Regulation of Canonical and Non-Canonical NF kappa B Signalling
in Lymphocytes by the Bcl10-MALT1 Complex

By

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Abstract of Thesis

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The NF-κB family of heterodimeric transcription factors is activated by many stimuli, and lead to the upregulation of countless genes. Not surprisingly, NF-κB plays a critical role in many aspects of cellular function. In T and B lymphocytes, antigen receptor stimulation leads to the activation of NF-κB through a signal transduction cascade involving the Bcl10-MALT1 complex. We hypothesized that this complex may be critical to signalling cascades other than those emanating from antigen receptors. B cell activation factor of the TNF family (BAFF) activates non-canonical NF-κB heterodimers that promote B cell survival. Here, we show that MALT1 is required for BAFF-induced phosphorylation of NF-κB2 (p100), p100 degradation and RelB nuclear translocation in B220^+ B cells. TRAF3, a known negative regulator of BAFF-R mediated signaling, interacts with MALT1 in a manner which is negatively regulated by BAFF, and TRAF3 levels are enhanced in MALT1^-/- B cells. MALT1^-/- CD21^{high}CD23^{low} (MZ) B cells show a defect in BAFF-induced survival and MALT1^-/- x BAFF-transgenic (Tg) mice have decreased MZ and B1 B cell levels compared to BAFF-Tg mice. In agreement with this in vitro data, phenotypes associated with over-expression of BAFF including
increased serum immunoglobulin titres, spontaneous germinal center (GC) formation, and immune complex deposition in the kidney were found to be dependent on B cell-intrinsic MALT1 expression. Our results demonstrate a novel role for MALT1 in biological outcomes induced by BAFF-mediated signal transduction.

The mechanism by which the Bcl10-MALT1 complex regulates antigen induced NF-κB activation in T cells remains controversial. To shed light on this regulatory network, we conducted biochemical purification of Bcl10, and identified Uev1a, a known regulator of antigen receptor mediated NF-κB activation. We hypothesized that mms2, and structurally similar molecule to Uev1a, may also impinge on NF-κB activation. Mms2 overexpression in 293T cells inhibited the Bcl10-induced activation of an NF-κB sensitive luciferase. Lymphocyte development and antigen receptor induced activation occurs normally mms2−/− mice. However, class switched serum immunoglobulins, and survival responses to DNA damage inducing γ-irradiation, are decreased in mms2−/− mice. Therefore, mms2 is dispensable in vivo for lymphocyte function and development, but is required for DNA damage responses.
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LIST OF ABBREVIATIONS

53BP1 – p53 binding protein 1
AP-1 – Activator protein 1
BAFF – B cell activating factor of the TNF superfamily
Bcl10 – B cell lymphoma protein 10
BCMA – B cell maturation antigen
BCR – B cell receptor
CARD – Caspase recruitment domain
CARMA – Caspase recruitment domain containing membrane-associated guanylate kinase
CBM – Carma-Bcl10-Malt1
CD – Cluster of differentiation
c-FLIP – c-Flice-like inhibitory protein
DAG – Diacylglycerol
EMSA – Electrophoretic mobility shift assay
ES cell – Embryonic stem cell
NF-κB – Nuclear factor that binds κB enhancers
DN – Double negative
FACS – Fluorescence activated cell sorting
FcεR1 – Fc ε receptor 1
FCS – Fetal calf serum
FO – Follicular
GC – Germinal center
H2AX – Histone 2AX
HA – Hemagglutinin
HRP – Horseradish peroxidase
IAP – Inhibitor of apoptosis
IκB – Inhibitor κB
IKK – IκB kinase
IL – Interleukin
Ig – Immunoglobulin
IP3 – Inositol triphosphate
i.p. – Intraperitoneally
i.v. – Intravenously
LPS – Lipopolysaccharide
MAGUK – Membrane-associated guanylate kinase
MALT1 – Mucosa-associated lymphoid tissue protein 1
MEF – Murine embryonic fibroblasts
MHC – Major histocompability complex
mms2 – methylmethane sulfonate 2
MZ – Marginal zone
NEMO – NF-κB essential modulator
NFAT – Nuclear factor of activated T cells
NIK – NF-κB inducing kinase

PCA – Passive cutaneous anaphylaxis

PCR – Polymerase chain reaction

PDK1 – 3-Phosphoinositide-dependent protein kinase

PLCγ1 – Phospholipase C γ1

PKCθ – Protein kinase C θ

PMA - Phorbol 12-myristate 13-acetate

PNA – Peanut agglutinin

Rel – Reticuloendotheliosis homology proteins

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TACI – Transmembrane activator and Calcium modulator and cyclophilin ligand

(CAML) interactor

TAP – Tandem affinity purification

TCR – T cell receptor

Td/Ti response – T cell dependent/ T independent response

TNFα – Tumour necrosis factor α

TRAF – TNFα receptor-associated factor

UBC13 – Ubiquitin conjugating enzyme 13

Uev1a – Ubiquitin enzyme variant 1a
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I. CHAPTER 1 - INTRODUCTION

The NF-κB molecules comprise a family of heterodimeric transcription factors that play critical roles in a multitude of cellular functions. By inducing the transcription of a wide variety of genes, NF-κB family members modulate immune responses, inflammation, cell survival and the cell cycle (Bonizzi and Karin, 2004; Hayden and Ghosh, 2004; Li and Verma, 2002). Deregulation of NF-κB activation can lead to disruption of immune system homeostasis and ultimately tumorigenesis. The past decade has seen the elucidation of many of the molecular elements involved in the intracellular signalling pathways leading to NF-κB activation. However, the means by which this regulation occurs in these pathways has remained obscure. In this thesis, I investigate how two crucial signalling elements, Bcl10 and MALT1, participate in a complex that regulates multiple routes of NF-κB activation. My results should lead to a greater understanding of how this crucial transcription factor is controlled, and may indicate molecules that can be targeted for future anti-cancer or anti-autoimmunity drug development.

1. NF-κB

NF-κB (nuclear factor of κB) was originally characterized by David Baltimore’s lab as a molecule that could bind to the enhancer regions of immunoglobulin light chain genes (Sen and Baltimore, 1986). Since that time, detailed examination has revealed that NF-κB heterodimers are made up of RelA, RelB and c-Rel transcription factors that activate the canonical NF-κB pathway, as well as the NFκB1 (translated as p105 precursor, then processed to p50) and NFκB2 (translated as p100 precursor, then
processed to p52) transcription factors that activate the non-canonical NF-κB pathway (Beinke and Ley, 2004). The defining feature of all NF-κB proteins is the presence of the Rel homology domain, which confers the ability to dimerize into homo- and heterodimers, and to bind DNA. The Rel domain was originally described as a viral oncogene associated with neoplastic progression of the lymphoreticular system in chickens through upregulation of NF-κB activity (Chen et al., 1981; Ghosh et al., 1990). This finding was an early and intriguing indication that NF-κB deregulation could cause disease and lead to cancerous transformation. In humans, aberrant NF-κB activation has been reported in cases of non-small cell lung carcinoma (NSCLC), colon carcinoma, breast cancer, lymphoma (such as MALT lymphoma), and other malignancies (Du and Isaccson, 2002; Gilmore et al., 2002; Li and Verma, 2002; Orlowski and Baldwin, 2002). NF-κB dimers regulate the transcription of a wide array of genes by binding the so-called κB consensus region within promoters or enhancers. The sequence that NF-κB heterodimers recognizes is 5’ GGGRNWYYCC 3’ (N – any base; R – purine; W – adenine or thymine; and Y – pyridimine) (Hoffmann et al., 2006). The NF-κB family members RelB, c-Rel and Rel A (p65) contain a transcription activation domain (TAD) which is the necessary element responsible of gene expression. Because p50 and p52 members lack the TAD, they cannot activate gene expression, and need to dimerize with TAD-containing RelA, RelB, and c-Rel members to do so. Interestingly, p50 and p52 homodimers have been shown to bind kB enhancer regions in the absence of NF-κB inducing stimuli, suggesting that they may serve to inhibit promiscuous NF-κB signaling (Hayden and Ghosh, 2008).
The mechanism by which the NF-κB family of proteins bind DNA has been elucidated using x-ray crystallographic studies (Hoffmann et al., 2006). Immunoglobulin like folds on the amino terminus of the protein interacts with κB consensus sequences, whereas largely hydrophobic residues on the carboxy terminus are responsible of the homo- and heterodimerization capabilities of the NF-κB family members.

2. Canonical and non-canonical NF-κB activation

Under resting conditions, NF-κB heterodimers are sequestered in the cytoplasm by the binding of various inhibitory molecules, known as inhibitors of κB signalling or IκBs (Figure 1). There are three typical IκB molecules that are known to interact with NF-κB in the cytosol, IκBα, IκBβ, and IκBε. The atypical IκBs, IκBζ, IκBγ and Bcl-3 do not have any NF-κB sequestration capabilities ascribed to them. The prototypical member of the IκB family is IκBα, which is degraded rapidly following NF-κB inducing stimuli, and reformulated, due to the fact that it is an NF-κB responsive gene (Hayden and Ghosh, 2004). The powerful nuclear export signal on IκBα allows it to bind to actively transcribing NF-κB heterodimers, and expel them from the nucleus, thereby shutting down gene activation. It is thought that IκBα inhibits NF-κB heterodimers from translocating to the nucleus by masking nuclear localization sequences on the heterodimers themselves. However, IκBα binding to p65:p50 was revealed by x-ray crystallographic studies to mask only one of the two nuclear localization sequences on the heterodimer. Furthermore, studies using the nuclear export inhibitor Leptomycin B
revealed that IκBα bound NF-κB is in a constant state of shuttling between the cytoplasm and the nucleus (Hayden and Ghosh, 2004). The reason for this energetically expensive shuttling process is unclear.

When the cell experiences an extracellular stimulus, the inhibition provided by IκBs is lifted and the NF-κB heterodimers are free to translocate into the nucleus and activate target gene transcription. The elements involved in the signalling pathways leading to so-called canonical or non-canonical NF-κB activation differ slightly, as do the target genes induced (Bonizzi and Karin, 2004). However, all these intracellular signals pass through a 700 kD complex called the IκB kinase (IKK) complex (Rothwarf and Karin, 1999). The IKK complex consists of three subunits: IKKα and IKKβ, which both have kinase capabilities, and IKKγ or NEMO (for NF-κB essential modulator), which is a critical regulator of IKKα and IKKβ. When an agent such as TNFα, IL-6, antigen or DNA damage activates the canonical NF-κB pathway, it is mainly the IKKβ subunit of the IKK complex that becomes activated. Activated IKKβ phosphorylates IκBs that have bound to and sequestered RelA in the cytoplasm on conserved serine residues (Ser 32 and 36 on IκBα, and Ser 19 and 23 on IκBβ) (Hatada et al., 1992). The phosphorylated IκB molecules are recognized by the βTrCP component of the Skp1-Cullin-Roc1/Rbx1/Hrt-1-F-box (SCF) E3 ubiquitin ligase complex which requires the E2 ubiquitin ligase UbcH5 for its function (Perkins, 2006). This complex catalyzes lysine 48 dependent ubiquitination of the IκB, which leads to its subsequent degradation by the proteosome freeing NF-κB heterodimers to translocate into the nucleus. Nuclear NF-κB then activates the transcription of a wide array of genes with anti-apoptotic (IAP, c-FLIP), proinflammatory (IL-6), or cell cycle-modulating (cyclin D1) functions. Other genes
induced by these NF-κB heterodimers, such as IκBα and A20, have negative regulatory effects on NF-κB signaling.

The non-canonical NF-κB pathway depends on activation of IKKα (Senftleben et al., 2001). In response to various stimuli, such as BAFF, NF-κB inducing kinase (NIK) phosphorylates and activates IKKα (Ling et al., 1998). NIK has also been shown to phosphorylate p100 at serine 866 and 870 (Xiao et al., 2001). Activated IKKα then drives the serine phosphorylation of IκB-like domains located within the NF-κB subunits themselves, such as those present in NF-κB2 p100 (Senftleben et al., 2001). Polyubiquitination and degradation of these phosphorylated domains in p100 allows the remaining p52 product to enter the nucleus, where it activates the transcription of anti-apoptotic genes such as Bcl-2 and Bcl-xL (Patke et al., 2004). Agents that are known to activate the non-canonical NF-κB signaling cascade are frequently members of the TNF superfamily, including lymphotoxins α and β, as well as B cell activating factor of the TNF superfamily (BAFF) (Bossen and Schneider, 2006) (Claudio et al., 2002; Kayagaki et al., 2002).
Figure 1. Canonical and Non-canonical NF-κB signal transduction.
3. BAFF

i) Sources of BAFF and its Regulation

BAFF (B cell activating cytokine of the TNF superfamily; also known as TALL-1, THANK, zTNF-4, and TNFSF13B), and a related cytokine, APRIL (a proliferation inducing ligand) are members of the TNF superfamily of cytokines and are known to be expressed by neutrophils, monocytes and dendritic cells (Nardelli et al., 2001). Recent studies have expanded the known producers of BAFF and APRIL to cells of non-hematopoetic origins, such as cytotrophoblasts in the placenta, epithelial cells of the tonsils and airways, breast adipocytes and carcinoma cells (Huard et al., 2008; Kato et al., 2006; Langat et al., 2008; Pelekanou et al., 2008). The expression of BAFF can induced by type I interferons, IL-10, G-CSF, and IFNγ (Mackay et al., 2003; Scapini et al., 2008). In addition, agonism of Toll-like family members, such as TLR4 with LPS, and TLR9 with CpG, can induce the expression of BAFF and/or APRIL (Boule et al., 2004). BAFF is known to promote B cell survival by up-regulating prosurvival factors Bcl-2 and Bcl-xL (Batten et al., 2000; Mackay et al., 1999; Schiemann et al., 2001). The importance of survival factor expression to the physiological function of BAFF was exemplified by the fact that the phenotype of the BAFF receptor (BAFF-R) deficient mouse (see later) can be rescued by transgenic expression of Bcl-xL (Amanna et al., 2003). APRIL, on the other hand, appears to be required for antigen presentation by B cells, long term survival of antibody secreting plasma cells, and antibody class switching to the IgA isotype (Dillon et al., 2006). BAFF can also induce cell cycle entry by triggering cyclin D2 synthesis (Huang et al., 2004). BAFF-dependent transitional B cell survival is required...
for the differentiation of mature B cell subsets, including CD21<sub>low</sub>CD23<sub>high</sub> follicular (FO) B cells and CD21<sub>high</sub>CD23<sub>low</sub> marginal zone (MZ) B cells (Batten et al., 2000).

**ii) BAFF and APRIL function in vivo – Lessons from genetically Modified Mice**

At the molecular level, BAFF binds to three different receptors: BCMA (B cell maturation antigen), TACI [transmembrane activator and calcium modulator and cyclophilin ligand (CAML) interactor], and BAFF-R (Marsters et al., 2000; Thompson et al., 2001; Thompson et al., 2000; Wu et al., 2000; Yan et al., 2001). APRIL is known only to bind to BCMA and TACI (Dillon et al., 2006). Knockout studies have shown that the functions of these receptors, as well as those ascribed to BAFF and APRIL, are distinct and non-overlapping. BAFF<sup>−/−</sup> mice display wide scale defects in immune homeostasis. These animals have greatly depressed immunoglobulin levels in the sera, a defect in B cell development beyond the Transitional T1 phase in the periphery, increase in allograft survival, and decreased T cell dependent and T cell independent immune responses (Mackay et al., 2003). April<sup>−/−</sup> mice indicate that this cytokine is critical for class switching to IgA, as these mice have depressed levels of IgA in the sera (Dillon et al., 2006). Interestingly, April appears to be necessary for the long term survival of plasma cells, as the persistence of tetanus toxoid specific bone marrow plasma cells is decreased significantly in the absence of this cytokine (Benson et al., 2008). Furthermore, T cell homeostasis is perturbed in the absence of April, as the level of effector memory T cells of the CD44<sup>hi</sup>CD62L<sup>low</sup> phenotype are increased in the April<sup>−/−</sup> mice (Belnoue et al., 2008). These data intimate that BAFF is required for B cell
development and survival whereas April plays more of a role in the long term survival of plasma cells, and plays some role in T cell function.

BAFF-transgenic mice display B cell hyperplasia, as evidenced by the greatly expanded levels of MZ B cells in the spleen and B1 B cells in the pleural and peritoneal cavities (Mackay et al., 1999). Furthermore, BAFF-transgenic mice develop autoimmune manifestations characterized by the destruction of the salivary glands, immunoglobulin deposition in the kidneys, and the production of autoantibodies (Mackay et al., 1999). Interestingly, this phenotype occurs in a manner that is independent of T-cell function, but dependent on MyD88, lending credence to the observation that TLR signaling is necessary for the expression of BAFF (Groom et al., 2007). The autoimmune symptoms observed in the BAFF-transgenic animal is perhaps due to the excessive survival signals provided by BAFF overriding the negative selection pressures that would normal delete autoreactive lymphocytes (ie- a breakdown in tolerance). It is thought, however, that BAFF selects for low affinity self reactive B cells of MZ origin, as high affinity autoreactive B cells are deleted long before the expression of the BAFF-R on developing B cells (Meyer-Bahlburg et al., 2008; Thien et al., 2004). Many patients with autoimmune diseases, such as SLE (System Lupus Erythematous), Rheumatoid Arthritis, Sjogren’s Syndrome, and multiple sclerosis, have greatly enhanced levels of BAFF in their serum (Sun et al., 2008). Whether this increased serum BAFF is leading to the manifestation of these pathologies, or is a by-product of the type I interferons released during autoimmune attack of self tissues, is unclear. However, BAFF depleting therapies, such as the fully humanized BAFF monoclonal antibody Lymphostat-B, and the TACI-Fc fusion protein Atacicept, have shown efficacy in the treatment of
Rheumatoid Arthritis and SLE (Carbonatto et al., 2008; Ding and Jones, 2006). Atacicept appears to be more efficacious, presumably due to the fact that it also depletes APRIL which is known to be necessary for the long term survival of autoantibody producing plasma cells (Mackay and Schneider, 2009). This could indicate that deregulation of BAFF levels could lead to autoimmunity, and that its depletion by serve to ameliorate disease.

The APRIL-transgenic mouse has also been reported. Like the Baff-transgenic mouse, these animals display B cell hyperplasia of B1 B cells in the peritoneum. However, in contrast to the BAFF-transgenic mouse, the overexpression of APRIL leads to increased T cell proliferation, and increased survival of CD4+ and CD8+ T cells (Dillon et al., 2006). This suggests that April plays a role in T cell survival, but a minor role in B cell survival compared to BAFF.

The BAFF-R was identified as the mutated factor in the naturally occurring mouse strain, A/WySnJ, which displayed immunodeficiency similar to, but not as severe as that observed in the BAFF−/− mouse (Yan et al., 2001). The mutation in this strain ablated a portion of the cytoplasmic tail of the BAFF-R suggesting that it may impinge on BAFF-mediated signal transduction. However, it was hypothesized that despite this mutation, there was still residual function downstream from the BAFF-R, given the lack of an exact phenocopy of the BAFF−/− mouse. The BAFF-R−/− mouse displayed a phenotype that was essentially identical to the to the BAFF−/− with additional defects in immunoglobulin class switching and the germinal center persistence in the former (Shulga-Morskaya et al., 2004).
The phenotype of the TACI\(^{-/-}\) mouse was a surprising one. Intriguingly, the TACI knockout displays a phenotype that, in many ways, is similar to that of the BAFF-Tg mouse. Perhaps most strikingly, TACI deficiency leads to severe splenomegaly that occurs as early as the Transitional T1 B cell stage (Seshasayee et al., 2003). TACI\(^{-/-}\) B cells hyperproliferate concomitant with the development of autoimmune glomerulonephritis, suggesting that signals emanating from TACI negative regulate BAFF induced responses. TACI is highly expressed on MZ B cells in the spleen, which are known to have a lower threshold of activation induced by repetitive, polymeric T cell independent antigens (Mantchev et al., 2007). In line with this observation, TACI\(^{-/-}\) mice display depressed antibody responses to T cell independent antigens. Although the mechanism by which TACI negatively regulates BAFF function is unclear at this point, it is speculated that TACI, due to fact that it binds oligomerized, 60-mer forms of BAFF, regulates levels of BAFF in the periphery (Bossen et al., 2008). Therefore, the absence of TACI allows for heightened levels of BAFF in the serum. It is also unknown whether TACI agonism elicits a specific signaling cascade that alters gene expression programs leading to negative regulation of BAFF responses.

