleolin in chronic lymphocytic leukemia cells induces stabilization of bcl2 mRNA. *Blood* 109: 3069-3075.

181. Cimmino, A., G. A. Calin, M. Fabbri, M. V. Iorio, M. Ferracin, M. Shimizu, S. E. Wojcik, R. I. Aqeilan, S. Zupo, M. Dono, L. Rassenti, H. Alder, S. Volinia, C. G. Liu, T. J. Kipps, M. Negrini, and C. M. Croce. 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A* 102: 13944-13949.

182. Deng, X., P. Ruvolo, B. Carr, and W. S. May, Jr. 2000. Survival function of ERK1/2 as IL-3-activated, staurosporine-resistant Bcl2 kinases. *Proc Natl Acad Sci U S A* 97: 1578-1583.

183. Dimmeler, S., K. Breitschopf, J. Haendeler, and A. M. Zeiher. 1999. Dephosphorylation targets Bcl-2 for ubiquitin-dependent degradation: a link between the apoptosome and the proteasome pathway. *J Exp Med* 189: 1815-1822.

184. Casola, S., K. L. Otipoby, M. Alimzhanov, S. Humme, N. Uyttersprot, J. L. Kutok, M. C. Carroll, and K. Rajewsky. 2004. B cell receptor signal strength determines B cell fate. *Nat Immunol* 5: 317-327.

185. Crotty, S. 2014. T follicular helper cell differentiation, function, and roles in disease. *Immunity* 41: 529-542.

186. Johnston, R. J., A. C. Poholek, D. DiToro, I. Yusuf, D. Eto, B. Barnett, A. L. Dent, J. Craft, and S. Crotty. 2009. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325: 1006-1010.

187. Yu, D., S. Rao, L. M. Tsai, S. K. Lee, Y. He, E. L. Sutcliffe, M. Srivastava, M. Linterman, L. Zheng, N. Simpson, J. I. Ellyard, I. A. Parish, C. S. Ma, Q. J. Li, C. R. Parish, C. R. Mackay, and C. G. Vinuesa. 2009. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 31: 457-468.

188. Deenick, E. K., A. Chan, C. S. Ma, D. Gatto, P. L. Schwartzberg, R. Brink, and S. G. Tangye. 2010. Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity* 33: 241-253.

189. Pratama, A., R. R. Ramiscal, D. G. Silva, S. K. Das, V. Athanasopoulos, J. Fitch, N. K. Botelho, P. P. Chang, X. Hu, J. J. Hogan, P. Mana, D. Bernal, H. Korner, D. Yu, C. C. Goodnow, M. C. Cook, and C. G. Vinuesa. 2013.

Roquin-2 shares functions with its paralog Roquin-1 in the repression of mRNAs controlling T follicular helper cells and systemic inflammation. *Immunity* 38: 669-680.

190. Hasbold, J., L. M. Corcoran, D. M. Tarlinton, S. G. Tangye, and P. D. Hodgkin. 2004. Evidence from the generation of immunoglobulin G-secreting cells that stochastic mechanisms regulate lymphocyte differentiation. *Nat Immunol* 5: 55-63.

191. Nutt, S. L., N. Taubenheim, J. Hasbold, L. M. Corcoran, and P. D. Hodgkin. 2011. The genetic network controlling plasma cell differentiation. *Semin Immunol* 23: 341-349.

192. Uren, A. G., K. O'Rourke, L. A. Aravind, M. T. Pisabarro, S. Seshagiri, E. V. Koonin, and V. M. Dixit. 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: 961-967.

193. Snipas, S. J., E. Wildfang, T. Nazif, L. Christensen, K. M. Boatright, M. Bogyo, H. R. Stennicke, and G. S. Salvesen. 2004. Characteristics of the caspase-like catalytic domain of human paracaspase. *Biol Chem* 385: 1093-1098.

194. Coornaert, B., M. Baens, K. Heyninck, T. Bekaert, M. Haegman, J. Staal, L. Sun, Z. J. Chen, P. Marynen, and R. Beyaert. 2008. T cell antigen receptor stimulation induces MALT1 paracaspase-mediated cleavage of the NF-kappaB inhibitor A20. *Nat Immunol* 9: 263-271.

195. Staal, J., Y. Driege, T. Bekaert, A. Demeyer, D. Muyllaert, P. Van Damme, K. Gevaert, and R. Beyaert. 2011. T-cell receptor-induced JNK activation requires proteolytic inactivation of CYLD by MALT1. *EMBO J* 30: 1742-1752.