Germline deletion of BCMA in mouse lead to a decidedly mild phenotype compared to BAFF-R or TACI knockouts. Initially, it was suggested that BCMA was necessary for antigen presentation by B cells, through its regulation of MHC class II expression (Yang et al., 2005). Recent studies suggest that signals transmitted through BCMA are essential for the long term persistence of plasma cells in the periphery (O'Connor et al., 2004).
Figure 2. BAFF, APRIL and their receptors.
**iii) BAFF-mediated signal transduction**

For many years, after the discovery that BAFF was a critical survival factor for B cells, the mechanism by which these signals were transmitted in the cytosol remained elusive. The groups of Vishva Dixit and Ulrich Siebenlist independently showed that BAFF signals through the non-canonical NF-κB pathway, leading to the processing of the p100 subunit to the transcriptionally active p52 factor in a manner that was dependent on IKKα, and was surprisingly independent of NEMO (Claudio et al., 2002; Kayagaki et al., 2002). The naturally occurring mouse mutant denoted *aly*, for alymphoplasia, display gross defects in B cell homeostasis. It was shown that the point mutation in these mice ablated the catalytic capability of the factor NIK, or NF-κB inducing kinase (Shinkura et al., 1999). Subsequent studies illustrated NIK phosphorylates and activates IKKα on serine 176 and can also directly phosphorylate NF-κB2 subunit p100 (Ling et al., 1998; Xiao et al., 2001). Thus, NIK is a critical kinase upstream of IKKα and NF-κB2 and is necessary for the propagation of BAFF signals leading to survival in B cells.
Figure 3. BAFF mediated activation of NF-κB2.
The mechanism by which BAFF transmitted signal to NIK was an area of intense research for many years. Due to the fact that it is a member of the TNF superfamily of receptors, it was speculated that TRAF family members were involved in this signal transduction cascade. Through yeast two hybrid studies, TRAF3 was shown to interact with the cytoplasmic tail of the BAFF-R, and its overexpression inhibited BAFF induced NF-κB activation and IL-10 production (Xu and Shu, 2002). This would suggest that TRAF3 is somehow negatively regulating signals emanating from the BAFF-R. In line with these in vitro observations, TRAF3<sup>−/−</sup> animals resemble BAFF-Tg animals, implicating TRAF3 as a negative regulator of BAFF signals (He et al., 2006). B cells from TRAF3-null mice exhibit constitutive NF-κB2 processing concomitant with ligand independent upregulation of ICAM-1, and extended survival in culture. In addition, the post natal mortality in TRAF3<sup>−/−</sup> mice is rescued in a p100 deficient background, indicating that the phenotype is dependent on ligand independent activation of NF-κB2. Interestingly, TRAF3 deficient B cells contain heightened levels of NIK, suggesting that the negative regulatory role of TRAF3 in BAFF mediated signal transduction stems from its negative regulation of NIK levels. An extension of this finding is that constitutive, non-canonical NF-κB2 activation due to the transgenic expression of NIK, is ablated when a TRAF3 binding mutant of NIK is overexpressed (Sasaki et al., 2008). B cell specific ablation of TRAF2 essentially phenocopies the TRAF3<sup>−/−</sup> mouse, suggesting that TRAF2 and TRAF3 function co-operatively to negatively regulate signals downstream of the BAFF-R (Gardam et al., 2008).
The mechanism by which TRAF2 and TRAF3 affect BAFF signal transduction was highlighted elegantly in three recent studies (Matsuzawa et al., 2008) (Vallabhapurapu et al., 2008) (Zarnegar et al., 2008). These studies suggest that in the absence of BAFF, a complex exists consisting of TRAF2, TRAF3, NIK and cIAP1/2. In this unstimulated context, NIK is polyubiquitinated in a Lysine 48 dependent manner through the Ubiquitin ligase function of cIAP1/2. This targets NIK for degradation by the proteasome, thereby inhibiting promiscuous NF-κB2 activation in the absence of extracellular stimuli. When BAFF binds to the BAFF-R, TRAF3 is recruited to the cytoplasmic tail of the receptor, freeing it from the TRAF2-NIK-cIAP complex. This recruitment activates the TRAF2 dependent polyubiquitination, and subsequent degradation of TRAF3 freeing NIK to phosphorylate and activate IKKα, which can subsequently activate NF-κB.
Figure 4. The role of TRAF2 and TRAF3 in BAFF induced NF-κB2 activation.
Agonism of the BAFF-R leads to the activation of NIK and IKKα, which leads to processing of the p100 subunit to p52, and its subsequent translocation to the nucleus in a heterodimer also containing RelB (Claudio et al., 2002; Kayagaki et al., 2002). However, mice with defects in RelB or NIK do not completely phenocopy BAFF−/− mice in terms of effects on B cell development (Shinkura et al., 1999; Weih et al., 2001). This observation suggests that various parallel pathways may emanate from BAFF-R and lead to the development of different B cell subsets.

In addition to the upregulation of survival factors through the activation of non-canonical NF-κB signaling, BAFF promotes metabolic fitness and cell growth through the Phosphoinositide-3-kinase (PI3K) dependent activation of mammalian target of rapamycin or mTOR (Patke et al., 2006) (Woodland et al., 2008). BAFF binding to the BAFF-R activates PI3K, was stimulation the accumulation of Phosphatidylinositol-3,4,5-trisphosphate at the membrane and the recruitment of Akt/PKB and PDK1. Activated Akt phosphorylates, thereby activating the mTOR complex 1 or mTORC1. Targets of mTORC1 in a BAFF mediated signaling cascade include ribosomal S6 Kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1) which function in ribosomal activation and mRNA translation, respectively. In addition, the antiapoptotic effects of BAFF can also be mediated by its ability to activate the phosphorylation of the pro-apoptotic BH3 only protein Bim in an ERK dependent fashion (Craxton et al., 2005). BAFF induced phosphorylation of Bim prevents its association with Bcl-2. Interestingly, Bim−/− mice have elevated levels T2 B cells, and FO B cells but reduced levels of MZ B
cells, suggesting that Bim levels has differential roles in different cell types (Craxton et al., 2005).

4. Bcl10-MALT1-Carma

i) Bcl10 and MALT1

MALT (mucosa-associated lymphoid tissue) lymphomas are malignancies of B cells normally resident in the body’s mucosae, most frequently in the stomach. This class of lymphomas is the most common arising at extranodal sites. Approximately 80% of cases arise due to chronic antigenic stimulation of resident B cells by acidophilic Helicobacter pylori bacteria in the stomach (Parsonnet et al., 1994). Therefore, antibiotic therapy directed towards the eradication of H. pylori in the gut is, by and large, an effective treatment for MALT lymphoma patients (Bayerdorffer et al., 1995). However, many patients were observed to be refractory to antibiotic mediated eradication therapy, and it was speculated that this non-responsiveness was due to a genetic aberration in these patients (Bayerdorffer et al., 1995). In the late 1990s, it was shown that antibiotic non-responsive MALT lymphoma patients exhibited two prominent chromosomal translocations that resulted in the overexpression of either Bcl10 (B cell lymphoma 10) or MALT1. Bcl10, also known as CIPER, CLAP, CARMEN, mE10 or cE10, was originally cloned from the chromosomal breakpoint t(1;14)(p22;q32) (Willis et al., 1999; Zhang et al., 1999). MALT1 was first identified in a t(11;18)(q21;q21) translocation that fused MALT1 with the IAP-2 gene and led to constituitive NF-κB activation (Akagi et al., 1999; Dierlamm et al., 1999). However, mice transgenic for MALT1 translocations do not develop lymphomas despite the fact that they exhibit
heightened NF-κB activation compared to their wild type (WT) counterparts (Baens et al., 2006). Overexpression studies in mouse and human cell lines further implicated MALT1 and Bcl10 in NF-κB activation (Srinivasula et al., 1999; Yan et al., 1999), and gene targeting studies in mice confirmed that Bcl10 and MALT1 are necessary for NF-κB activation and immune homeostasis (Rueflin-Brasse et al., 2003; Ruland et al., 2001; Ruland et al., 2003).

Bcl10−/− mice display reduced numbers of MZ B cells in the spleen and B-1 B cells in the peritoneum. In the thymus, CD4 and CD8 double negative thymocytes pass through a coordinated sequence of developmental steps denoted DN1 to DN4 based on the expressed of cell surface markers CD25 and CD44. Survival of DN3 and DN4 thymocytes, the so-called β-checkpoint stage, is dependent on NF-κB activation downstream of the pre-TCR (Siebenlist et al., 2005). T cell development is also impaired in the Bcl10−/− mouse, as evidenced by the increased numbers of DN4 thymocytes present, further underscoring the need for NF-κB activation at the β-selection stage. In addition, basal immunoglobulin levels, as well as T-dependent and T-independent humoral responses, are depressed in Bcl10−/− animals. In vitro, Bcl10−/− B and T lymphocytes stimulated by antigen receptor engagement or treatment with PMA (phorbol 12-myristate 13-acetate)/ionophore fail to activate NF-κB and thus are unable to proliferate (Ruland et al., 2001).

MALT1−/− T cells also fail to proliferate in vitro and cannot activate NF-κB signaling pathways in response to stimulation of the T cell receptor (TCR) with anti-CD3/anti-28 antibodies. MALT1−/− mice show defects in MZ B cell and B1 B cell development, but, in contrast to Bcl10−/− mice, NF-κB activation in response to engagement of the B cell
receptor (BCR) appears to be intact. These results suggest that MALT1 participates in an additional pathway in B cells that is necessary for proper B cell development. MALT1 deficient mice display normal distribution of CD4 and CD8 populations in the spleen and lymph nodes. Furthermore, the CD4/CD8 profile in the thymus is normal in the absence of MALT1. However, T cells in the periphery of MALT1−/− mice display lower percentages of CD25, CD44 and CD69 activation markers (Ruland et al., 2003). These data suggest that MALT1 is dispensible for T cell development, but is required for T cell activation.

ii) Antigen receptor mediated signal transduction

Antigen, presented in the context of Major histocompatibility complexes (MHC) on antigen presenting cells, and appropriate co-stimulatory cues, activates several parallel signal transduction cascades in T and B cells that alter gene expression programs and confer effector function. An early event in these pathways is the reorganization of signaling molecules into supramolecular activation clusters or “SMACs” at the cell surface. The so-called immunological synapse forms between the T cell and antigen presenting cell, and can be divided into the central SMAC (c-SMAC) and the peripheral SMAC (p-SMAC) (Monks et al., 1998). The c-SMAC contains many critical signaling molecules such as the CD3 associated TCR and CD28. Also, enriched in the c-SMAC are lipid rafts, which are cholesterol and sphingolipid enriched microdomains where many critical signalling components are known to cluster. Although somewhat controversial, it is thought that localization to the lipid raft is critical to the transduction of signal. The observation that the overexpression of NEMO that specifically localizes to
the membrane causes constitutive NF-κB activation lends credence to this hypothesis (Schulze-Luehrmann and Ghosh, 2006). It should be noted that the molecular events downstream of antigen binding to a B or T cell are highly similar. The description of antigen receptor signaling will focus on that emanating from the TCR.

Upon binding to antigen, a rapid wave of receptor proximal tyrosine phosphorylation events is elicited. CD4 or CD8 molecules expressed on the surface of helper T cells and cytotoxic T cells, respectively, interact through their cytoplasmic tails with various members of Src tyrosine kinase family. Lck and Fyn are known to be critical for T cell activation. These molecules are maintained in an inactive state by phosphorylation. In the presence of antigen, Lck and Fyn are dephosphorylated by the action of the membrane associated phosphatase CD45. Activated Lck and Fyn then recognize and phosphorylate Immunoreceptor related tyrosine activation motifs (ITAMS) on the cytosolic tails of TCR associated CD3ζ. Phosphorylated ITAM motifs are then recognized by Zeta associated protein of 70 kD, or ZAP70, which upon activation, phosphorylates factors such LAT and SLP76 which serve as docking sites for many downstream factors. One such factor is phospholipase C γ1 (PLCγ1) which generates diacylglycerol (DAG) and inositol-1,4,5-triphosphate. DAG activates members of the PKC family (see later) which leads to activation of NF-κB. IP₃ leads to increases in intracellular calcium, which activates NFAT transcription factors downstream of the phosphatase calcineurin. Ras family members also localize to LAT/SLP76 related complexes which lead to the MAPK dependent activation of AP-1 transcription factors.
Figure 5. Signaling Pathways downstream of the TCR
NF-κB activation downstream of the TCR is rather weak and does not lead to the full activation of a T cell. Costimulatory cues, through the interaction of B7.1 and B7.2 molecules on the surface of antigen presenting cells and CD28 expressed on the surface of T cells, are required for full activation of NF-κB. Indeed, one of the main mechanisms for the induction of anergy or unresponsiveness of a T cell and tolerance is through T cell agonism in the absence of costimulation. It is thought that signals through CD28 do not necessarily provide a specific signal to NF-κB, but rather, simply augment signals delivered through the TCR (Schmitz and Krappmann, 2006). The first pathway involves the activation of PI3K, and the generation of lipid metabolites that recruit factors such as Akt, PDK1 and Vav to the cytosolic face of CD28 (Lemmon, 2005). Akt has been shown to activate the IKK complex through the recruitment of the serine kinase Cot (Kane et al., 2002). In addition, the transgenic expression of Akt leads to constitutive NF-κB activation (Jones et al., 2000). CD28 agonism by the B7 family of molecules activates NF-κB also through the recruitment of the Vav, which has been shown to form a complex with SLP76. This complex, in turn leads to the activation of PLCγ1 which stimulates the production of DAG and activation of PKC (Villalba et al., 2000).

PKCθ is the member of the PKC family that is critical for signal transduction to NF-κB downstream of the TCR. Although T cells are known to express several isoforms of PKC, PKCθ is the only one that inducibly localizes to the cSMAC upon antigen stimulation (Monks et al., 1998). Although the exact mechanism whereby PKCθ becomes activated is a matter of some debate, it is thought to be recruited to lipid rafts by PDK1, which phosphorylates PKCθ on several residues (Villalba et al., 2002).
Interestingly, PDK1 simultaneously recruits PKCθ and CARMA1 after antigen stimulation suggesting a critical role in antigen receptor mediated NF-κB activation (Lee et al., 2005). Although there is a surfeit of biochemical data suggesting that PKCθ is a crucial intermediary in NF-κB signal transduction, data from genetic studies are somewhat controversial. The first PKCθ knockout reported exhibited defects in TCR induced NF-κB activation (Sun et al., 2000). A subsequent PKCθ−/− mouse displayed a markedly different phenotype, with defects in NFAT activation but not in activation of NF-κB or AP-1 (Pfeifhofer et al., 2003). The differences between these two mouse strains may stem from the different targeting strategies employed by the two groups.

Our lab (and others) then showed that BCL10, MALT1 and a third component called CARMA (caspase recruitment domain containing membrane-associated guanylate kinase) form a trimolecular complex (the CBM complex) that conveys the signal from PKCθ to IKK(Thome, 2004),(Lucas et al., 2001). In vitro studies have subsequently shown that the overexpression of Bcl10, CARMA or MALT1 can activate NF-κB in a process that may involve higher order oligomerization of all these factors (Schulze-Luehrmann and Ghosh, 2006).
Figure 6. TCR-induced NF-κB activation
iii) Components of the CBM complex

CARMA is thought to act downstream of PKCθ and upstream of Bcl10. This conclusion is based on studies showing that: 1) CARMA constitutively associates with lipid rafts; 2) Carma overexpression fails to induce NF-κB activation in Bcl10−/− MEFs; and 3) the overexpression of a constitutively active form of PKCθ fails to activate NF-κB in Carma−/− T cells (Egawa et al., 2003; Gaide et al., 2002; Gaide et al., 2001; Ruland et al., 2001; Wang et al., 2002). At a structural level, Carma belongs to the membrane-associated guanylate kinase (MAGUK) family of proteins and is the only MAGUK protein exclusively expressed in lymphocytes (Egawa et al., 2003). Carma consists of an N-terminal caspase recruitment domain (CARD), a coiled-coil (CC) domain, a PDZ domain, an SH3 domain, and a GUK domain (Egawa et al., 2003). In response to TCR or BCR engagement, Carma is recruited to lipid rafts by PDK1, and the region of Carma between the CC and PDZ domains is phosphorylated on three critical serine residues termed the PKC regulated domain, or PRD, by PKCθ in T cells or by PKCβ in B cells (Gaide et al., 2001; Matsumoto et al., 2005; Sommer et al., 2005). Interestingly, reconstitution of CARMA-deficient Jurkat cells with a ΔPRD-CARMA leads to constitutive activation of NF-κB, suggesting that this region somehow negatively regulates the pathway (Sommer et al., 2005). PRD phosphorylation appears to be critical for the recruitment of Bcl10 and subsequent NF-κB activation, as mutagenesis of the CARMA phosphorylation sites abolishes these events (Sommer et al., 2005). Furthermore, mutagenesis of the CARD domain of CARMA abolishes inducible binding
to Bcl10, and NF-κB activation, suggesting that these two molecules must form a complex to transduce signal (Gaide et al., 2002).

The Bcl10 gene encodes a protein with an N-terminal CARD domain that facilitates its interaction with CARMA through CARD-CARD interactions. Deletion of the CARD domain of Bcl10 ablates antigen receptor induced The C-terminus of BCL10 contains a domain of unknown structure that possesses a large number of serine and threonine residues, suggesting that this region may have a signaling function (Thome and Tschopp, 2002).

MALT1 functions directly downstream of Bcl10 during antigen induced NF-κB activation (Lucas et al., 2001; Uren et al., 2000). MALT1 interacts with the a region slightly C terminal to the CARD domain of Bcl10 and acts synergistically with this molecule to activate NF-κB (Lucas et al., 2001; Ruland et al., 2003). Structurally, MALT1 contains an N-terminal death domain, followed by two Ig-like domains that mediate interaction with Bcl10, and a C-terminal caspase-like “paracaspase” domain (Lucas et al., 2001; Ruland et al., 2003). Whether or not the paracaspase domain of MALT1 has any physiologically relevant catalytic activity has been a matter of debate for years (see later).
Figure 7. Molecular interactions within the CBM complex (Thome, 2004)
iv) Bcl10 and MALT1 in signalling associated with non-antigen receptors

In addition to their roles in antigen receptor signaling, Bcl10 and MALT1 are pivotal signal integrators that act downstream of several other receptors. It is not yet clear whether CARMA is also involved in this signal integration. It is known that Bcl10 and MALT1 act in a complex that is required for some (but not all) of the biochemical events and cellular outcomes triggered by FcεRI engagement. When FcεRI on mast cells is engaged by its cognate ligand IgE, preformed mediators (including histamine and leukotrienes) are released from mast cell granules and the de novo synthesis of proinflammatory factors (including TNFα) is initiated (Rivera, 2002; Siraganian, 2003). These mediators then combine to trigger and sustain allergic inflammation (Galli et al., 2005). The FcεRI-associated complex containing Bcl10 and MALT1 is required for the late phase passive cutaneous anaphylaxis (PCA) response, IL-6 and TNFα production, and NF-κB activation, but Bcl10 and MALT1 are dispensable for mast cell degranulation, IgE-induced mast cell survival, and mast cell development (Klemm et al., 2006).

Bcl10 and MALT1 are also critical for FcγR signalling, acting to positively regulate actin polymerization induced by a variety of stimuli. For example, when siRNA was used to silence Bcl10 expression in the THP-1 monocytic cell line, IgG-induced actin polymerization and phagocytosis by these cells was inhibited. Similarly, Bcl10 ablation in the Jurkat T cell line abolished actin polymerization induced by anti-CD3/anti-CD28 treatment (Rueda et al., 2007). Bcl10 and MALT1 also appear to be necessary for NF-κB activation downstream of G-coupled protein receptors, including the lysophosphatidic
acid (LPA) receptor and the angiotensin II receptor (Klemm et al., 2007; McAllister-Lucas et al., 2007). CCR3 is a chemokine receptor specific for Eotaxin and is expressed on the surface of eosinophils and Th2 cells (Pease, 2006). Eotaxin induced eosinophilia in an air pouch model is ablated in the absence of Bcl10 or MALT1, suggesting that these factors may have some signaling functionality downstream of CCR3 (M. Tusche, and G. Duncan, unpublished observation). Lastly, Bcl10 interacts with the scaffolding protein CARD9, and, along with MALT1, is necessary for signals transmitted through Dectin-1, a receptor critical for anti-fungal immunity (Gross et al., 2006).
Figure 8. MALT1 in non-antigen receptor signal transduction
5. Unanswered questions

i) Physiological function of the MALT1 paracaspase domain

As mentioned above, whether or not the paracaspase domain of MALT1 is important for its role in promoting NF-κB activation has been a matter of heated discussion. It has been shown that the mutagenesis of a particular cysteine residue, whose equivalent is critical for the function of caspases, results in a reduction (but not abolition) of the ability of MALT1 to activate NF-κB (Lucas et al., 2001). This result suggests that MALT1 may in fact have a catalytic function of some importance. Furthermore, the purified MALT1 paracaspase domain binds to ketoxymethyl probes. Since these chemical entities bind to the active nucleophillic residue in proteolytic enzymes, this suggests that it contains the necessary active structure for proteolytic activity. Nevertheless, MALT1 does not cleave generic tetrapeptide substrates that are hydrolyzed by most proteolytic enzymes (Snipas et al., 2004). These observations suggest that if MALT1 is catalytically active, it acts on a specific substrate of unique or unusual structure.

Several studies have recently demonstrated that MALT1 is, in fact, an active protease with the catalytic site positioned at C464 in the human protein and at C461 in the mouse version. Rudi Beyeart and colleagues have identified the MALT1 substrate as A20, also known as TNFα inducible protein 3 (TNFAIP3). In Jurkat cells (a human T cell line), the cleavage of A20 at a specific arginine residue was necessary for the complete activation of NF-κB induced by TCR engagement (Coornaert et al., 2008).
A20 is a deubiquitinating enzyme with a C-terminal OTU-type DUB domain (Krikos et al., 1992). It is known to negatively regulate NF-κB activation induced by TNFα or LPS (Skaug et al., 2009). A20−/− mice die postnatally due to multi-organ inflammation and leukocyte infiltration (Lee et al., 2000). Recent studies have indicated that A20 is deregulated in SLE and is deleted in MALT lymphoma, the latter observation being perhaps significant in that A20 is thought to be cleaved by MALT1 (Musone et al., 2008) (Novak et al., 2009). Dixit and colleagues demonstrated that A20 actually serves as a dual function Ubiquitin editing factor, both adding and removing polyubiquitin chains from RIP1, a critical signaling intermediate downstream of TNF-R (Wertz et al., 2004). Furthermore, A20 can de-ubiquitinate and thus negatively regulate CBM complex formation (Stilo et al., 2008).

The group of Margot Thome has suggested that Bcl10 is cleaved by MALT1 in response to TCR agonism, and that this Bcl10 cleavage is necessary for T cell adhesion to fibronectin (Rebeaud et al., 2008). In neither report of A20 or Bcl10 cleavage by MALT1 is the relevance of MALT proteolytic function addressed *in vivo*. It is also possible that MALT1 acts on additional substrates whose cleavage may fine-tune the signalling pathway leading to NF-κB activation in T cells.

### ii. *Mechanism of action of CBM complex*

Many studies, both *in vitro* and *in vivo* in knockout mice, have shown that the CBM complex is required for NF-κB activation in murine lymphocytes (Egawa et al.,
2003; Hara et al., 2003; Ruefli-Brasse et al., 2003; Ruland et al., 2001; Ruland et al., 2003; Xue et al., 2003). However, until recently, the mechanism by which this complex functions was mysterious. Recently, it has been suggested that polyubiquitination plays a crucial role in NF-κB activation downstream of many receptors such as antigen receptor, Toll-like receptors, TNFR, intracellular peptidoglycan sensors NOD1 and NOD2, the viral RNA receptor RIG-1, and BAFF-R (Skaug et al., 2009).

Bcl10 can be induced to interact with an E2 ubiquitin ligase complex that consists of Mms2 and Ubc13 and mediates the Lys63-dependent polyubiquitination of IKKγ (Sun et al., 2004; Zhou et al., 2004) (Figure 3). In another report, the authors suggested that Bcl10 interacted with Uev1a, a molecule known to functionally interact with UBC13 (Zhou et al., 2004). Unlike polyubiquitination of Lys48, which targets proteins for degradation by the proteasome, polyubiquitination of Lys63 facilitates protein-protein interactions and acts as a positive regulator of cell signalling (Haglund and Dikic, 2005). The exact biochemical reason why the two different polyubiquitin linkages lead to such disparate cellular outcomes is not clear at this point. It is speculated that lysine 48 linkages allow for inter-ubiquitin interactions which lead to a globular polyubiquitin structures. No such inter-ubiquitin interactions are possible with lysine 63 linked chains allowing for an extended structure. Therefore, the different arrangement in three dimensional space of the different types of polyubiquitin chains may allow for the different outcomes observed (Skaug et al., 2009).

The Ubc13/Uev1A-dependent polyubiquitination of IKKγ has been shown in vitro to depend on the E3 ligase function of TRAF6, which recruits TAK1 and its binding partners TAB1-3 (Wang et al., 2001). Within this E3 ligase complex, activated TAK1
phosphorylates IKKβ at Ser177 and Ser181 and thus activates the IKK complex (Wang et al., 2001). This so-called TAK1 complex has been shown biochemically to be critical for NF-κB activation downstream of many receptors. Although this model is compelling, in vivo studies have indicated that there may be some redundancy in this pathway: 1) T cell-specific deletion of TRAF6 does not affect NF-κB activation in these cells but, curiously, causes widespread inflammation and a resistance of these T cells to suppression mediated by CD4+CD25+ regulatory T cells (King et al., 2006). 2) In vitro studies have suggested that MALT1, rather than TRAF6, may play the role of the E3 ubiquitin ligase during Lys63-dependent ubiquitination of IKKγ in T cells, despite the fact that MALT1 lacks any domains classically associated with ubiquitin ligase function (Zhou et al., 2004). 3) UBC13 is dispensable for antigen receptor-induced NF-κB activation in B cells but required for this process in T cells (Yamamoto et al., 2006a; Yamamoto et al., 2006b). 4) Conditional knockout studies have shown that TAK1 is not essential for NF-κB activation in B cells or effector T cells but is required for NF-κB activation in thymocytes and naïve T cells (Liu et al., 2006; Sato et al., 2005; Sato et al., 2006; Wan et al., 2006). Therefore, it remains unclear exactly how the CBM complex function in vivo to influence NF-κB activation. 5) A NEMO knockin mouse harboring a mutation that was hypothesized to abolish polyubiquitination, shows that NEMO polyubiquitination is dispensable for antigen receptor induced NF-kB activation but is required for Toll-like receptor signaling (Ni et al., 2008).
Figure 9. Mechanisms of IKK activation by the CBM complex
iii) **Negative regulation of NF-κB activation**

NF-κB activation is a powerful stimulus of cell survival and proliferation, events that can set the stage for autoimmunity or tumorigenesis if not closely regulated. Therefore, NF-κB signaling downstream of the TCR can be downmodulated in a number of ways. PKCθ is involved in the TCR recycling cascade, through phosphorylation of 2 conserved serine residues, which functions to internalize the TCR to turn off signaling (von Essen et al., 2006). The inhibition of CBM complex formation by MALT1 substrates may represent one means by which signals transmitted from antigen receptors to NF-κB can be negatively regulated. Indeed, *in vitro*, the formation of the CBM complex can be inhibited by A20, a putative substrate of MALT1 as described above. However, other negative regulatory loops appear to exist, including TCR-dependent degradation of Bcl10. Israel and colleagues have shown that, in the presence of TCR agonism or PMA/ionophore treatment, Bcl10 is phosphorylated at Thr81 and Ser85 in an IKKβ-dependent manner (Lobry et al., 2007). These phosphorylation events allow Bcl10 to be ubiquitinated and proteasomally degraded. *In vitro* studies suggest that Bcl10 degradation negatively regulates T cell activation, as Jurkat T cells overexpressing a non-degradable, mutated form of Bcl10 (Bcl10 T81A/S85A) show enhanced and prolonged IL-2 production following stimulation. This non-degradable Bcl10 protein accumulates in the nuclei of these Jurkat cells, reminiscent of the abnormal nuclear localization of Bcl10 in MALT lymphoma patients (Ye et al., 2006). These observations suggest that a defect in Bcl10 degradation, which would lead to increased NF-κB activation, may act as a molecular trigger of tumorigenesis. An accumulation of non-degradable Bcl10 protein
might also lead to autoimmunity due to this protein’s role in controlling lymphocyte homeostasis. Another study suggested that Bcl10 inducibly interacts with cIAP, NEDD4, and Itch after stimulation. This interaction leads to the monoubiquitination of Bcl10 and its lysosomal degradation (Wegener et al., 2006). Which Bcl10 degradation pathway is relevant \textit{in vivo}, is a question that is yet to be addressed.

\textit{iv) The Role of MALT1 in B cell activation and homeostasis}

The role of MALT1 in B cells remains controversial. Despite no defects in NF-κB activation downstream of the BCR, MZ B cells in the spleen and B1 B cells in the peritoneum fail to develop in MALT1\textsuperscript{−/−} mice. This phenotype is reciprocal to that of the BAFF-Tg animal, which display enhanced frequencies of MZ B cells and B1 cells (Mackay et al., 1999). Interestingly, MALT1 lymphoma samples exhibit heightened expression of BAFF (Kuo et al., 2008), suggesting that perhaps both deregulation of MALT1 and BAFF could contribute to lymphomagenesis.

\textbf{6. Thesis Structure}

In this thesis, I describe my work dissecting the mechanisms by which the CBM complex modulates non-canonical and canonical NF-κB activation and immune homeostasis. I will first describe the methods that I used throughout this thesis. In the first Results chapter, I will discuss \textit{in vitro} data describing how MALT1 regulates non-canonical NF-κB activation induced by BAFF in B cells. In the second Results chapter, I
will discuss how MALT1 modulates the activity of BAFF \textit{in vivo} by describing the phenotype of a MALT1\textsuperscript{-/-} mouse crossed into a BAFF transgenic background. In the third Results chapter, I will discuss the role of mms2 in Bcl10-mediated NF-\kappa B activation \textit{in vitro}, and the somewhat surprising phenotype of the mms2 knockout mouse.
II. CHAPTER 2 – MATERIAL AND METHODS

Mice

The generation of Bcl10−/−, MALT1−/− and BAFF-Tg mice has been described previously (Mackay et al., 1999; Ruland et al., 2001; Ruland et al., 2003). The expression of BAFF in the BAFF-Tg mouse is under the control of a liver specific promoter, thereby allowing for a systemic upregulation of BAFF levels (Mackay et al., 1999). Bcl10−/−xBAFF-Tg and MALT1−/−xBAFF-Tg mice were maintained with the transgene expressed heterozygously so as to generate littermate controls. BAFF-Tg+/− and BAFF-Tg+/+ mice have equivalent phenotypes (McCarthy et al., 2006). Jh−/− mice were obtained from Dr. Simon Fillatreau, DRFZ, Berlin, Germany (Gu et al., 1993). Mice were analyzed at 8-10 weeks of age, and were all backcrossed for at least 6 generations into C57BL/6 backgrounds. All animal experiments were conducted using protocols approved by the Animal Use Committee of the Ontario Cancer Institute and the University of Toronto.

Generation of the mms2 knockout mouse

To explore an in vivo role for mms2 in NF-κB signaling, we sought to generate a targeted deletion of the mms2 gene using conventional methods of homologous recombination in murine embryonic stem (ES) cells that would allow the eventual development of a “knockout” animal. For these purposes, a targeting construct was generated that targeted exons 3 and 4 of the murine mms2 locus and was predicted to result in a frameshift mutation and a premature stop codon. The construct was electroporated into ES cells and recombinants were selected in 250 μg/ml G418. Positive clones were confirmed by
southern blot in which BglII digestion of genomic DNA from recombinants yielded a 5.7 kB mutant fragment and 23.4 kB wild type (WT) fragment when hybridized to a flanking probe. Three +/- ES cell clones were confirmed by hybridization to the flanking probe and a neo-specific probe that confirmed a single site of integration for the targeting construct. Recombinant ES clones heterozygous for the targeted mutation were microinjected into C57BL/6 blastocytes and implanted into pseudopregnant females. Agouti pups were crossed into C57BL/6 mice and the offspring were assessed for germline transmission of the targeted allele by southern blotting as described above. F1 progeny were backcrossed for 6 generations into the C57BL/6 background before phenotypic analysis.

*Cell lines*

Jurkat human T cell lines were obtained from ATCC and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 5 mM L-glutamine, 55 µM 2-mercaptoethanol (ME) and antibiotics (R10 medium). The BJAB human Burkitt lymphoma cell line (the kind gift of S. Kalled, Biogen-Idec, Inc, Cambridge, Ma), was maintained in R10 medium. HEK293T human embryonic kidney cells were cultured in DMEM medium supplemented with 10% FCS, 5 mM L-glutamine, 55 µM ME and antibiotics (D10 medium). All murine embryonic fibroblast (MEF) strains, namely NEMO⁺/⁺, NEMO⁻/⁻, MALT1⁺/⁺, and MALT1⁻/⁻ MEFs, were cultured in D10 medium. MEFs were isolated from embryonic day 14 (E14) embryos generated from timed heterozygous intercrossings and were immortalized by repeated passage in culture.
Purification of T cells and B cells

Spleens from WT, Bcl10<sup>−/−</sup>, MALT1<sup>−/−</sup> and Mms2<sup>−/−</sup> mice were homogenized through steel mesh into PBS containing 1% FCS to obtain single cell suspensions. Erythrocytes were removed by incubating cells in 1ml Red Blood Cell Lysis Buffer (Sigma-Aldrich, St. Louis, MO) for 7 minutes at room temperature (RT). B220<sup>+</sup> B cells were isolated by negative depletion using biotinylated anti-CD11b, anti-Thy 1.2 and anti-Ter119 antibodies plus streptavidin-conjugated magnetic beads (Becton Dickinson). Purity of B220<sup>+</sup> cells was confirmed to be >95% using flow cytometry. T cells were isolated also by negative depletion using biotinylated anti-CD11b, anti-B220 and anti-Ter119 antibodies plus streptavidin-conjugated magnetic beads (Becton Dickinson). CD3<sup>+</sup> T cells were consistently >95% pure.

T cell and B cell stimulation

T cells or B cells were cultured in 96-well plates (2 x 10<sup>5</sup> cells/well) in R10 medium. B cells were stimulated at 37°C with 2 μg/ml recombinant BAFF (Peprotech, Rocky Hills, NJ) or 5 μg/ml anti-IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), or 5 μg/ml anti-CD40 (Jackson), or 20 μg/ml LPS (Sigma) for the time points appropriate for each type of experiment. T cells were stimulated at 37°C with 10 ng/ml phorbol myristate acetate (PMA) plus 50 ng/ml ionomycin (both from Sigma), or 100ng/ml plate-bound anti-CD3 antibody (Becton Dickinson), or 50ng/ml plate-bound anti-CD3 plus 50ng/ml plate-bound anti-CD28 (Becton Dickinson) for the time points appropriate for each type of experiment.
**T cell and B cell proliferation**

To assay proliferation, 1.0 µCi $^3$H-thymidine (Perkin Elmer, Boston, MA) was added to cultures of stimulated T or B cells for the last 8 hours of culture. Thymidine incorporation was measured using a Top Count NXT microplate scintillation counter (Packard).

**Immunoblotting**

B cells or T cells ($10^7$ cells) were serum-starved for 4 hours prior to stimulation in OptiMEM medium as described above. For the preparation of cytosolic extracts, stimulated cells were resuspended in lysis buffer [50 mM Tris-Cl pH 7.4, 250 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, 1 mM NaF, 1 mM Na$_4$VO$_3$, complete protease inhibitor cocktail (Roche, Mannheim, Germany)]. For the preparation of nuclear lysates, stimulated cells were resuspended in Buffer A (10 mM HEPES pH 7.4, 10mM KCl, 0.1mM EDTA supplemented with 1 mM PMSF, 1 mM DTT, and phosphatase and protease inhibitor cocktails [from Roche]) and incubated on ice for 15 minutes with occasional agitation. NP-40 was added to a final concentration of 0.65% and incubation was continued for a further 15 minutes. Samples were centrifuged at 10,000 rpm for 1 minute at 4°C to pellet nuclei. Nuclear pellets were resuspended in Buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA supplemented as above) for 30 minutes on ice with occasional agitation.

Cytosolic or nuclear lysates were cleared by centrifugation at 14,000 rpm for 10 minutes at 4°C. Protein content was normalized and western blotting was performed according to standard protocols. Blots were probed with the appropriate antibodies and
proteins were visualized using enhanced chemiluminescence according to the manufacturer’s protocol (GE Healthcare, Buckinghamshire, UK). Blots were stripped and re-probed with anti-actin or anti-tubulin antibody (Sigma-Aldrich, St. Louis, MO) as a loading control. Antibodies against phospho-IκBα, phospho-ERK1/2, ERK, phospho-p38, NF-κB2 (p100/p52), RelB and phospho-p100 (Ser 866/870) were obtained from Cell Signaling Technologies (La Jolla, CA). Antibodies against IκBα, HA, TRAF3, TRAF2, Bcl10, MALT1, PKCδ and USF2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mms2 and anti-CBP were obtained from Zymed (Carlsbad, CA).

**Immunoprecipitation (IP)**

Stimulated or unstimulated cells were washed once with cold PBS, resuspended in 1 ml modified RIPA buffer (50 mM Tris-Cl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, protease and phosphatase inhibitor cocktail tablets), incubated at 4°C with constant rotation 20 minutes, and centrifuged 14000 rpm for 10 minutes to remove debris. Lysates were precleared by adding 30 µl Protein G-sepharose 4 Fast Flow (GE Healthcare) followed by rotation at 4°C for 30 minutes. Precleared lysates were incubated with a 1:100 dilution of anti-Bcl10, anti-NEMO or anti-MALT1 (all from Santa Cruz) for 18 hours at 4°C with constant rotation. Immune complexes were precipitated using 30 µl Protein G-Sepharose 4 Fast Flow with rotation at 4°C for 30 minutes, eluted by boiling in Laemmli buffer, fractionated on SDS-PAGE gels, and immunoblotted according to standard protocols.
**EMSA**

B cells (10^7 cells) were serum-starved, stimulated for 8 hours with 5 µg/ml anti-IgM, and nuclear extracts were prepared as described above. Nuclear extract samples (2.5 µg) were analyzed for DNA binding using the Odyssey IR EMSA kit (Licor, Lincoln, NE). For supershift assays, 1 µl anti-p65 (Santa Cruz) was added to the DNA binding reaction prior to the addition of labeled oligo followed by incubation at RT for 20 minutes. Binding reactions were fractionated on TBE gels (Biorad) and visualized using an Odyssey Infared imager.