196. Hailfinger, S., H. Nogai, C. Pelzer, M. Jaworski, K. Cabalzar, J. E. Charton, M. Guzzardi, C. Decaillet, M. Grau, B. Dorken, P. Lenz, G. Lenz, and M. Thome. 2011. Malt1-dependent RelB cleavage promotes canonical NF-kappaB activation in lymphocytes and lymphoma cell lines. *Proc Natl Acad Sci U S A* 108: 14596-14601.

197. Lucas, P. C., M. Yonezumi, N. Inohara, L. M. McAllister-Lucas, M. E. Abazeed, F. F. Chen, S. Yamaoka, M. Seto, and G. Nunez. 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: 19012-19019.

198. Hailfinger, S., G. Lenz, V. Ngo, A. Posvitz-Fejfar, F. Rebeaud, M. Guzzardi, E. M. Penas, J. Dierlamm, W. C. Chan, L. M. Staudt, and M. Thome. 2009.

Essential role of MALT1 protease activity in activated B cell-like diffuse large B-cell lymphoma. *Proc Natl Acad Sci U S A* 106: 19946-19951.

199. Ferch, U., B. Kloo, A. Gewies, V. Pfander, M. Duwel, C. Peschel, D. Krappmann, and J. Ruland. 2009. Inhibition of MALT1 protease activity is selectively toxic for activated B cell-like diffuse large B cell lymphoma cells. *J Exp Med* 206: 2313-2320.

200. Nagel, D., S. Spranger, M. Vincendeau, M. Grau, S. Raffegerst, B. Kloo, D. Hlahla, M. Neuenschwander, J. Peter von Kries, K. Hadian, B. Dorken, P. Lenz, G. Lenz, D. J. Schendel, and D. Krappmann. 2012. Pharmacologic inhibition of MALT1 protease by phenothiazines as a therapeutic approach for the treatment of aggressive ABC-DLBCL. *Cancer Cell* 22: 825-837.

201. Fontan, L., C. Yang, V. Kabaleeswaran, L. Volpon, M. J. Osborne, E. Beltran, M. Garcia, L. Cerchietti, R. Shaknovich, S. N. Yang, F. Fang, R. D. Gascoyne, J. A. Martinez-Climent, J. F. Glickman, K. Borden, H. Wu, and A. Melnick. 2012. MALT1 small molecule inhibitors specifically suppress ABC-DLBCL in vitro and in vivo. *Cancer Cell* 22: 812-824.

202. Rebeaud, F., S. Hailfinger, A. Posevitz-Fejfar, M. Tapernoux, R. Moser, D. Rueda, O. Gaide, M. Guzzardi, E. M. Iancu, N. Rufer, N. Fasel, and M. Thome. 2008. The proteolytic activity of the paracaspase MALT1 is key in T cell activation. *Nat Immunol* 9: 272-281.

203. Uehata, T., H. Iwasaki, A. Vandenbon, K. Matsushita, E. Hernandez-Cuellar, K. Kuniyoshi, T. Satoh, T. Mino, Y. Suzuki, D. M. Standley, T. Tsujimura, H. Rakugi, Y. Isaka, O. Takeuchi, and S. Akira. 2013. Malt1-induced cleavage of regnase-1 in CD4(+) helper T cells regulates immune activation. *Cell* 153: 1036-1049.

204. Jeltsch, K. M., D. Hu, S. Brenner, J. Zoller, G. A. Heinz, D. Nagel, K. U. Vogel, N. Rehage, S. C. Warth, S. L. Edelmann, R. Gloury, N. Martin, C. Lohs, M. Lech, J. E. Stehklein, A. Geerlof, E. Kremmer, A. Weber, H. J. Anders, I. Schmitz, M. Schmidt-Supprian, M. Fu, H. Holtmann, D. Krappmann, J. Ruland, A. Kallies, M. Heikenwalder, and V. Heissmeyer. 2014. Cleavage of roquin and regnase-1 by the paracaspase MALT1 releases their cooperatively repressed targets to promote T(H)17 differentiation. *Nat Immunol* 15: 1079-1089.