**B cell survival assays**

Marginal zone (CD21^{high}CD23^{low}) and FO (CD21^{low}CD23^{high}) B cells were isolated from total B220^+ B cells using anti-CD21/23 staining and a FACSARia cell sorter (Becton Dickinson). Unfractionated B220^+ B cells or sorted B cells were cultured with or without 2 µg/ml recombinant BAFF, or with 5 µg/ml anti-IgM for the indicated time points. Viability was assessed using Annexin V/propidium iodide (PI) staining and a FACSCalibur Flow Cytometer (Becton Dickinson). Viability was expressed as the fold increase in viable cells (AnnexinV/PI negative) cells over untreated controls or % Viable cells. The PI3K inhibitor LY294002 (Calbiochem, Gibbstown, NJ) was resuspended in DMSO and used at a final concentration of 5 µM.

**Serum Ig ELISA**

Immunoglobulin isotypes were determined using standard ELISA of serially diluted mouse serum samples. Blood was collected from the tail vein into 1.5 ml tubes and
centrifuged for 14,000 rpm for 1 minute at 4°C. Primary antibodies recognizing mouse 
Igs and HRP-linked secondary antibodies recognizing specific isotypes were obtained 
from Southern Biotechnology (Birmingham, AL). Standard curve immunoglobulins were 
from eBioscience (San Diego, CA).

**Flow cytometry**

Peritoneal lavage was conducted by injecting 8 ml PBS into the peritoneum of a 
euthanized mouse and massaging gently for 2 minutes. Recovered peritoneal cells were 
resuspended in FACS buffer (PBS containing 3%FCS and 0.09% sodium azide) and 
incubated with anti-CD11b-FITC, anti-B220-APC (both from eBioscience), anti-CD5-PE 
and anti-IgM-Cyc (both from Becton Dickinson). Total splenocytes were collected from 
spleens as described above, resuspended in FACS buffer, and incubated with anti-CD21-
FITC, anti-IgM-CyC, anti-B220-PE (all from Becton Dickinson), anti-CD1d-PE and anti-
CD23-biotin followed by incubation with streptavidin-APC (all from eBioscience). For 
BAFF-R staining, whole splenocytes were incubated with anti-CD4-PE, anti-B220-FITC 
(both from Becton Dickinson) and BAFF-R-biotin (the kind gift of S. Kalled, Biogen-
Idec, Inc., Cambridge, MA.) followed by incubation with streptavidin-APC. Thymocytes 
were prepared as described and incubated with antibody cocktails consisting of CD4-
FITC, CD8-PE and CD3-APC for CD4/8 profiling, or B220-FITC, CD4-FITC, CD8-
FITC, TCRγδ-FITC, NK1.1-FITC, CD11b-FITC, CD25-APC and CD44-PE for the 
analysis of DN1-4 populations in the Lin⁻ population (all from Becton Dickinson). Single 
cell suspensions from pooled lymph nodes (mesenteric, inguinal, mandibular, paraaortic, 
axillary) were prepared as described and stained with CD4-FITC and CD8-PE (Becton
Dickinson). Immunostained cells were washed in FACS buffer and resuspended in 2% paraformaldehyde. Samples were processed using FACScalibur or FACSCanto (Becton Dickinson) and analyzed using FlowJo software.

**Luciferase reporter assays**

MALT1+/+ MEFS, MALT1−/− MEFs, or HEK293T cells were maintained in D10 medium. At 24 hours before transfection, cells (2x10^5/well) were plated in 6 well plates. On the day of transfection, the medium was replaced with Optimem without antibiotics. Transfection of pcDNA3-NIK, pcDNA3-Bcl10, pCMV-Bcl10-TAP, pCMV-TAP, or pcDNA3-mms2 (1.8 µg), pBV-Luc (0.2 µg) or pRTLK (20 ng), or empty vector controls, was performed using Fugene 6 transfection reagent (Roche, Mannheim, Germany) according to the manufacturer’s protocol. Luciferase activity was measured 72 hours after transfection using the Dual Luciferase Assay system (Promega, Madison, WI) and a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). Data were expressed as fold increase over empty vector-transfected controls.

**Intracellular phospho-flow cytometry**

B220⁺ B cells (5x10^5) were stimulated for 5 minutes at 37°C with 2 µg/ml BAFF. Cells were fixed with paraformaldehyde and permeabilized in 1 ml BD Phosphoflow Perm Buffer III (90% methanol-based; BD Biosciences Pharmingen, San Diego, CA). After washing, cells were stained with an antibody cocktail consisting of 1/100 dilutions of anti-CD21-PE, anti-CD23-FITC (both from Becton Dickinson) and anti-phospho-p100 (Ser866/870; Cell Signaling, La Jolla, CA), and incubated on ice for 30 minutes.
Following washing in FACS buffer (PBS containing 3%FCS and 0.09% sodium azide), cells were stained with a 1/1000 dilution of anti-rabbit Alexa 647 (Molecular Probes, Eugene, OR) for 1 hour on ice. Cells were washed, resuspended in FACS buffer, and collected using a FACSCanto Flow Cytometer (Becton Dickinson). Samples were analyzed using FlowJo software.

**RNAi mediated knockdown**

Cells (5 x 10^6) were washed once in PBS and resuspended in 100 µl Nucleofector Solution T (Amaxa Biosystems, Gaithersburg, MD) containing 125 pmoles siGenome Smartpool MALT1 or NF-κB2 siRNA (Dharmacon). Nucleofection was performed using program T-016 on a Nucleofector II device (Amaxa Biosystems). Cells were collected for analysis 48 hours post-nucleofection.

**Real-time PCR**

FO and MZ B cells were FACS-sorted as described above and stimulated with 2 µg/ml BAFF for 12 hours. Stimulated cells were lysed in TRIzol reagent (Invitrogen, Carlsbad, CA) and total RNA was isolated using the manufacturer’s protocol. cDNA was synthesized using 500 ng RNA, oligo(dT)20 primers, and a cDNA first strand synthesis kit (Invitrogen). cDNAs were amplified using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900T Real Time PCR system (Applied Biosystems), and were normalized against GAPDH as an internal control. Data were expressed as fold increase over unstimulated controls. Oligos used for real time PCR were as follows: Bel-2 (sense: ATGCCTTTGTGGAACTATATGGC; antisense:
GGTATGCACCCAGAGTGATGC), Bcl-xL (sense: AGGCGATGAGTTTGAACTGC;
antisense: AAAGCTCTGATACGCGGTCC), Cyclin D1 (sense: GCGTACCTGACACCAATCTC;
antisense: CTCTCTTCGCACTTCTGCTC), and Cyclin D2 (sense: GAGTGGGAACTGGTAGTGTTG;
antisense: CGCACAGAGCGATGAAGGT).

_Td and Ti humoral responses_

For assessment of Td responses in mice, 100 µg NP-CCG (Biosearch Technologies,
Novato CA) was mixed 1:1 with Imject alum (Pierce) and administered intraperitonally
(i.p.). Spleens and serum were harvested at 11 days post-immunization. Germinal center
B cells in the B220+ splenic population were assessed using anti-GL7-FITC and anti-
Fas-biotin (both from eBioscience), followed by Streptavidin-CyC (Becton Dickinson).
The production of high and low affinity anti-NP antibodies was assessed by applying
serum to ELISA plates coated with either NP<sub>30</sub> or NP<sub>3</sub> (Biosearch Technologies).
Detection was performed using the secondary reagents described above for serum Ig
ELISAs. For assessment of Ti responses in mice, 30µg NP-Ficoll (Biosearch
Technologies) was administered i.p., and spleens and serum were harvested and analyzed
at 7 days post-immunization as described for Td responses.

_Immunohistochemistry and immunofluorescence_

Immunohistochemistry and immunofluorescence were performed according to standard
protocols. Briefly, spleen sections were stained with PNA-biotin (Vector Laboratories)
plus anti-B220-FITC to detect GCs, or with MAdCAM-1-biotin (eBioscience) plus anti-
B220-FITC to visualize the MZ. Sections were washed and incubated with streptavidin-HRP (Prozyme, San Leandro, CA) and anti-FITC-AP (Roche, Laval, QC). Colorimetric development was achieved using Novared HRP and Alkaline Phosphatase Substrate development kits (Vector Laboratories) according to the manufacturer’s protocols. Deposition of various Ig isotypes in the kidneys was visualized by staining with anti-IgA-FITC, anti-IgM-FITC or anti-IgG FITC (Becton Dickinson). Images of stained sections were acquired using a Leica DM-R digital fluorescent microscope and Openlab software.

**Bone marrow chimeras**

Recipient mice were lethally irradiated by two exposures to 550 rad γ-irradiation. Mixed donor bone marrow cells (BMC) (1x10⁶) were injected into the tail veins of the irradiated recipients and the transplanted mice were fed drinking water containing 2mg/ml neomycin sulfate (Bioshop Canada Inc.) for 2 weeks. After 10-12 weeks of bone marrow reconstitution, analyses of serum Ig and B cell subsets, immunohistochemistry and immunofluorescence were conducted as described above.

**Generation of mms2 flanking probe for southern blotting**

The flanking probe for mms2 was isolated using PCR of ES cell genomic DNA mediated by Hot Star Taq polymerase (Qiagen, Mississauga, ON). Amplification was conducted using the following mms2-specific oligos: forward (5’-ACA CCA GA CAG TA CAG TAT ACC-3’); reverse (5’-GTA CTC ACG AGT ACG TTT CTC TTG-3’). Conditions for amplification were as follows:

95°C 15 minutes
95°C  30 seconds  
58°C  15 seconds  x 35 cycles  
72°C  45 seconds  
72°C  10 minutes  
4°C  Hold

An approximately 600 bp PCR fragment was cloned into pCR2.1-TOPO using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). Positive clones were selected using blue/white screening and confirmed by sequencing. The mms2 flanking probe was excised from the vector by overnight digestion with EcoRI, and extracted using the QiaQuik Gel extraction kit (Qiagen). Purified probe (25 ng) was radiolabelled with 50µCi of Easytides Deoxyctydine 5-triphosphate [α-32P] (Perkin Elmer, Boston, MA) using the Amersham Rediprime Random Prime Labelling System (GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s suggested protocol, and used directly in southern blot analyses.

Southern blotting
Genomic DNA was isolated from mouse tails by overnight digestion with proteinase K at 55°C, followed by phenol/chloroform extraction and cold ethanol/sodium acetate precipitation. DNA was quantified using a NanoDrop Spectrophotometer (Wilmington, DE). A total of 10 µg DNA was incubated overnight at 37°C with 100 units of BglII (New England Biolabs) and digested DNA was fractionated on a 0.8% Agarose gel. The gel was washed for 1 hour at RT in solution I (0.5M NaOH, 1.5M NaCl), extensively rinsed in water, incubated at RT for 1 hour with constant rotation in solution II (1.0M Tris pH 7.0, 1.5M NaCl), and finally incubated for 10 minute in 2XSSC solution (30 mM
Sodium Citrate, 300 mM NaCl). DNA was transferred onto Hybond N+ nitrocellulose membranes (GE Healthcare) overnight at room temperature. Membranes were UV-crosslinked at 120,000 µJoules using a UV Stratalinker 2400 (Stratagene, La Jolla, CA) and incubated in Salmon Sperm Pre-hybridization buffer (Pre-Hyb; 1M NaCl, 50 mM Tris pH 7.5, 10% dextran sulphate, 1% SDS, 250 µg/ml salmon sperm DNA) for 3 hours at 65°C with constant rotation. Radiolabelled probe (25 ng) was added to membranes in Pre-Hyb buffer followed by incubation overnight at 65°C with constant rotation. Membranes were washed for 1 hour each in wash solution I (2xSSC, 0.1% SDS), wash solution II (0.5xSSC, 0.5% SDS) and wash solution III (0.1xSSC, 1% SDS). Washed membranes were exposed overnight using a photobleached enhancer screen in a closed radiography cassette. Southern blots were visualized using a Typhoon Tri Variable Mode Phosphoimager and analyzed by Image Quant software (GE Healthcare).

In vitro Ig class switching analysis

B220+ B cells were isolated from spleens using negative depletion as described above. Purified B cells were cultured in R10 medium supplemented with 5 ng/ml IL-4 (Peprotech) and 25 ng/ml LPS (Sigma) at a density of 10^6/ml for 4 days. Cells were then stained with anti-IgG1-FITC (Becton Dickinson), collected on a FACSCalibur, and analyzed using FloJo software.

Thymocyte death assay

Mice (6-10 weeks old) were sacrificed and their thymi were extracted. Single cell suspensions were obtained by mashing thymi through steel mesh. Thymocytes were
cultured in R10 medium at a density of 10⁶ cells/ml, and subjected to 0, 2, or 4 Gy γ-irradiation. After 24 hours, cell viability was assessed by Annexin-V/PI staining as described above.

*Generation of the pCMV-Bcl10-TAP vector*

Human Bcl10 was amplified from a Jurkat cDNA library using the Expand High Fidelity PCR system (Roche, Mannheim, Germany) and a nested approach. Initially, an external forward oligo (5’-CCGGAAGGACGCCATCCCGCC) and an external reverse oligo (5’-GTCACCGTGGTTTGTATAGAGATGCC) were used for amplification under the following conditions:

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<tr>
<th>Temp (°C)</th>
<th>Time</th>
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<tbody>
<tr>
<td>94</td>
<td>2 min</td>
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<tr>
<td>94</td>
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<td>59</td>
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<td>60 sec</td>
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<td>72</td>
<td>7 min</td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
</tr>
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</table>

A total of 2 µl from this PCR reaction was used as the template for a second PCR. A reverse oligo with a BspE1 restriction site (5’ – GGGTCCGGAGAGGCTCCGCCCCTCCGTCC) and a forward oligo with a BamH1 restriction site (5’ – GGGGGATGCTCATGGGGTAAAGAGCCCG) was used to amplify Bcl10 using the conditions outlined above, except that annealing was at 65° C.
The PCR fragment was then cloned into pCR2.1-TOPO as described above and its sequence confirmed. Bcl10 was excised from TOPO using sequential BamH1 and BspE1 digestion and gel-extracted. pCMV-TAP (the kind gift of E. Izaurralde and M. Wilm, EMBL) was also digested with BamH1 and BspE1, dephosphorylated at 37°C for 30 minutes using 2.5 units calf intestine alkaline phosphatase (Roche), and gel-extracted. Bcl10 was ligated into pCMV-TAP using the DNA Rapid Ligation kit (Roche).

**Tandem affinity purification (TAP) of Bcl10**

pCMV-TAP or pCMV-Bcl10-TAP (2 µg) was nucleofected into Jurkat cells using Transfection Solution V and program X-001 on a Nucleofector II device (Amaxa Biosystems). At 48 hours post-nucleofection, cells were counted and a limiting dilution was performed in 1 mg/ml G418 in R10 medium. Individual clones were tested or Bcl10-TAP expression using anti-CBP antibody? explain?. Positive clones were expanded, and pCMV-TAP- or pCMV-Bcl10-TAP-expressing cells (5 x 10⁷) were left unstimulated, or stimulated with 10 ng/ml PMA plus 50 ng/ml ionomycin for 30 minutes at 37°C. Cells were washed in ice cold PBS and lysed using 3 freeze/thaw cycles at -80°C in TAP lysis buffer (10 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.1%NP-40, 1 mM NaF, 1 mM Na₃VO₄, complete protease inhibitor cocktail tablet). IgG-sepharose beads (Amersham) (80 µl) were washed 3 times in 1 ml TAP lysis buffer. Lysates were added to washed Ig-Sepharose beads and incubated with rotation at 4°C for 3 hours. Beads were centrifuged at 12,000 rpm for 1 minute at 4°C, washed twice with TAP lysis buffer, and twice with 1X TEV protease buffer (10 mM Tris pH 8.0, 100 mM NaCl, 0.1% Triton X-100, 0.5 mM EDTA, 10% glycerol). Beads were then resuspended in 200 µl TEV
protease buffer with 30 units TEV protease (Invitrogen) and incubated overnight at 4°C with constant rotation. Calmodulin-Sepharose beads (80 µl) were washed twice with calmodulin binding (CB) buffer (10 mM Tris pH 8, 100 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM imidazole, 1 mM Mg-acetate). TEV cleaved supernatants from IgG beads were combined with the pre-washed calmodulin beads and CB buffer supplemented with 2mM CaCl₂ was added to a total volume of 1 ml. Beads were incubated at 4°C with rotation for 3 hours, washed 3 times with CB buffer, and complexes were eluted by incubating beads for 10 minutes in CB buffer supplemented with 1 mM EGTA. Beads were collected by centrifugation and supernatants were subjected to immunoblotting analysis as described above.

Statistical analyses

Statistical analyses were conducted using the Student’s t test for variance.
III. CHAPTER 3 – The Role of MALT1 in BAFF-induced non-canonical NF-κB signal transduction and cell survival

MALT1<sup>−/−</sup> mice have a defect in MZ and B1 B cell development, and serum Ig levels without any gross abnormalities in anti-IgM induced signal transduction or proliferation. Because BAFF-Tg mice have a specific increase in MZ and B1 B cells, and serum Ig, we speculated that MALT1 may play a role in BAFF mediated signal transduction. The results of these studies are reported in this chapter. This work was conducted solely by the author of this thesis.

i. MALT1 is dispensable for B cell proliferation, IκBα degradation and NF-κB activation induced by BCR engagement

To clarify whether MALT1 is required for B cell proliferation triggered by BCR engagement, we stimulated purified B220<sup>+</sup> B cells from WT, MALT1<sup>−/−</sup> and Bcl10<sup>−/−</sup> mice with anti-IgM or anti-CD40 and monitored <sup>3</sup>H-thymidine incorporation. As expected, the proliferation of Bcl10<sup>−/−</sup> B cells was significantly reduced, but no statistically significant difference in proliferation was noted between WT and MALT1<sup>−/−</sup> B cells (Figure 10A to 10C). Anti-IgM stimulation of FO and MZ B cells sorted from MALT1<sup>−/−</sup> mice upregulated B7 similar to that of wild type controls (Figure 10D).
Figure 10. MALT1 is dispensable for B cell proliferation and B7.2 upregulation

A) B220⁺ B cells were isolated from spleens of WT (white bars), Bcl10⁻/⁻ (black bars) and MALT1⁻/⁻ (grey bars) mice and stimulated for 24 or 48 hours with 5 µg/ml anti-IgM. Proliferation was assessed by ³H-thymidine incorporation. Results shown are the mean ± S.D. of 3 replicates/genotype and are representative of 3 independent analyses. B220⁺ B cells of the indicated genotypes were stimulated in vitro with the indicated increasing doses of anti-IgM (B) or anti-CD40 (C). Proliferation was measured using ³H-Thymidine incorporation. D) Splenocytes from the indicated genotypes were cultured for 12 hours with 5 µg/ml anti-IgM, and B7 upregulation was assessed in the MZ and FO compartments as described in Materials and Methods. Red histograms are unstained, green histograms are media treated samples, and blue histograms represent anti-IgM (5 µg/ml; 12 hours) treated samples.
Consistent with the results presented in Figure 4, anti-IgM treatment stimulated IκBα degradation in WT and MALT1−/− B cells but not in Bcl10−/− B cells (Figure 11A). Anti-IgM stimulation of B cells is known to activate the binding to DNA of NF-κB heterodimers containing p65. We assessed the role of MALT1 in this process by performing NF-κB-specific electrophoretic mobility shift assays (EMSAs). Anti-IgM induced the up-regulation of p65-dependent DNA binding in WT and MALT1−/− B cells but not in Bcl10−/− B cells (Figure 11B). Thus, unlike Bcl10, MALT1 is not required for overall B cell proliferation, IκBα degradation, or DNA binding to NF-κB consensus sequences induced by BCR engagement.
Figure 11. MALT1 is dispensable for B cell proliferation and canonical NF-κB activation induced by BCR engagement. A). Purified splenic B cells from WT, Bcl10−/− and MALT1−/− mice were stimulated with 5 µg/ml IgM for the indicated times and lysates were western-blotted to detect IκBα. Actin, loading control. Results shown are representative of 3 independent analyses. B) Nuclear lysates were prepared from unstimulated (-) B cells of the indicated genotypes, or from B cells that had been treated for 8 hours with 5 µg/ml anti-IgM. Lysates were subjected to EMSA to detect up-regulation of binding to NF-κB DNA consensus sequences (p65). Results shown are representative of at least 3 independent analyses.
ii. BAFF-induced p100 phosphorylation and degradation are diminished in MALT1−/− B cells

We next evaluated the role of MALT1 in non-canonical NF-κB signaling. First, we confirmed that MALT1−/− splenic B cells expressed WT levels of BAFF-R (Figure 12A). We then stimulated purified WT and MALT1−/− B220+ B cells for 4 hours with recombinant BAFF and examined NF-κB2 (p100) phosphorylation and degradation. Immunoblotting showed that, in BAFF-stimulated WT B220+ B cells, p100 was degraded to yield the transcriptionally active p52 subunit (Figure 12B, cyt.). In contrast, MALT1−/− B cells failed to generate p52 in response to BAFF (Figure 12B, cyt.). BAFF stimulation also led to the nuclear accumulation of RelB in WT B cells, but not in MALT1−/− B cells (Figure 12B, nuc.). Certain B cell populations are known to be under-represented (but not completely absent) in MALT1−/− mice, in particular CD21highCD23low MZ B cells. To exclude the possibility that the p100 degradation defect was merely due to an absence of certain BAFF-responsive B cell subsets, we used phospho-flow cytometry to assess p100 phosphorylation in unfractionated as well as sorted MZ versus FO B cell populations. Although CD23low B cells were rare in MALT1−/− mice, we were able to gate sufficient numbers of them to confirm that they expressed high levels of CD21 and thus were not T1 cells (Figure 12C). Upon stimulation with BAFF, WT total B cells exhibited an increase in phospho-p100 levels compared to unstimulated controls (Figure 12D, top). This increase was also evident in WT FO B cells (CD23highCD21low) and WT MZ B cells (CD23lowCD21high). However, BAFF-induced p100 phosphorylation was not observed in the MALT1−/− total, MZ or FO populations (Figure 12D, bottom). Thus, MALT1 is...
required for BAFF-induced p100 phosphorylation and degradation in the MZ and FO B cell compartments.
Figure 12. BAFF-induced p100 phosphorylation and degradation are diminished in MALT1\textsuperscript{-/-} B cells. A) Normal BAFF-R expression on B cell subsets. Splenocytes obtained from mice of the indicated genotypes were stained with a cocktail of antibodies to identify B cell subsets, and with or without anti-BAFF-R-biotin to detect BAFF-R expression. B cells were separated based on B220, IgM, CD23 and CD21 expression: T1 = IgM\textsuperscript{high}, CD21\textsuperscript{low}, CD23\textsuperscript{neg}; T2 = IgM\textsuperscript{high}, CD21\textsuperscript{high}, CD23\textsuperscript{+}; MZ = IgM\textsuperscript{high}, CD21\textsuperscript{high}, CD23\textsuperscript{+}; Follicular = IgM\textsuperscript{low}, CD21\textsuperscript{low}, CD23\textsuperscript{high}. B) Reduced p100 degradation and RelB nuclear translocation. Highly purified B220\textsuperscript{+} cells from spleens of WT and MALT1\textsuperscript{-/-} mice were stimulated with 2 µg/ml BAFF for 4 hours. Cytoplasmic lysates were western-blotted to detect p100 and p52, and nuclear lysates were probed with anti-RelB. Actin and USF2, loading controls. Cyt. = cytoplasmic lysate; Nuc. = nuclear lysate. Results shown are representative of 4 independent analyses. C) Altered MZ and FO B cell development. Splenocytes from mice of the indicated genotypes were isolated and stained with anti-B220-APC, anti-CD21-FITC and anti-CD23-PE. The percentages of MZ (CD21\textsuperscript{high}CD23\textsuperscript{-}) and FO (CD21\textsuperscript{low}CD23\textsuperscript{high}) B cell subsets in the B220\textsuperscript{+} population are indicated. D) Reduced p100 phosphorylation. BAFF-stimulated total splenic B cells from WT and MALT1\textsuperscript{-/-} mice were immunostained with anti-CD21-PE and anti-CD23-FITC, fixed and permeabilized. The permeabilized cells were then stained with anti-phospho-p100, which was visualized using anti-rabbit Alexa 467. Results shown are a flow cytometric analysis of phospho-p100 expression. The dotted line indicates the BAFF-induced shift in phospho-p100 levels in WT cells; this shift does not occur in MALT1\textsuperscript{-/-} B cells. Results shown are representative of 5 independent analyses (n=5 mice/genotype).
To further elucidate the role of MALT1 in BAFF signal transduction, we used RNAi-mediated gene silencing to ablate MALT1 expression in BJAB cells, which are a Burkitt lymphoma cell line that expresses BAFF-R but not BCMA or TACI (Thompson et al., 2001). This cell line is also refractory (in terms of p100 phosphorylation), to Mega-APRIL, a cytokine that binds to both BCMA and TACI, but not BAFF-R (M. Tusche, unpublished observation). Introduction of siMALT1 into these cells resulted in near-complete silencing of MALT1 expression (Figure 13A, cyt.) in comparison to cells transfected with scrambled (SCR) siRNA control. In addition, nuclear translocation of RelB in response to BAFF was impaired in BJAB MALT1 knockdown cells compared to the siSCR control (Figure 13A, nuc.). Furthermore, the increase in phospho-p100 induced by BAFF pre-treatment of control siSCR BJAB cells was absent in MALT1 knockdown BJAB cells (Figure 13B). This BAFF-induced phospho-p100 shift was not observed in BJAB cells in which NF-κB2 had been ablated by RNAi-mediated silencing, indicating that the antibody utilized for this analysis was specific (Figure 13B, right panel).

Studies have shown that BAFF treatment of whole splenic B cells inhibits the nuclear translocation of PKCδ (Mecklenbrauker et al., 2004). Therefore, we tested the role of MALT1 in BAFF induced inhibition of PKCδ nuclear translocation. MALT1+/+ and MALT1−/− B cells were stimulated with BAFF in vitro and nuclear lysates were western-blotted to detect PKCδ. No differences in BAFF-induced inhibition of PKCδ nuclear translocation were seen (Figure 13C). Taken together, these data suggest that MALT1 is necessary for BAFF-induced activation of the non-canonical NF-κB pathway, but is dispensable for the regulation of PKCδ localization.
Figure 13. BAFF-induced p100 phosphorylation and degradation are diminished in MALT1−/− B cells. A) BJAB cells were nucleofected with either siSCR (control) or siMALT1 duplexes and cultured for 48 hours. Cells were then collected and stimulated with 2 µg/ml BAFF for 4 hours. Cytoplasmic lysates were western-blotted to detect MALT1, and nuclear lysates were western-blotted to detect RelB. Tubulin and USF2, loading controls. Results shown are each representative of 2 independent analyses. B) MALT1 and NF-κB2 was knocked down in BJAB cells and intracellular phosphoflow to detect p100 phosphorylation in response to BAFF was conducted. Results shown are representative of 3 independent analyses. C) B220+ B cells of the indicated genotypes were left unstimulated or stimulated with 2 µg/ml BAFF for 4 hours. Nuclear lysates were western-blotted to detect PKCδ. No differences were observed.
iii. BAFF-induced survival, anti-apoptotic gene induction and TRAF3 degradation are reduced in MALT1\(^{-/-}\) CD21\(^{hi}\)CD23\(^{lo}\) MZ B cells

BAFF promotes B cell survival by activating the transcription of various anti-apoptotic genes. To assess the role of MALT1 in BAFF-mediated survival, we treated purified B220\(^{+}\) B cells with recombinant BAFF and assessed their viability over time by Annexin/PI staining. After 48 hours of culture with 2 \(\mu\)g/ml BAFF, no differences were observed between WT and MALT1\(^{-/-}\) B cells in either fold increase in survival over unstimulated controls (Figure 14A) or in total percentage of viable cells (Figure 14B). After 72 hours of culture, BAFF-stimulated WT B cells exhibited approximately a 7.5 fold increase in survival over non-stimulated controls, whereas MALT1\(^{-/-}\) B cells showed only a 3 fold increase in survival (Figure 14A). Since protracted (>72 hours) BAFF treatment does not result in expansion of the FO B cell population in culture (Batten et al., 2000), we postulated that the differences we observed at this time point were due to subset-specific effects of BAFF. We therefore isolated FO and MZ B cells from WT and MALT1\(^{-/-}\) mice and examined their viability in the absence and presence of BAFF. Interestingly, we found that MALT1 was dispensable for the BAFF-induced increase in survival of FO B cells but required for increased MZ B cell survival (Figure 14C).

Inhibition of PI3K inhibited BAFF induced survival in both the MZ and FO compartments (Figure 14D). There were no statistically significant differences in the survival of MALT1\(^{+/+}\) and MALT1\(^{-/-}\) MZ B cells or FO B cells stimulated with anti-IgM (Figure 14E). Consistent with these data and with published reports (Batten et al., 2000; Mackay et al., 1999; Schiemann et al., 2001; Weih et al., 2001), BAFF treatment up-regulated mRNAs for Bcl-2, Bcl-x\(_L\), cyclin D1 and cyclin D2 in FACS-sorted FO and
MZ B cells from WT mice but failed to induce these pro-survival factors in MALT1$^{-/-}$ MZ B cells (Figure 14F). In contrast, levels of Bcl-2, Bcl-xL and cyclin D1 in MALT1$^{-/-}$ FO B cells were similar to those in WT controls. Lastly, TRAF3 deficiency in B cells has been shown to augment responses to BAFF stimulation, as well as to expand the MZ compartment (Gardam et al., 2008). These observations suggest that TRAF3 acts as a negative regulator of signals downstream of BAFF-R. We compared TRAF3 levels in WT and MALT1$^{-/-}$ MZ and FO B cells and found that TRAF3 was elevated in unstimulated MALT1$^{-/-}$ MZ cells compared to the WT, but that TRAF3 levels were similar in unstimulated WT and MALT1$^{-/-}$ FO B cells (Figure 14G). Thus, MALT1 is required for BAFF-induced survival, the up-regulation of anti-apoptotic genes, and TRAF3-mediated regulation of BAFF signaling specifically in MZ B cells.
Figure 14. **BAFF-induced survival and anti-apoptotic gene induction are reduced in MALT1−/− B cells.** A). Highly purified B220+ B cells from WT and MALT1+/− mice were cultured for the indicated time points in medium alone or medium supplemented with 2 µg/ml BAFF. Viability was assessed using Annexin V/PI staining. Data are expressed as (A) fold increase in survival over unstimulated controls, and (B) percentage viable cells. Results shown are the mean ± S.D. of 5 mice/genotype and are representative of 3 independent analyses. C) FO B cells (CD21highCD23low) and MZ B cells (CD21lowCD23high) were FACS sorted from splenocytes were stimulated with BAFF and stained to assess viability as in A. Results shown are the mean viability ± S.D. of at least 5 mice/genotype and are representative of 4 independent analyses. D) FO and MZ B cells from the indicate genotypes were pretreated with 5 µM LY294002 or DMSO and incubated with either media or 2 µg/ml BAFF for 48 hours. Survival is expressed as the fold increase in surviving cells as compared to non-stimulated controls. Data is representative of 3 independent analyses. E) FO (top) and MZ B cells (bottom) of the indicated genotypes were stimulated with 5 µg/ml anti-IgM and viability was assessed after the indicated times in culture as for A. Cells from two different mice/genotype were examined. F) FO and MZ B cells from WT and MALT1−/− mice were unstimulated (medium) or stimulated with 2 µg/ml BAFF and total RNA was isolated. cDNAs for Bcl2 (white), Bcl-xL (red), cyclin D1 (blue) and cyclinD2 (green) were amplified by PCR. Results shown are the mean fold increase in specific mRNA induction over unstimulated controls and are representative of 3 independent analyses. G) Resting MZ and FO B cells of the indicated genotypes were western-blotted to detect TRAF3. Results shown are representative of 3 independent analyses.
iv. MALT1 is required for NIK-mediated NF-κB activation and interacts with TRAF3 in the absence of BAFF stimulation

NIK is a critical mediator of non-canonical NF-κB signaling (Shinkura et al., 1999). Although Bcl10 is known to be necessary for NIK-induced NF-κB activation, it is unclear whether MALT1 is also involved in this process. Because levels of NIK in primary cells are extremely low (Gardam et al., 2008; Xie et al., 2007), we used overexpression of NIK and a luciferase reporter system in MEFs to examine NIK function in the absence of MALT1. In WT MEFs, NIK over-expression induced a 50-fold increase in NF-κB activity compared to vector-transfected controls (Figure 15A). However, this increase in NF-κB reporter activity did not occur in NIK-overexpressing MALT1−/− MEFs. To determine whether the kinase activity of NIK required MALT1, we transiently transfected WT and MALT1−/− MEFs with a NIK-expressing plasmid and assessed p100 phosphorylation at serine 866 and 870, residues known to be targeted by NIK(Xiao et al., 2001). WT MEFs exhibited robust up-regulation of phospho-p100 in response to NIK transfection compared to vector-transfected controls (Figure 15B), whereas NIK-mediated phosphorylation of p100 was minimal in MALT1−/− MEFs (Figure 15B). These data indicate that MALT1 is involved in NIK-mediated NF-κB activation.

We observed an increase in TRAF3 levels in unstimulated MALT1−/− MZ B cells. Because TRAF3 is known to be a negative regulator of signals emanating from the BAFF-R, we theorized that MALT1 and TRAF3 could form a complex in a resting state, and that this interaction could be downregulated in the presence of BAFF. To test this hypothesis, we stimulated serum starved BJAB cells with BAFF, immunoprecipitated
lysates of these cells with anti-MALT1 antibody, and probed a western blot of these immunoprecipitated proteins with anti-TRAF3 antibody. In the absence of BAFF, MALT1 interacted with TRAF3, as expected (Figure 15C). However, if BJAB cells received BAFF treatment for 2 or 4 hours, the interaction between MALT1 and TRAF3 was ablated. Interestingly, MALT1 re-associated with TRAF3 in cells that received BAFF stimulation for 24 hours. These data indicate that MALT1 and TRAF3 do form a complex in a manner that is regulated by BAFF.
Figure 15. MALT1 is required for NIK-mediated NF-κB activation and interacts with TRAF3 in the absence of BAFF stimulation. A) Decreased NF-κB activation. WT and MALT1−/− immortalized MEFs were transfected with empty vector or a NIK-expressing plasmid and assayed for NF-κB activation using a luciferase reporter system. Results are expressed as the mean fold increase in luciferase activity ± S.D of triplicate samples and are representative of 4 independent analyses. B) Abrogated p100 phosphorylation. The MEFs in A were western-blotted to detect phosphorylated p100. Results shown are representative of 3 independent analyses. C) BAFF treatment disrupts MALT1-TRAF3 interaction. BJAB cells were serum-starved and stimulated with 2 µg/ml BAFF for the indicated time points. Lysates were immunoprecipitated with anti-MALT1 or anti-TRAF3, and western-blotted to detect the indicated proteins. WCE = whole cell extracts.
In conclusion, we have shown in this chapter that MALT1 is a signalling intermediate downstream of the BAFF-R, and is specifically necessary for the survival of MZ B cells.
IV. CHAPTER 4 – The Role of MALT1 in BAFF function in vivo

Because we observed a defect in BAFF induced signal transduction in MALT1 deficient cells, we sought to establish a model to address the role of MALT1 in BAFF function in vivo. To this end, we generated MALT1−/−xBAFF-Tg mice. The phenotype of this cross is presented in this chapter. This work was done in collaboration with the Dr. Jennifer Gommerman in the Department of Immunology at the University of Toronto and members of her group.

i. MALT1 is required for BAFF-induced increases in serum Ig, MZ B cells and B1 B cells in vivo

Because BAFF-Tg mice exhibit increases in some serum Ig isotypes, we first conducted sandwich ELISAs on serum samples obtained from WT, BAFF-Tg, MALT1−/− and MALT1−/−xBAFF-Tg animals to assess if this altered Ig profile was a MALT1-dependent phenomenon. Bcl10−/− and Bcl10−/−xBAFF-Tg mice, which were expected to be refractory to non-limiting levels of BAFF due to the critical role of Bcl10 downstream of the BCR22,23, were analyzed in parallel as negative controls. The serum of BAFF-Tg mice contained significant increases in IgA, IgM, IgG2c and IgG3 compared to non-transgenic WT controls (Figure 16A). More moderate increases in IgG1 and IgG2b were also observed in these animals. Sera from MALT1−/− and Bcl10−/− mice exhibited a marked reduction in all Ig isotypes analyzed, consistent with published reports22-25. Strikingly, deficiency of either MALT1 or Bcl10 in the BAFF-Tg background led to significant decreases in all isotypes compared with BAFF-Tg controls (Figure 16A). Thus, the enhanced Ig production observed in BAFF-Tg mice requires not only Bcl10 but also
MALT1, and excess BAFF cannot restore the defect in Ig production in Bcl10\(^{-/-}\) and MALT1\(^{-/-}\) mice.

B1 B cells are greatly reduced in Bcl10\(^{-/-}\) and MALT1\(^{-/-}\) mice\(^{22,24}\) and our flow cytometric analysis revealed that Bcl10\(^{-/-}\) mice harbour an unusual population of CD5\(^{low}\)IgM\(^{low}\) cells in the peritoneal cavity (Figure 16B, top row). We asked whether excess BAFF could rescue B1 B cell numbers in the peritoneal cavity of Bcl10\(^{-/-}\) and MALT1\(^{-/-}\) mice. However, deficiency of either Bcl10 or MALT1 in a BAFF-Tg background did not restore normal frequencies (Figure 16B, top; Figure 16C, left) of B1 B cells. Therefore, excess BAFF cannot restore the B1 cell population in Bcl10\(^{-/-}\) and MALT1\(^{-/-}\) mice.