205. Gewies, A., O. Gorka, H. Bergmann, K. Pechloff, F. Petermann, K. M. Jeltsch, M. Rudelius, M. Kriegsmann, W. Weichert, M. Horsch, J. Beckers, W. Wurst, M. Heikenwalder, T. Korn, V. Heissmeyer, and J. Ruland. 2014. Uncoupling Malt1 threshold function from paracaspase activity results in destructive autoimmune inflammation. *Cell Rep* 9: 1292-1305.

206. Jaworski, M., B. J. Marsland, J. Gehrig, W. Held, S. Favre, S. A. Luther, M. Perroud, D. Golshayan, O. Gaide, and M. Thome. 2014. Malt1 protease inactivation efficiently dampens immune responses but causes spontaneous autoimmunity. *EMBO J* 33: 2765-2781.

207. Hachmann, J., S. J. Snipas, B. J. van Raam, E. M. Cancino, E. J. Houlihan, M. Poreba, P. Kasperkiewicz, M. Drag, and G. S. Salvesen. 2012. Mechanism and specificity of the human paracaspase MALT1. *Biochem J* 443: 287-295.

208. Hachmann, J., L. E. Edgington-Mitchell, M. Poreba, L. E. Sanman, M. Drag, M. Bogyo, and G. S. Salvesen. 2015. Probes to monitor activity of the paracaspase MALT1. *Chem Biol* 22: 139-147.

209. Eitelhuber, A. C., O. Vosyka, D. Nagel, M. Bognar, D. Lenze, K. Lammens, F. Schlauderer, D. Hlahla, K. P. Hopfner, G. Lenz, M. Hummel, S. H. Verhelst, and D. Krappmann. 2015. Activity-based probes for detection of active MALT1 paracaspase in immune cells and lymphomas. *Chem Biol* 22: 129-138.

210. Shinall, S. M., M. Gonzalez-Fernandez, R. J. Noelle, and T. J. Waldschmidt. 2000. Identification of murine germinal center B cell subsets defined by the expression of surface isotypes and differentiation antigens. *J Immunol* 164: 5729-5738.

211. Kerfoot, S. M., G. Yaari, J. R. Patel, K. L. Johnson, D. G. Gonzalez, S. H. Kleinstein, and A. M. Haberman. 2011. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity* 34: 947-960.

212. Kloo, B., D. Nagel, M. Pfeifer, M. Grau, M. Duwel, M. Vincendeau, B. Dorken, P. Lenz, G. Lenz, and D. Krappmann. 2011. Critical role of PI3K signaling for NF-kappaB-dependent survival in a subset of activated B-cell-like diffuse large B-cell lymphoma cells. *Proc Natl Acad Sci U S A* 108: 272-277.

213. Baracho, G. V., M. H. Cato, Z. Zhu, O. R. Jaren, E. Hobeika, M. Reth, and R. C. Rickert. 2014. PDK1 regulates B cell differentiation and homeostasis. *Proc Natl Acad Sci U S A* 111: 9573-9578.

214. Davis, R. E., V. N. Ngo, G. Lenz, P. Tolar, R. M. Young, P. B. Romesser, H. Kohlhammer, L. Lamy, H. Zhao, Y. Yang, W. Xu, A. L. Shaffer, G. Wright, W. Xiao, J. Powell, J. K. Jiang, C. J. Thomas, A. Rosenwald, G. Ott, H. K. Muller-Hermelink, R. D. Gascoyne, J. M. Connors, N. A. Johnson, L. M. Rimsza, E. Campo, E. S. Jaffe, W. H. Wilson, J. Delabie, E. B. Smeland, R. I. Fisher, R. M. Braziel, R. R. Tubbs, J. R. Cook, D. D. Weisenburger, W. C. Chan, S. K. Pierce, and L. M. Staudt. 2010. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. *Nature* 463: 88-92.

215. Wang, D., Y. You, S. M. Case, L. M. McAllister-Lucas, L. Wang, P. S. DiStefano, G. Nunez, J. Bertin, and X. Lin. 2002. A requirement for CARMA1 in TCR-induced NF-kappa B activation. *Nat Immunol* 3: 830-835.

216. Wiesmann, C., L. Leder, J. Blank, A. Bernardi, S. Melkko, A. Decock, A. D'Arcy, F. Villard, P. Erbel, N. Hughes, F. Freuler, R. Nikolay, J. Alves, F. Bornancin, and M. Renatus. 2012. Structural determinants of MALT1 protease activity. *J Mol Biol* 419: 4-21.