Although a lack of MALT1 did not alter total B220\(^{+}\)CD23\(^{+}\) B cell numbers in the spleen (Table 1), flow cytometric analysis revealed altered homeostasis of the MZ B cell subset (B220\(^{high}\)IgM\(^{high}\)CD21\(^{high}\)CD23\(^{low}\)CD1d\(^{high}\)). Our BAFF-Tg mice showed a greatly expanded MZ B cell compartment (Figure 16B, bottom; Figure 16C, right) as well as a 7-fold increase in the absolute numbers of these cells (Table 1). We asked whether over-expression of BAFF could restore normal numbers of MZ B cells in MALT1\(^{-/-}\) mice. Bcl10\(^{-/-}\)xBAFF-Tg and MALT1\(^{-/-}\)xBAFF-Tg animals showed both a lower percentage (Figure 16B, bottom; Figure 16C, right) of splenic MZ B cells compared to BAFF-Tg mice. No significant differences in MZ B cell frequencies were observed between MALT1\(^{-/-}\) and MALT1\(^{-/-}\)xBAFF-Tg mice, or between Bcl10\(^{-/-}\) and Bcl10\(^{-/-}\)xBAFF-Tg mice. In addition, a marked absence of CD23\(^{low}\)CD21\(^{high}\)CD1d\(^{high}\) B cells was apparent in the Bcl10\(^{-/-}\) background, with or without the BAFF transgene (Figure 16B, bottom; Figure 16C, right). Interestingly, as was observed for the “atypical” CD5\(^{low}\)IgM\(^{low}\)
peritoneal B cell subset, MALT1−/− mice did not display the unusual population of CD23lowCD21low cells that was noted in Bcl10−/− mice (with or without the transgene) (Figure 16B, bottom). Taken together, our data confirm a significant reduction of MZ B cells and B1 B cells in MALT1−/− and Bcl10−/− mice, and demonstrate that over-expression of BAFF cannot rescue these defects. These results further corroborate our findings that BAFF-R signaling requires MALT1.
Figure 16. MALT1 is required for BAFF-induced increases in basal serum Ig, MZ B cells and B1 B cells. A) Decreased serum Iggs. Serum samples from 8-10 week old mice of the indicated genotypes (B-Tg=BAFF-Tg) were analyzed by ELISA to detect the indicated Ig isotypes. Data shown are levels for individual mice (n=3-9/genotype) and the horizontal bar is the mean. Results shown are representative of 4 independent analyses. B) Decreased MZ B and B1 B cells. Top: Peritoneal cells from 8-10 week old mice of the indicated genotypes were pre-gated on B220 and the B1 B cell population was isolated as IgM$^{\text{high}}$CD5$^{\text{high}}$. Bottom: Splenocytes from 8-10 week old mice of the indicated genotypes were pre-gated on B220, CD23 and IgM. The IgM$^{\text{high}}$CD23$^{\text{low}}$ population was isolated and further fractionated to examine MZ B cells (CD21$^{\text{high}}$CD1d$^{\text{high}}$). FACS plots shown are representative of 6 independent analyses. C) Quantitation of B1 and MZ B cells. The cells in B were quantitated and the results expressed as the mean % of B220$^{+}$ cells $\pm$ S.D of 4 independent analyses (n=6-14 mice/genotype).
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*Table 1 – Absolute cell numbers of mice used.*
ii. Impaired T-dependent and T-independent humoral responses in the absence of MALT1

BAFF-R is required for T-dependent (Td) B cell responses, and T-independent (Ti) responses are augmented in BAFF-Tg mice (Mackay et al., 1999). To assess whether MALT1 is important for the effects of BAFF on Td responses, we immunized WT, MALT1^{-/-} and Bcl10^{-/-} mice and their BAFF-Tg counterparts with the Td antigen NP-CGG. NP-CGG immunization induced a modest increase in the frequency of GC B cells (Fas^{+}GL7^{+}) in WT mice (Figure 17A, B). Numbers of GC B cells were already elevated in unimmunized BAFF-Tg mice, with a further increase observed when these mice were immunized (Figure 17A, B). However, even in the presence of excess BAFF (which provokes augmented Td and Ti responses), an absence of MALT1 abrogated both Td and Ti responses, as judged by the frequency and absolute number of total GC B cells (Figure 17A, B). These results were confirmed by measuring titres of high affinity (NP-30) and low affinity (NP-3) antibodies directed against NP-CGG (Figure 17C) or NP-Ficoll (Figure 17D). As expected, Bcl10^{-/-}xBAFF-Tg mice also failed to mount a Td response in the presence or absence of the BAFF transgene (Figure 17A, B). Thus, even though excess BAFF augments Td/Ti responses in a WT background, Bcl10^{-/-} and MALT1^{-/-} mice fail to mount Td/Ti responses and the defect cannot be rescued by BAFF overexpression.
Figure 17. Td and Ti humoral responses are dysfunctional in the absence of MALT1. (A,B) Decreased GC B cells. Mice of the indicated genotypes (n=4/genotype) were left unimmunized (unim) or immunized (imm) with the Td antigen NP-CGG. GC B cells in the spleens of these mice were detected by immunostaining plus flow cytometry. The quantification of the results of two such independent analyses are shown in (B). Decreased Igs in response to Td (C) and Ti (D) antigens. Serum samples from the mice in A and from mice immunized with the Ti antigen NP-Ficoll were analyzed by sandwich ELISA for the presence of high affinity (NP-30) and low affinity (NP-3) anti-NP antibodies. One of two representative experiments is shown (n=4/genotype for each experiment). For B-D, white bars represent unimmunized controls and black bars represent immunized mice.
iii. B cell-intrinsic expression of MALT1 is required for BAFF induced increases in serum Ig production in vivo

It is possible that the failure to recover Td responses in MALT1<sup>−/−</sup> x BAFF-Tg mice could have been due to an absence of T cell help, since MALT1<sup>−/−</sup> mice have a profound defect in T cell activation (Ruland et al., 2003). Likewise, the T cell defects in MALT1<sup>−/−</sup> mice could explain the low serum Ig production in these mutants (Fig. 16A). To dissect the functions of MALT1 in T cells versus B cells, we carried out mixed bone marrow (BM) chimera experiments in Jh<sup>−/−</sup> x BAFF-Tg recipients. Jh<sup>−/−</sup> mice contain no B cells due to a defect in the Ig heavy chain locus, but T cell numbers and functions are normal in these animals. We mixed WT, Bcl10<sup>−/−</sup> or MALT1<sup>−/−</sup> BM with Jh<sup>−/−</sup> BM and injected it intravenously into lethally-irradiated Jh<sup>−/−</sup> x BAFF-Tg hosts to generate chimeric mice with WT T cells and WT, Bcl10<sup>−/−</sup> or MALT1<sup>−/−</sup> B cells. We then analyzed serum Igs in these mutants and found that introduction of mixed WT+Jh<sup>−/−</sup> BM → Jh<sup>−/−</sup> x BAFF-Tg recipients resulted in a significant increase in the production of all Ig isotypes compared to WT → WT controls (Figure 18). However, MALT1<sup>−/−</sup>+Jh<sup>−/−</sup> → Jh<sup>−/−</sup> x BAFF-Tg mice exhibited a significant decrease in all Ig isotypes compared to WT+Jh<sup>−/−</sup> → Jh<sup>−/−</sup> x BAFF-Tg mice, as did Bcl10<sup>−/−</sup>+Jh<sup>−/−</sup> → Jh<sup>−/−</sup> x BAFF-Tg mice. Thus, MALT1 expression in B cells is required for the steady-state production of Ig, and the elevated Ig titres observed in BAFF-Tg mice, in particular IgA, require the expression of MALT1 in B cells.
Figure 18. B cell-intrinsic expression of MALT1 is required for BAFF induced elevation of serum Ig production. Mixed bone marrow chimeras were generated with indicated donor bone marrow and lethally irradiated recipient mice, as described in the Materials and Methods. At 10 weeks post-transfer, serum was collected and subjected to ELISA to measure serum Ig levels.
iv. BAFF-induced GC formation in the spleen and Ig deposition in the kidney
require B cell-intrinsic MALT1

BAFF-Tg mice exhibit a high frequency of PNA+ GC networks in the absence of immunization, a phenomenon we also observed (Figure 19A, top row) (Mackay et al., 1999). In contrast, no PNA+ cells were identified in sections of spleens from MALT1−/− xBAFF-Tg animals, indicating that the formation of BAFF-induced spontaneous GCs requires MALT1. Similar results were obtained for Bcl10−/−xBAFF-Tg mice. An increase in PNA+ cells was also observed in sections of spleen from WT+Jh+/− BM → Jh−/−xBAFF-Tg recipients but not in spleens of either MALT−/− or Bcl10−/− BM → Jh−/−xBAFF-Tg recipients (Figure 20A, top row). In addition, BAFF-Tg spleens (from either the WT or mixed BM chimeric backgrounds) that were stained with MAdCAM-1 to delineate the MZ sinus showed that the MZ was increased in size (Figure 19A, bottom row). This MZ expansion was not observed in MALT1−/−xBAFF-Tg or Bcl10−/−xBAFF-Tg mice, or in MALT1−/− and Bcl10−/− mixed BM chimeras (Figure 20A, bottom row). A disruption in the MZ sinus that was apparent in Bcl10−/−xBAFF-Tg mice was not present in MALT1−/− xBAFF-Tg animals (Figure 19A, bottom row).

BAFF-Tg mice exhibit mild glomerulonephritis characterized by Ig deposition in the kidney mesangium (Mackay et al., 1999). Using immunofluorescence, we detected deposits of IgM, IgG and IgA in sections of kidneys from BAFF-Tg animals and WT+Jh+/− BM → Jh−/−xBAFF-Tg recipients (Figure 19B, 20B). These Ig deposits were not present in kidney sections obtained from MALT1−/−xBAFF-Tg, Bcl10−/−xBAFF-Tg, MALT1−/−
+Jh^+/ BM → Jh^-xBAFF-Tg, or Bcl10^-/ + Jh^-BM → Jh^-xBAFF-Tg mice. Thus, the major histological abnormalities associated with BAFF over-expression are B cell-intrinsic and require MALT1 for their manifestation.
Figure 19. **BAFF-induced GC formation in the spleen and Ig deposition in the kidney require MALT1.** A) Spleen sections from mice of the indicated genotypes were immunostained to detect B220 (blue) plus the GC marker PNA (red; top), or B220 (blue) plus the MZ sinus marker MAdCAM-1 (red; bottom). B) Kidney sections from the mice in A were stained with FITC-conjugated antibodies specific for the indicated Ig isotypes and visualized using fluorescence microscopy. For A and B, images shown are representative of 4 independent analyses of at least 8 mice/genotype.
Figure 20. B-cell intrinsic expression of MALT1 is required for BAFF induced GC expression and Ig deposition in the kidneys. A) Spleen sections from mice of the indicated bone marrow chimeric mice were immunostained to detect B220 (blue) plus the GC marker PNA (red; top), or B220 (blue) plus the MZ sinus marker MAdCAM-1 (red; bottom). B) Kidney sections from the mice in A were stained with FITC-conjugated antibodies specific for the indicated Ig isotypes and visualized using fluorescence microscopy. For A and B, images shown are representative of 4 independent analyses of at least 8 mice/genotype.
In this chapter, we have shown, in collaboration with the laboratory of Jennifer Gommerman, that MALT1 is necessary for many of the effects of BAFF overexpression \textit{in vivo}.
V. CHAPTER 5 – The in vitro and in vivo role of the E2 ubiquitin ligase co-factor

**Mms2**

This work was conducted solely by the author of the thesis.

A trimolecular complex of Bcl10, MALT1, and CARMA1 has been shown biochemically and genetically to be a critical regulator of NF-κB activation downstream of TCR and BCR engagement (Thome, 2004). To identify novel proteins interacting with this complex, we conducted a Tandem Affinity Purification of Bcl10 from Jurkat cells stably transfected with pCMV-Bcl10-TAP. Bcl10 tagged with TAP activated NF-κB-sensitive luciferase activity to the same level as untagged Bcl10 (M. Tusche, unpublished observation). In agreement with the studies of Zhou and colleagues (Zhou et al., 2004), we identified Uev1a as an interacting partner of Bcl10 (Figure 21).
**Figure 21. Uev1a physically associates with Bcl10.** TAP purification. Jurkat T cells stably transfected with either pCMV-Bcl10-TAP or pCMV-TAP were stimulated for 30 minutes with PMA/ionophore. TAP purification to isolate Bcl10 was conducted as described in the Materials and Methods, and TAP elutants were western-blotted to detect the indicated proteins.
We hypothesized that Mms2, a molecule that is structurally similar to Uev1a, may play a role in Bcl10-induced NF-κB signalling. Therefore, we overexpressed Mms2 in 293T cells in the presence or absence of Bcl10 and carried out NF-κB-dependent luciferase reporter assays. Bcl10 overexpression alone resulted in a robust upregulation of luciferase reporter activity, whereas Mms2 overexpression alone did not produce significant levels of luciferase signals (Figure 22A). Intriguingly, co-expression of mms2 with Bcl10 resulted in a dose-dependent downregulation of NF-κB-dependent reporter activity. We repeated these experiments using tumour necrosis factor α (TNFα), a cytokine known to activate canonical NF-κB signaling. We found that TNFα-induced NF-κB reporter activity was downregulated when mms2 was overexpressed in HEK293T cells (Figure 22B).

We next explored whether the negative inhibitory effect that mms2 appeared to exert on the Bcl10 pathway in any way impinged on Bcl10-induced ubiquitination of NEMO. Polyubiquitination of NEMO by the Bcl10 complex is necessary for canonical NF-κB activation (Zhou et al., 2004). We transfected HEK293T cells with an HA-tagged ubiquitin construct, along with Bcl10 or mms2, alone or in tandem. NEMO was then immunoprecipitated and the immunoprecipitates were western-blotted to detect the HA epitope. As shown in Figure 22C, overexpression of Bcl10 alone induced high levels of polyubiquitinated NEMO. However, when mms2 was co-expressed with Bcl10, the polyubiquitination of NEMO was abolished. Mms2 is thus a negative regulator of Bcl10-mediated NEMO polyubiquitination.
Figure 22. Mms2 negatively regulates NF-κB activation by decreasing Bcl10-mediated polyubiquitination of NEMO. A) Mms2 decreases Bcl10-mediated NF-κB activation. HEK293T cells were transfected with NF-κB luciferase reporter vectors either alone, or in combination with an empty vector, or 1 µg vectors expressing Bcl10 or and 10 ng to 1 µg mms2. Amount of DNA was adjusted to 2 µg using empty vector. Luciferase activity was assessed 48 hours later. Final luciferase readings were obtained by subtracting background luciferase readings. Data shown are the mean±S.D. of triplicate samples and are representative of 4 independent analyses. B) Mms2 decreases TNFα-induced NF-κB activation. HEK293T cells were transfected as in A. After 48 hours, these cells were serum-starved for 5 hours, followed by the addition of TNFα to the culture for 6 hours and assessment of luciferase activity as for A. C) Mms2 decreases polyubiquitination of NEMO. 293T cells were transfected with vectors expressing Bcl10, mms2 or HA-ubiquitin (HA-Ub) either alone, or in the indicated combinations. After 48 hours, NEMO was immunoprecipitated from lysates and immunoprecipitates were western-blotted to detect HA (ubiquitin) and NEMO. Results shown are representative of 4 independent transfections and indicate that Bcl10 expression induces ubiquitination of NEMO that is suppressed in the presence of mms2.
It is well known that several negative regulators of NF-κB, including IκBα, are induced by NF-κB, establishing negative feedback loops that prevent prolonged NF-κB signaling. Because our data pointed to a role for mms2 as a negative regulator of NF-κB, we investigated whether Mms2 transcription was dependent on NF-κB signaling. To this end, we conducted RT-PCR analyses of RNA isolated from NEMO⁺/⁺ and NEMO⁻/⁻ immortalized MEFs stimulated for various times with TNFα. NEMO is a critical component of the IKK complex, and NEMO-deficient MEFs completely lack NF-κB signaling induced by TNFα. We found that WT MEFs treated with TNFα for up to 6 hours exhibited robust upregulation of Mms2 mRNA (Figure 23, left). Furthermore, induction of IκBα, a gene whose transcription is known to be controlled by NF-κB, was also observed. The upregulation of neither mms2 nor IκBα was observed in NEMO⁻/⁻ MEFs (Figure 23, right). These data indicate that mms2 induction is dependent on NF-κB signaling.
Figure 23. Upregulation of mms2 mRNA is dependent on NF-κB signaling. NEMO \(^{+/+}\) and NEMO\(^{-/-}\) immortalized MEFs were serum-starved overnight prior to stimulation with 10 ng/ml TNF\(\alpha\) for the indicated times. Total RNA was prepared from lysates and subjected to RT-PCR analysis to detect mms2 and \(\mathrm{I\kappaB}\alpha\) mRNA expression. GAPDH, loading control. Results shown are representative of 3 independent analyses.
To determine the *in vivo* role of Mms2 in NF-κB signaling, we generated a targeted deletion of the mms2 gene using conventional methods of homologous recombination in murine ES cells. A construct was created that targeted exons 3 and 4 of the murine *mms2* locus and was predicted to cause a frameshift mutation and a premature stop codon (Figure 24).
Figure 24. Targetting of the murine mms2 locus. A genomic Mms2 clone was isolated from a 129J library and was used to construct a targeting vector according to standard methods. Briefly, exons 2 and 3 were replaced by a neomycin resistance cassette (neo), causing a frameshift mutation and a premature stop codon. B, BglI site; F, flanking probe.
The targeting construct was electroporated into 129J ES cells, and recombinants were selected in G418. Positive clones were confirmed by PCR and southern blot using a flanking probe (Figure 25A) or a neo-specific probe (Figure 25B), the latter confirmed a single site of integration of the targeting construct. Chimeric pups were bred into the C57BL/6 background and germline transmission of the mutant allele into progeny was confirmed by southern blotting with the flanking probe (Figure 25C).

Mms2<sup>+/−</sup> mice were backcrossed for 6 generations into the C57BL/6 background and were genotyped by PCR (Figure 25D) or southern blotting (Figure 25E) of tail DNA. RT-PCR analyses of cDNA from Mms2<sup>+/+</sup> and Mms2<sup>−/−</sup> ES cells revealed that no Mms2 RNA was present in the knockout cells (Figure 25F). When Mms2<sup>+/−</sup> mice were intercrossed, mms2<sup>−/−</sup> mice were born at the expected Mendelian ratio: ++ - 22.15%; +/- - 49.00%; −− - 28.85%; n = 157. However, by 6 weeks of age, Mms2<sup>−/−</sup> mice appeared runted compared with their mms2<sup>+/+</sup> littermates (Figure 25G).
Figure 25. Generation of the mms2 knockout mouse. The Mms2 targeting construct was electroporated into 129J ES cells and homologous recombinants were selected in G418 and screened for heterozygosity using southern blotting. Genomic DNA from screened clones was digested with BglII and southern-blotted using the flanking probe (A), or a neo-specific probe (B). Correctly targeted ES cells were injected into C57BL6 blastocytes and implanted into pseudopregnant females. Chimeric pups were intercrossed into C57BL6 animals, and germline transmission in the progeny was confirmed by southern-blotting of BglII-digested genomic DNA as for A. (C) * - germline transmission into progeny. Each lane represents a different pup. Heterozygous animals were intercrossed to generate knockout mice, which were assessed by PCR (D), southern blotting (E), and RT-PCR (F). Mendelian ratios (G) were determined from the progeny of heterozygous intercrossings. The weights (G) of males of the indicated genotypes were determined at 6 weeks of age. Horizontal bar, mean weight.
Our previous studies showed that Mms2 is a negative regulator of NF-κB activation induced by Bcl10. Because NF-κB activation downstream of the Bcl10 complex is necessary for immune cell activation and homeostasis, we focused our efforts on analyzing the immune system of the Mms2−/− mouse. We initially assessed the role of Mms2 in lymphocyte proliferation. To this end, we purified T cells and B cells from spleens of Mms2+/+ and Mms2−/− mice by negative depletion. T cells were stimulated with PMA/ionophore, plate-bound anti-CD3, or plate-bound anti-CD3/CD28, and proliferative capacity was assessed using 3H-thymidine incorporation. Under all stimulation conditions tested, no significant differences in proliferation were observed between Mms2+/+ and Mms2−/− T cells (Figure 26A). We then stimulated B cells with anti-IgM, LPS, or anti-CD40 and assessed proliferation. As for T cells, there were no significant differences in proliferation between Mms2+/+ and Mms2−/− B cells (Figure 26B). Therefore, Mms2 is dispensable for lymphocyte proliferation.
Figure 26. Mms2 is dispensable for lymphocyte proliferation. T cells (A) and B cells (B) were purified from the spleens of Mms2\textsuperscript{+/+} and Mms2\textsuperscript{-/-} mice and proliferation in response to stimulation was determined by 3H-thymidine incorporation. T cells were stimulated for 48 hours with plate-bound anti-CD3, anti-CD3/28, or PMA/ionomycin. B cells were stimulated for 48 hours with anti-IgM, LPS or anti-CD40. Results shown are the mean±S.D. of triplicate samples and are representative of 3 independent analyses.
We next examined the role of Mms2 in signal transduction downstream of the TCR. We purified T cells from Mms2\(^{+/+}\) and Mms2\(^{-/-}\) spleens, starved the T cells in Optimem medium for 2 hours, and stimulated the cells with PMA/ionophore for 5 or 45 minutes. We first assessed the phosphorylation/degradation status of IκBα, a well-known indicator of IKK activity downstream of Bcl10. After 5 minutes stimulation, robust upregulation of IκBα phosphorylation was observed in both Mms2\(^{+/+}\) and Mms2\(^{-/-}\) T cells (Figure 27A). After 45 minutes of stimulation, IκBα phosphorylation was downmodulated with similar kinetics to baseline levels in both Mms2\(^{+/+}\) and Mms2\(^{-/-}\) T cells. We then evaluated MAPK kinase activation in Mms2\(^{+/+}\) and Mms2\(^{-/-}\) T cells and found that phosphorylation levels of ERK and p38 (upper band) were upregulated to a similar extent (Figure 27B). Thus, Mms2 is dispensable for signal transduction leading to IKK activity and MAPK activation in T cells.
Figure 27. T cell signaling to IKK and MAPK are normal in the absence of Mms2. T cells were purified from spleens of mms2<sup>+</sup> and mms2<sup>-/-</sup> mice, serum-starved for 2 hours, and stimulated for the indicated times with PMA/ionomycin. Lysates were western-blotted to detect IkBα and phospho-IkBα (A), or phospho-ERK and phospho-p38 (upper band) (B). Total ERK were used as loading controls for A and B, respectively. ns = non-specific band.
We next assessed the role of Mms2 in lymphocyte development using flow cytometry. We first compared thymocyte development in Mms2\(^{+/+}\) and Mms2\(^{-/-}\) mice. Mms2\(^{-/-}\) thymi were similar to those of wild type in appearance, size, and cell number. Thymi were homogenized through steel mesh. Red blood cells were removed and stained with antibody cocktails in FACS buffer as outlined in Materials and Methods. CD4/CD8 profiles in the thymus were similar in Mms2\(^{+/+}\) and Mms2\(^{-/-}\) mice (Figure 28A). To address the DN1 to DN4 progression of thymocytes, we compared the CD25/CD44 staining profiles of thymic CD4-CD8- populations in Mms2\(^{+/+}\) and Mms2\(^{-/-}\) mice but found no significant differences (Figure 28B). When we determined percentages of peripheral T cells by staining cell suspensions from lymph nodes with anti-CD4/CD8 antibodies, no appreciable differences were observed between Mms2\(^{+/+}\) and Mms2\(^{-/-}\) mice (Figure 28C). Furthermore, lymph nodes were similar macroscopically between wild type and knockout. Mature B cells in the periphery were also unchanged by Mms2 deficiency, as determined by B220 staining (Figure 28D). Lastly, the development of MZ and FO B cells spleen was not affected by Mms2 deficiency, based on CD21/CD23 staining of the B220\(^{+}\) population in the spleen (Figure 28E). Wild type and knockout spleens were similar in appearance and contained a similar amount of cells. Therefore, Mms2 appears to be dispensable for T and B lymphocyte development.
Figure 28. Lymphocyte development is normal in Mms2<sup>−/−</sup> mice. Spleens, thymi, lymph nodes and bone marrow were extracted from Mms2<sup>+/+</sup> and Mms2<sup>−/−</sup> mice and cell suspensions prepared. Thymic (A) and lymph node (C) suspensions were stained with anti-CD3, anti-CD4 and anti-CD8 antibodies, and CD4/CD8 expression was assessed on the CD3<sup>+</sup> population by flow cytometry. (B) CD25 and CD44 expression levels were assessed on the CD4<sup>−</sup>CD8<sup>−</sup> population in the thymus. (D) Flow cytometric determination of B220 and IgM expression on bone marrow. E) Flow cytometric determination of CD21 and CD23 expression to evaluate FO and MZ B cell populations in spleen.
Using sandwich ELISA, we compared Ig levels in the serum of Mms2^{+/+} and Mms2^{-/-} mice. Serum IgM levels were comparable but serum levels of IgA, IgG1, and IgG3 were significantly decreased in Mms2^{-/-} mice compared to the WT ($p<0.05$) (Figure 29A). Because we suspected a defect in Ig class switch recombination, we cultured Mms2^{+/+} and Mms2^{-/-} B cells with LPS and IL4 for 4 days to induce class switching in vitro, and then assessed numbers of IgG1+ B cells using flow cytometry. We observed an approximate 2-fold reduction in the percentage of cells able to produce IgG1 antibodies in Mms2^{-/-} cultures (Figure 29B). Therefore, Mms2 is required for efficient Ig class switch recombination.
Figure 29. Mms2<sup>−/−</sup> mice have a defect in class switch recombination. (A) Serum samples prepared from mms2<sup>+/+</sup> and mms2<sup>−/−</sup> mice (3/group) were subjected to ELISA to determine levels of the indicated Ig isotypes. Results shown are individual values (horizontal bar is the mean) and are representative of 3 analyses with 3 mice in each group. B) Decreased Ig class switching in vitro. B cells purified from Mms2<sup>+/+</sup> and Mms2<sup>−/−</sup> mice (2/group) were cultured for 4 days in the presence of LPS plus IL4. The percentage of IgG1<sup>+</sup> B cells was assessed by flow cytometry. Results shown are the mean±S.D. of triplicate samples and are representative of 4 independent analyses.
Mice with defects in DNA repair pathways, such as H2AX and 53BP1 knockout mice, also appear to be runted and have deficiencies in class switch recombination, much like Mms2−/− mice (Celeste et al., 2002; Ward et al., 2003). We therefore determined whether mms2 deficiency impinged on DNA damage responses. To this end, we isolated Mms2+/+ and Mms2−/− thymocytes and subjected them to 2 or 4 Gy γ-irradiation, followed by determination of cell viability using Annexin-V/PI staining (Figure 30). At 24 hours after 2 Gy or 4 Gy irradiation, Mms2−/− thymocytes were less viable than Mms2+/+ thymocytes (11.4% viable vs. 29.1% viable, and 3.4% viable vs. 26.1% viable, respectively). These results implicate Mms2 in signaling pathways that ameliorate DNA damage.

Figure 30. Mms2−/− thymocytes are more sensitive to DNA damage-induced cell death. Thymocytes extracted from Mms2+/+ and Mms2−/− mice (3/group) were left untreated or treated for 24 hours with the indicated doses of γ-irradiation. Cells were cultured for an additional 24 hours before being assessed for viability using Annexin-V/PI staining. Results shown are the mean ± S.D. of triplicate samples and are representative of 4 independent analyses.
VI. CHAPTER 6 - DISCUSSION

The objectives of this thesis were to investigate novel signal transduction pathways that are affected by the CBM complex, and to identify novel means by which the CBM is regulated. To this end, we have shown that MALT1 is necessary for the propagation of signals downstream of the BAFF-R. Furthermore, we have shown in vitro that Mms2 functionally interacts with Bcl10, and downregulates Bcl10 induced NF-κB activation through the inhibition of Bcl10 induced NEMO polyubiquitination. Surprisingly, Mms2 appears dispensible for PMA/Ionophore induced NF-κB signalling, and antigen receptor induced activation. Studies are ongoing to determine whether signalling to NF-kB induced by TNFα, or Toll-like receptor (TLR)-ligands are altered in the absence of mms2. Our data suggests that mms2 plays a role in responses to DNA damage. I shall discuss the implications of these findings here.

i. Role of MALT1 in BAFF effects in vitro and in vivo

Knockout studies have shown that both MALT1 and Bcl10 are essential for canonical NF-κB activation downstream of the TCR (Ruefli-Brasse et al., 2003; Ruland et al., 2001; Ruland et al., 2003). However, unlike Bcl10, MALT1 is largely dispensable for NF-κB activation and proliferation induced by engagement of the BCR or CD40 (Ruland et al., 2003). Although modest defects in signaling downstream of the BCR have been reported for MALT1−/− mice (Ferch et al., 2007), in our hands, these defects do not impair B cell activation in terms of IgM induced proliferation, B7 upregulation, or NF-κB activation (Figure 10-11). Nevertheless, MZ B cells, as well as serum Ig titres, are
significantly decreased in our MALT1−/− mice. Since BAFF over-expression has been reported to drive the expansion of MZ B cells (Bossen and Schneider, 2006), we investigated whether MALT1 plays a role in BAFF signaling.

Our work demonstrates for the first time that MALT1 is critically required for a novel signaling pathway induced by BAFF, and thus for many BAFF-dependent in vivo effects. Specifically, we have shown that BAFF-induced phosphorylation of NF-κB2 (p100) in total B220+B cells, FO B cells and MZ B cells, is dependent on MALT1. Furthermore, we demonstrate that BAFF-induced degradation of p100 and RelB nuclear translocation is diminished in the absence of MALT1, despite the presence of normal B cell proliferation and canonical NF-κB activation. BAFF-induced signaling events were arrested in the BJAB cell line following siRNA-mediated knockdown of MALT1, thus corroborating our knockout mouse findings. Since the BJAB cell line is derived from a human Burkitt’s lymphoma, MALT1 may also play a role in BAFF-mediated activation of NF-κB2 in humans. Based on the block in B cell maturation in the MALT1−/− mice, and the fact that signals downstream of BAFF-R are necessary for B cell survival, we place our new signaling node downstream of BAFF-R, rather than TACI or BCMA which also bind BAFF (Marsters et al., 2000; Ruefli-Brasse et al., 2003; Ruland et al., 2003; Thompson et al., 2001; Thompson et al., 2000; Wu et al., 2000; Yan et al., 2001). This hypothesis is supported by the observation that siRNA-mediated MALT1 knockdown in BJAB cells (which express only BAFF-R, and not TACI nor BCMA (Thompson et al., 2001)) arrests BAFF-induced signal transduction.

Although MALT1 is essential for BAFF-driven NF-κB2 activation in all B cell subsets, the requirement for MALT1 for BAFF-mediated B cell survival is only
manifested in CD21\textsuperscript{high}CD23\textsuperscript{low} MZ B cells. Specifically, in response to BAFF stimulation, MALT1\textsuperscript{−/−} FO B cells exhibited normal survival in vitro accompanied by up-regulation of Bcl-2, BclxL and cyclin D1. However, all of these parameters are MALT1-dependent in MZ B cells. The only exception to this pattern was cyclin D2, which seems to require MALT1 in both the MZ and FO B cell subsets. Thus, presumably there are other cell cycle entry mediators that can compensate for cyclin D2 in MALT\textsuperscript{−/−} FO B cells. Nevertheless, these findings potentially explain why MALT1\textsuperscript{−/−}, as well as RelB\textsuperscript{−/−} and aly/aly mice (a naturally occurring mouse strain that has non-functional NIK), do not precisely phenocopy BAFF\textsuperscript{−/−} mice but share some similarities (Shinkura et al., 1999; Weih et al., 2001). The incomplete phenocopy may arise because the MALT1/NF-κB2 axis induces a survival program that is essential in MZ B cells but redundant in FO B cells. This could be due to the heightened levels of TRAF3 that we observe in MALT1\textsuperscript{−/−} MZ B cells. This BAFF-induced survival program, which involves the up-regulation of Bcl2 family members, may also be elicited in part by the cytoplasmic retention of PKCδ (Mecklenbrauker et al., 2004). Indeed, we have observed that BAFF-induced cytoplasmic retention of PKCδ in MALT1\textsuperscript{−/−} B220\textsuperscript{+} B cells (unsorted B cells, predominantly FO B cells) occurs normally. Future work will be required to investigate whether cytoplasmic PKCδ retention in MALT\textsuperscript{−/−} MZ B cells is impaired. The fact that BAFF-Tg mice have a selective and dramatic increase in MZ B cells with only a modest increase in FO B cells raises the possibility that the MALT1/NF-κB2 survival pathway may be exquisitely sensitive to levels of circulating BAFF, and perhaps more so than alternative survival mechanisms operating in FO B cells (Mackay et al., 1999). The PI3K pathway (Patke et al., 2006) is a possible candidate for the alternative BAFF-dependent
survival pathway that sustains MALT1−/− FO B cells. In support of this concept, we found that pharmacologic inhibition of PI3K abrogated the BAFF-dependent survival of both WT and MALT1−/− FO and MZ B cells (Figure 14). Furthermore, knockin mice harboring a catalytically inactive p110δ subunit of PI3K have a selective defect in FO B cell development, while MZ B cell development is intact. (K. Okkenaug, personal communication).

BAFF-Tg animals exhibit pathological features such as hyperglobulinaemia, spontaneous GC formation, and Ig deposition in the kidneys (Mackay et al., 1999), and we show that this phenotype does not occur in the absence of MALT1. It was known that both Bcl10 and MALT1 are required for TCR signaling, but it was important to determine whether Bcl10 and MALT1 functions were also required in B cells for the appearance of these BAFF-induced effects. Indeed, we showed that BAFF-induced increases in MZ B cells, Ig titres, GC formation and kidney Ig deposition all required B cell-intrinsic expression of MALT1 (and Bcl10). The expansion of the B1 B cell compartment in the peritoneum has also been reported in BAFF-Tg animals, albeit to varying degrees in different studies (Gavin et al., 2005; Khare et al., 2000; Mackay et al., 1999). In our hands, we see reproducible increases in the B1 B cell population in the presence of the BAFF transgene. Although these alterations fail to reach statistical significance, they are ablated in the context of either MALT1 or Bcl10 deficiency. Therefore, the finding that the pathological features associated with excess BAFF are abrogated in mice that do not express B cell-intrinsic MALT1 further supports the hypothesis that BAFF-R signals require MALT1 in vivo.
We have shown that MALT1 is necessary for signals emanating from BAFF-R that lead to the survival of MZ B cells (Figures 10-15). Furthermore, BAFF over-expression cannot rescue the defects that we observe in MALT1−/− mice (Figures 16-18). Likewise, the manifestation of the pathologies associated with BAFF over-expression requires MALT1 (Figure 19-20). Our in vivo findings are particularly compelling given that BCR-mediated proliferation, canonical NF-κB activation, IgM induced B7 upregulation in FO and MZ B cells, and FO and T1 B cell development are intact in MALT1−/− mice (Figure 10). However, cues delivered via the BCR are critical for the survival and differentiation of various B cell subsets (Lam et al., 1997). Bcl10−/− mice exhibit non-functional NF-κB signaling, impaired proliferation, and a CD23low compartment that is dominated by T1 (CD21low) B cells, suggesting an underlying BCR lesion (Figures 10, 11 and 16B). Therefore, it is impossible to tell whether the lack of MZ and B1 B cells in Bcl10−/−xBAFF-Tg mice is due to perturbations downstream of the BCR, or to signaling defects in the BAFF-induced non-canonical NF-κB pathway. Sasaki and colleagues have reported that constitutively active canonical NF-κB signaling in B cells can rescue MZ and FO B cell development in a BAFF-R−/− background (Sasaki et al., 2006). The implication of this finding is that, in situations of enforced activation of BAFF- or BCR-initiated pathways, BAFF and BCR signaling activities can compensate for one another. Since MALT1−/− mice show functional anti-IgM-induced proliferation, we attribute the defects in MZ and B1 B cell development in MALT1−/− and MALT1−/− xBAFF-Tg mice to impaired BAFF signaling. However, the possibility exists that an unknown factor is non-functional in MALT1−/− mice and generates a phenotype that cannot be rescued by BAFF over-expression. The reported block in MALT1−/− mice of the
activation of c-Rel-containing NF-κB heterodimers in response to BCR signaling could represent such a possibility (Ferch et al., 2007).

Gardam and colleagues (Gardam et al., 2008) showed that B cell specific ablation of TRAF2 and TRAF3 results in a phenotype similar to that of BAFF-Tg animals, in that MZ B cell accumulation and NF-κB2 degradation are enhanced. These workers proposed the existence of a TRAF2-TRAF3 axis that negatively regulates a signaling pathway that emanates from BAFF-R and leads to B cell survival. We have observed that TRAF3 is elevated in MZ B cells obtained from a MALT1+/− animal, perhaps providing a mechanism by which MALT1 regulates signals specifically in the MZ. Furthermore, we have shown that BAFF negatively modulates the interaction of TRAF3 with MALT1 in BJAB cells. Interestingly, MALT1 can use its C-terminal paracaspase domain to interact with TRAF2 (M. Tusche, unpublished observations). Therefore, MALT1 may potentiate TRAF2-mediated down-modulation of TRAF3 in a signaling pathway downstream of BAFF-R. Such antagonism could result in NF-κB2 activation and the initiation of a B cell survival program that involves Bcl2 family members and possibly cytoplasmic retention of PKCδ. Since mice with B cell-specific ablations of TRAF2/3 have an expanded MZ B cell compartment, it may be that MZ B cells are particularly sensitive to TRAF3 degradation and respond by activating the MALT1/NF-κB2 axis. Indeed, TRAF3 levels are low in FO B cells (Gardam et al., 2008), suggesting that TRAF3 may be more relevant for the “fine-tuning” of BAFF-R signals in the MZ compartment than in the FO compartment. When TRAF2/TRAF3 doubly deficient mice are crossed into the BAFF−/− background, MZ B cells are restored to full function (Gardam et al., 2008). This finding indicates that TRAF2/3 degradation occurs downstream of BAFF-R signaling. FO B cells
are also partially rescued in TRAF2-/-TRAF3-/-BAFF-/- mice, suggesting that TRAF3 degradation is sufficient for FO B cell survival. However, since these results have been obtained in the absence of any other BAFF-R mediated survival signals (such as PI3K activation), we cannot conclude that TRAF2 and TRAF3 are essential for WT FO B cell survival. Nevertheless, a signaling pathway downstream of BAFF-R in MZ B cells is beginning to emerge: we propose that the triggering of BAFF-R results in a MALT1-dependent targeting of TRAF2/TRAF3 degradation in MZ B cells. The resulting downregulation of TRAF3 through proteosomal degradation promotes the activation of NF-κB2 and the initiation of a cell survival program. It has been postulated that TRAF2/TRAF3 may act to promote NIK-mediated ubiquitination and degradation (Gardam et al., 2008). Interestingly, we have found that BAFF negatively regulates MALT1 interaction with endogenous TRAF3 in BJAB cells (Figure 15C). Therefore, we propose that in the absence of positive signal through the BAFF-R, MALT1 sequesters TRAF3. BAFF-R signaling dissociates this complex, allowing for TRAF2 mediated down-modulation of TRAF3 in a signaling pathway downstream of BAFF-R, resulting in NF-κB2 activation and the initiation of a B cell survival program. Given that TRAF3 is elevated in MALT1-/- B cells, perhaps TRAF2 is dependent on MALT1 for the ubiquitination and subsequent degradation of TRAF3. The interaction of TRAF2 with the paracaspase domain of MALT1 that we observe lends credence to this hypothesis. Our data suggest that NIK activity is compromised in MALT1-/- cells, which could be due to their elevated TRAF3 levels. Thus, NIK may represent the intermediary kinase in a BAFF-R signaling cascade that is positioned between MALT1-dependent TRAF3 downregulation and activation of NF-κB2.
Both Bcl10 and MALT1 are frequently involved in translocations associated with extranodal MZ B cell lymphomas of mucosa-associated lymphoid tissues (MALT lymphomas) (Akagi et al., 1999; Dierlamm et al., 1999; Willis et al., 1999; Zhang et al., 1999). However, mice transgenic for the API2-MALT1 translocation do not develop lymphoma after 50 weeks (Baens et al., 2006), implying that the presence of the fusion protein alone is not sufficient to drive neoplastic progression. BAFF levels are elevated in the serum of patients with a variety of B cell malignancies, and autocrine BAFF production is crucial for the survival of some B cell lymphomas (Fu et al., 2006; Ng et al., 2005). One possibility is that MALT lymphomagenesis is driven by MALT1 translocations because they deregulate BAFF activity. It would be intriguing to investigate whether MALT lymphoma patients have elevated BAFF levels in their serum.