217. Oeckinghaus, A., E. Wegener, V. Welteke, U. Ferch, S. C. Arslan, J. Ruland, C. Scheidereit, and D. Krappmann. 2007. Malt1 ubiquitination triggers NF-kappaB signaling upon T-cell activation. *EMBO J* 26: 4634-4645.

218. Pelzer, C., K. Cabalzar, A. Wolf, M. Gonzalez, G. Lenz, and M. Thome. 2013. The protease activity of the paracaspase MALT1 is controlled by monoubiquitination. *Nat Immunol* 14: 337-345.

219. Mueller, J., M. Matloubian, and J. Zikherman. 2015. Cutting edge: An in vivo reporter reveals active B cell receptor signaling in the germinal center. *J Immunol* 194: 2993-2997.

220. Hauser, A. E., T. Junt, T. R. Mempel, M. W. Sneddon, S. H. Kleinstein, S. E. Henrickson, U. H. von Andrian, M. J. Shlomchik, and A. M. Haberman. 2007. Definition of germinal-center B cell migration in vivo reveals predominant intrazonal circulation patterns. *Immunity* 26: 655-667.

221. Dierlamm, J., M. Baens, I. Wlodarska, M. Stefanova-Ouzounova, J. M. Hernandez, D. K. Hossfeld, C. De Wolf-Peeters, A. Hagemeijer, H. Van den Berghe, and P. Marynen. 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: 3601-3609.

222. Lucas, P. C., P. Kuffa, S. Gu, D. Kohrt, D. S. Kim, K. Siu, X. Jin, J. Swenson, and L. M. McAllister-Lucas. 2007. A dual role for the API2 moiety in API2-MALT1-dependent NF-kappaB activation: heterotypic oligomerization and TRAF2 recruitment. *Oncogene* 26: 5643-5654.

223. Rosebeck, S., L. Madden, X. Jin, S. Gu, I. J. Apel, A. Appert, R. A. Hamoudi, H. Noels, X. Sagaert, P. Van Loo, M. Baens, M. Q. Du, P. C. Lucas, and L. M. McAllister-Lucas. 2011. Cleavage of NIK by the API2-MALT1 fusion oncoprotein leads to noncanonical NF-kappaB activation. *Science* 331: 468-472.

224. Nie, Z., M. Q. Du, L. M. McAllister-Lucas, P. C. Lucas, N. G. Bailey, C. M. Hogaboam, M. S. Lim, and K. S. Elenitoba-Johnson. 2015. Conversion of the

LIMA1 tumour suppressor into an oncogenic LMO-like protein by API2-MALT1 in MALT lymphoma. *Nat Commun* 6: 5908.

225. Lee, K. Y., F. D'Acquisto, M. S. Hayden, J. H. Shim, and S. Ghosh. 2005. PDK1 nucleates T cell receptor-induced signaling complex for NF-kappaB activation. *Science* 308: 114-118.

226. Bauquet, A. T., H. Jin, A. M. Paterson, M. Mitsdoerffer, I. C. Ho, A. H. Sharpe, and V. K. Kuchroo. 2009. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol* 10: 167-175.

227. Choi, Y. S., R. Kageyama, D. Eto, T. C. Escobar, R. J. Johnston, L. Monticelli, C. Lao, and S. Crotty. 2011. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* 34: 932-946.

228. Fahey, L. M., E. B. Wilson, H. Elsaesser, C. D. Fistonich, D. B. McGavern, and D. G. Brooks. 2011. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J Exp Med* 208: 987-999.

229. Yunis, J. J., G. Frizzera, M. M. Oken, J. McKenna, A. Theologides, and M. Arnesen. 1987. Multiple recurrent genomic defects in follicular lymphoma. A possible model for cancer. *N Engl J Med* 316: 79-84.

230. Iqbal, J., W. G. Sanger, D. E. Horsman, A. Rosenwald, D. L. Pickering, B. Dave, S. Dave, L. Xiao, K. Cao, Q. Zhu, S. Sherman, C. P. Hans, D. D. Weisenburger, T. C. Greiner, R. D. Gascoyne, G. Ott, H. K. Muller-Hermelink, J. Delabie, R. M. Braziel, E. S. Jaffe, E. Campo, J. C. Lynch, J. M. Connors, J. M. Vose, J. O. Armitage, T. M. Grogan, L. M. Staudt, and W. C. Chan. 2004. BCL2 translocation defines a unique tumor subset within the germinal center B-cell-like diffuse large B-cell lymphoma. *Am J Pathol* 165: 159-166.