In conclusion, we have shown both in vitro and in vivo that MALT1 is crucial for many of the biological effects mediated by BAFF. Our results suggest that MALT1 is a critical intermediate in a novel signaling pathway that emanates from BAFF-R and drives the survival and expansion of MZ B cells. MALT1 may thus represent an attractive target for therapeutic intervention in the treatment of lymphoma or autoimmunity.

ii. Mms2 as a negative regulator of NF-κB activation

The CBM complex has emerged as a critical signaling component that acts downstream of diverse receptors to trigger the activation and subsequent nuclear translocation of NF-κB heterodimers. Furthermore, deregulation of the CBM complex, such as is caused by chromosomal translocations involving Bcl10 and MALT1, can
promote the pathogenesis of various neoplastic states through the constitutive activation of NF-κB. Therefore, studies aimed at identifying novel regulators of the CBM complex could shed light on the fine molecular details of NF-κB signaling, as well as reveal molecules of therapeutic relevance.

We conducted a biochemical purification of Bcl10 from Jurkat T cells stably expressing Bcl10-TAP. In agreement with a published report (Zhou et al., 2004), we identified Uev1a as an interacting partner of Bcl10 (Figure 21). Uev1a is thought to be a critical co-factor for Ubc13, which catalyzes the lysine -63 polyubiquitination of NEMO (Zhou et al., 2004). Uev1a shares a high homology with another E2 ubiquitin ligase, Mms2 (Andersen et al., 2005). Therefore, we postulated that Mms2 might impinge on NF-κB signalling downstream of the CBM complex. Interestingly, Mms2 overexpression inhibited Bcl10- and TNFα-induced activation of NF-κB-sensitive luciferase constructs in a dose-dependent manner (Figure 22A, B).

We speculated that the mechanism by which Mms2 inhibits the transduction of signals culminating in NF-κB activation was through the inhibition of NEMO polyubiquitination. Both Mms2 and the related protein Uev1a can interact with UBC13 to form E2 ubiquitin ligase heterodimers that catalyze lysine-63 dependent polyubiquitination, depending on the cellular context. We envisaged a situation in which Mms2 competes with Uev1a for binding to Ubc13, thereby inhibiting Ubc13-Uev1a interaction and impairing the catalytic activity of this heterodimer. Our work has shown that overexpression of Mms2 inhibits Bcl10-mediated polyubiquitination of NEMO, indicating that Mms2 is a negative regulator of this process (Figure 22C). Alternatively, it might be possible that overexpression of Mms2 alters the stoichiometry of factors
within the CBM complex, thereby impinging on their ability to transduce signal to NF-κB.

Several well-known negative regulators of NF-κB activation, including IκBα and A20, are activated by NF-κB heterodimers. We therefore speculated that Mms2 might be an NF-κB-inducible gene. Indeed, we show that Mms2 is strongly induced by TNFα in WT MEFs but not in NEMO−/− MEFs (in which NF-κB cannot be activated) (Figure 23). Interestingly, the kinetics of Mms2 induction differs slightly from those of the classical NF-κB regulator, IκBα. Whereas the peak of IκBα upregulation is reached by 1 hour post-NF-κB activation, Mms2 is only modestly upregulated at 1 hour and is not strongly upregulated until 4 hours post-NF-κB activation. We attribute this discrepancy to the inherent differences in the means by which these factors modulate NF-κB signaling. IκBα interacts directly with promoter-bound NF-κB heterodimers and, by virtue of its nuclear export signal, expels activated NF-κB from the nucleus, thereby dampening NF-κB activation. Rapid IκBα upregulation is thus required to successfully downregulate NF-κB. Mms2, on the other hand, negatively regulates the polyubiquitination of NEMO, thereby inhibiting NF-κB activation at the level of the IKK complex. The negative regulatory function of Mms2 is thus required at a later time point than that of IκBα.

iii. Mms2 as a positive regulator of DNA damage responses

Confusion abounds in the literature over whether Ubc13’s binding partners, mms2 and Uev1a have distinct roles in lysine-63-dependent polyubiquitination. Even in the
most recent of reviews by leaders in the field of NF-κB-dependent signal transduction and polyubiquitination, Mms2 and Uev1a are inaccurately cited as being the same molecule (Chiu et al., 2009). In fact, Uev1a contains a 30 amino acid region in its N-terminus that does not appear in the mms2 protein (Pelzer et al., 2009). Thus, although Uev1a and Mms2 are structurally very similar and bind to Ubc13 to mediate ubiquitination, our work shows that these molecules have divergent physiological functions manifested as differing effects on various cellular processes.

To delineate the roles of these molecules, we have generated knockout mice deficient for either Mms2 or Uev1a. Our studies of the Uev1a knockout mice are beyond the scope of this thesis but shall be touched upon briefly in the Future Directions section. Our Mms2 knockout mouse was created using standard homologous recombination in ES cells (Figure 24). These mutants are born at the expected Mendelian ratio but are slightly runted by weaning age (Figure 25).

Our observation that Mms2 affected the ability of Bcl10 to transduce signals to NF-κB prompted us to focus on the role of Mms2 in lymphocyte signaling and homeostasis. Components of the CBM complex have been shown genetically to be necessary for NF-κB activation downstream of antigen receptors, as well as for various other aspects of lymphocyte development. We postulated that, since Mms2 appears to be a component of the CBM complex, it too could be involved in lymphocyte signaling and homeostasis. Because we found that Mms2 negatively regulates NEMO polyubiquitination and NF-κB signaling in general, we postulated that Mms2−/− lymphocytes might be hyperactivated by antigen signaling, and might overpopulate immune compartments compared to WT controls. Surprisingly, however, we found that
lymphocyte signaling and development proceed normally in the absence of mms2 (Figures 26-28). These data suggest that Mms2 may play a role in lysine-63 polyubiquitination in a cellular context other than lymphocyte homeostasis. Genetic studies defining the phenotypes of mice with T cell- or B cell-specific deletions of Ubc13 suggest that this E2 ubiquitin ligase heterodimer is necessary for lymphocyte development and activation (Yamamoto et al., 2006a; Yamamoto et al., 2006b). The Ubc13 conventional knockout mouse is embryonic lethal due to severely impaired genomic stability, while Ubc13^{+/−} mice exhibit massive defects in LPS signaling and are resistant in models of endotoxic shock, suggesting that multiple signaling pathways depend on Ubc13 (Fukushima et al., 2007). Significantly, proliferation induced by LPS is normal in Mms2^{−/−} B cells. However, preliminary studies suggest that knockdown of Uev1a using siRNA prevents activation of NF-κB by LPS treatment (Andersen et al., 2005). These results imply that UBC13 may require Uev1a, but not Mms2, for the propagation of signals downstream of TLR4 and antigen receptor.

Our in vitro results point towards a function for Mms2 in the negative regulation of Bcl10-mediated NF-κB activation and imply that Mms2 affects lymphocyte signaling downstream of antigen receptor engagement. However, the phenotype of Mms2^{+/−} mice indicates that Mms2 does not play a unique role in the propagation of antigen receptor-derived signals. The reason for this discrepancy between our in vitro and in vivo findings is not clear. We speculate that, in vivo, Ubc13 may require Uev1a, rather than Mms2, to regulate signals derived from antigen receptors. In addition, our observations may be due to inherent differences in NF-κB signaling pathways between human and mouse. Like many other similar reports in the literature, our in vitro studies examined human-derived
cell lines such as HEK293 or Jurkat cells, and perhaps observations obtained from such studies are not translatable to the mouse. Numerous instances of differences in NF-κB signaling between human and mouse are known. For example, several elegant biochemical studies in human cell lines have shown that, to activate NF-κB in T cells, TRAF6 is the E3 ubiquitin ligase that must associate with the E2 ligase complex consisting of Uev1a/UBC13. However, T cell-specific TRAF6 deletions do not impair NF-κB activation downstream of TCR engagement (King et al., 2006). Another example of this discrepancy has been demonstrated in studies of the role of caspase-8 in T cell signaling. Many reports, both in human and mouse, have proposed that caspase-8 is necessary for the propagation of signals downstream of the TCR to NF-κB activation. In humans, the mechanism seems to involve the recruitment of caspase-8 to lipid rafts and its interaction with the CBM complex. However, caspase-8 is not known to interact with lipid rafts or the CBM in murine T cells (M. Pellegrini, personal communication).

Mms2 was originally cloned from a yeast strain that was sensitive to treatment with the potent DNA alkylating agent methanemethylsulfonate (MMS), suggesting that Mms2 plays a role in responses to DNA damage (Broomfield et al., 1998). Furthermore, the Ubc13-Uev1a heterodimer has been shown to activate NF-κB signaling, whereas the Ubc13-Mms2 heterodimer is reported to be necessary for the DNA damage responses (Andersen et al., 2005). We observed that Mms2−/− mice were significantly runted compared to littermate controls, and had significantly less class-switched Ig in their serum. These defects are reminiscent of impairments displayed by mutants with deficiencies in DNA damage repair pathways, such as histone H2AX and 53BP1 knockout mice (Celeste et al., 2002; Ward et al., 2003). Indeed, at the cellular level,
Mms2−/− thymocytes are significantly more sensitive to γ-irradiation-induced death than are their WT counterparts. We suspect that Mms2 may be involved in DNA damage focus formation because recent reports have suggested that Ubc13, Mms2’s binding partner, is necessary for γ-irradiation-induced 53BP1 focus formation at sites of DNA damage (Kolas et al., 2007). Ubc13’s function in this process is the polyubiquitination of the E3 ubiquitin ligase RNF8 (Kolas et al., 2007).

In conclusion, we have identified the E2 ubiquitin ligase Mms2 as a novel interacting partner of Bcl10, and a negative regulator of NF-κB activation in vitro. In vivo, Mms2 is dispensable for lymphocyte activation and development but is important for Ig class switch recombination and DNA damage responses. Our data highlight the divergent roles of Mms2 in NF-κB activation and DNA damage responses.
VII. CHAPTER 7 - FUTURE DIRECTIONS

In this thesis, I have described my work on dissecting the non-canonical and canonical pathways of NF-κB activation, as well delineating the phenotype of the mms2\(^{-/-}\) mouse. In this section, I shall discuss our future plans for deciphering the regulation of the CBM complex during NF-κB activation.

i. MALT1

The first two data chapters of this thesis described our work defining the role of MALT1 in BAFF-mediated processes *in vivo* and *in vitro*. Although MALT1 is a critical signal mediator acting downstream of a wide array of receptors, the exact molecular details as to how MALT1 exerts its effects are not known. Several groups have reported that the catalytic function of MALT1 is critical to its effects *in vitro* but the function of MALT1’s paracaspase domain has yet to be studied *in vivo* (Coornaert et al., 2008; Rebeaud et al., 2008). We have preliminary data acquired from reporter assays that reveal that MALT1\(^{-/-}\) MEFs expressing a MALT1 protein with a mutated paracaspase domain [MALT1(C461A)] show a significant decrease in Bcl10-induced NF-κB activation compared to MALT1\(^{-/-}\) MEFs reconstituted with WT MALT1. Furthermore, MALT1\(^{-/-}\) T cells reconstituted with MALT1(C461A) show impaired PMA/ionomycin-induced IκBα degradation compared to MALT1\(^{-/-}\) T cells reconstituted with WT MALT1. These results suggest that the paracaspase function of MALT1 is crucial for IKK activity and thus NF-κB activation (M. Tusche, unpublished observations). We are currently engaged in a program to generate bone marrow chimeric mice reconstituted with BMC infected with
retrovirus encoding MALT1 WT or MALT1(C461A). We plan to explore the role of MALT1 catalytic activity in lymphocyte homeostasis and activation in vivo.

Thus far, two targets of MALT1 catalytic activity have been identified: A20 and Bcl10. However, whether these factors are truly downregulated in a MALT1-dependent manner is a matter of some debate. To identify other substrates of MALT1-mediated proteolysis, we will employ Stable Isotope Labelling with Amino Acids in Cell Culture, or SILAC. SILAC is a quantitative proteomic technique based on mass spectrometry that reveals differences in the abundance of a particular protein in two different cell populations (Mann, 2006). Briefly, half of the cell population of interest is cultured in medium containing dialyzed serum and amino acids composed of normal (non-radioactive) isotopes (“light” medium). The other half of the cell population of interest is grown in medium containing amino acids labelled with heavy isotopes (“heavy” medium); in our case, we will use medium containing $^{13}$C$_6$ L-lysine and $^{13}$C$_6^{15}$N$_4$ L-arginine. The cells are cultured in their respective media for one week to permit full incorporation of the heavy and light amino acids into cellular proteins. The cells are then lysed and the lysates digested with trypsin to generate peptides. Peptides containing arginine are 6 daltons heavier when derived from cells cultured in the heavy medium compared to the same peptide isolated from cells grown in the light medium. The peptides are fractionated according to hydrophobicity by passage over a C18 reverse-phase column, and analyzed using an Agilent 1100 series HPLC operating in-line with a Thermo-Fisher LTQ-Orbitrap hybrid mass spectrometer. The Mascot database search algorithm is then applied to identify the original proteins.
The attraction of the SILAC technique is that peptides obtained from cells grown under two different culture conditions (heavy and light) can be analyzed simultaneously. Pairs of chemically identical peptides can be distinguished by the mass spectrometer based on the mass differences caused by heavy vs. light isotope incorporation. The ratio of the intensities of various peaks in the mass spectra produced by such peptide pairs accurately reflects the relative abundance ratio for the heavy and light forms of the original protein found in the two cultures. We believe that SILAC is a highly suitable technique for our proposed study of putative MALT1 substrates, and we intend to make use of it on MALT1+/+ and MALT1−/− primary murine T cells, as well as human Jurkat T cells in which MALT1 has been stably knocked down using lentivirally-delivered shRNA. Putative targets identified using the SILAC approach will be tested directly using an in vitro MALT1 proteolysis assay with purified, 35S-labelled MALT1 paracaspase (residues 334-824; MALT1C) fused to a fragment of bacterial gyrase B (MALT1C-gyrase), which allows the formation of MALT1 oligomers. Cleavage products will be assessed using autoradiography. The MALT1C-gyrase fusion protein has been shown to cleave A20, as well as activate IKK, in in vitro assays (Coornaert et al., 2008). Any cleavage of 35S-labelled targets incubated with MALT1C-gyrase will be identified by autoradiography.

The deletion of MALT1 in the germline of mice has effects that are largely limited to NF-κB signal transduction in the immune system, such that these mutants are essentially healthy. If MALT1 does prove to have catalytic activity that is critical to its function in vivo, the therapeutic targeting of MALT1 with small molecule inhibitors could represent a novel mode of therapy for lymphoma or autoimmunity. To increase our
understanding of the basic properties of the MALT1 enzyme, we will determine the
crystal structure of MALT1C using X-ray crystallography. To date, we have purified
milligram amounts of MALT1C and obtained crystals. We also intend to co-crystallize
MALT1C with each putative substrate identified in our SILAC screen and validated in
our in vitro proteolytic assay. We should obtain structural data, such as the geometry of
the MALT1C active site, which will yield insights into the mechanism of catalysis
mediated by this paracaspase.

Based on our crystallographic data, we will use computational chemistry and
molecular modelling to screen for small molecules that can fit into the MALT1C active
site and thereby impair its function. Any potential small molecule inhibitors identified
using this approach will then be tested for their effects on MALT1-mediated cleavage
using the SILAC approach in activated Jurkat cells, as well as by the in vitro proteolysis
assay using MALT1C-gyrase B. It will also be important to determine whether any of the
putative MALT1 inhibitors affects T cell function. To this end, we will pre-treat purified
murine T cells in vitro with a candidate inhibitor, activate these cells by anti-CD3/CD28
treatment, and assess T cell proliferation, IL-2 production, Ca^{2+} mobilization, and
upregulation of T cell activation markers CD25 and CD69.

A useful inhibitor of MALT1 proteolytic function should specifically inhibit the
signalling pathway leading from TCR engagement to NF-κB activation but leave other
TCR-induced pathways untouched. To ensure that a candidate inhibitor affects only NF-
κB activation, we will use western blotting to compare IκBα levels and phosphorylation
status in inhibitor-treated and untreated cells. In addition, we will use EMSA to assess the
effect of a candidate inhibitor on NF-κB’s ability to bind to the appropriate consensus
sequences in DNA. Finally, we will attempt to determine whether treatment with MALT1 inhibitors prevents or slows the development of MALT lymphomas in mouse models. One such model is *Helicobacter heilmanni* infection in mice, in which infected animals develop MALT lymphomas that closely resemble the human disease (Nakamura et al., 2007).

**ii. Bcl10**

The phosphorylation and subsequent degradation of Bcl10 may be another means of negatively regulating the formation of the CBM complex and NF-κB activation downstream of antigen receptors. *In vitro*, non-degradable Bcl10 accumulates in the nuclei of cells (Lobry et al., 2007). A similar observation has been made in human MALT1 lymphoma cells harboring the Bcl10-IgH translocation (Nakamura et al., 2008). Whether the ability of Bcl10 to be phosphorylated and/or degraded is relevant to lymphocyte development or homeostasis is currently not known.

To investigate the role of Bcl10 degradation *in vivo*, we will generate bone marrow chimeric mice reconstituted with Bcl10−/− BMC expressing a non-degradable form of Bcl10 (T81A/T85A). Briefly, we will infect BMC from Bcl10−/− mice with MSCV engineered to express either WT Bcl10 or Bcl10 T81A/S85A. We will evaluate the activation and proliferation of T and B cells that were isolated from these mutants and stimulated *ex vivo* through their TCRs or BCRs, respectively. We will also monitor NF-κB activation in these lymphocytes using the various assays described above. We anticipate that, given the reported role of Bcl10 degradation in the downmodulation of NF-κB signalling, that lymphocytes harboring the non-degradable Bcl10 protein will
exhibit enhanced and prolonged activation of elements in the NF-κB pathway. These cells will also likely show increased proliferation or IL-2 production, and may exhibit nuclear accumulation of non-degradable Bcl10 protein. Lastly, we will use flow cytometry to assess potential lymphocyte expansion in the spleen, thymus or lymph nodes of chimeric mice expressing non-degradable Bcl10.

iii. Mms2

We have shown that Mms2−/− thymocytes are more susceptible to DNA damage than their WT counterparts. In the future, we will clarify the mechanism underlying this vulnerability. Because Ubc13, a known binding partner of Mms2, localizes to DNA damage foci, we speculate that Mms2 will also localize at these sites, and may affect the localization of other factors relevant to the formation or function of damage-induced foci. To address these issues, we will irradiate Mms2−/− MEFs and Mms2−/− B cells and use immunofluorescence to detect the presence of DNA damage-related proteins such as 53BP1, Nbs1, Rad51 and Brca1 in DNA damage foci. We will also assess the subcellular localization of mms2 in WT cells. The Brca1 protein, which is encoded by the breast cancer susceptibility gene Brca1, co-crystallizes with Mms2, suggesting that this E2 ligase may affect Brca1 function (Christensen et al., 2007). We will therefore explore whether mutation of dysregulation of Mms2 contributes to mammary tumor formation. We will cross Mms2−/− mice to Erbb2 transgenic mice, which spontaneously develop mammary cancers (Quaglino et al., 2008). Tumour latency and mortality will be analyzed in these double mutants to determine whether mms2 deficiency promotes mammary cancer development.
iv. Uev1a

Recent studies suggest that Uev1a is necessary for NF-κB activation downstream of TNF receptor (TNFR) engagement (Andersen et al., 2005). We have established in a program to generate conventional Uev1a knockout and tissue-specific Uev1a knockout mice using the Cre/loxP system. We anticipate that complete Uev1a deficiency will result in embryonic lethality based on the fact that many knockouts of intermediates of TNFR signaling, including TRAF2 and NEMO, die in utero due to massive liver degeneration. Targeting constructs for conventional and tissue-specific Uev1a deletion have been generated and electroporated into mouse ES cells, and we are currently screening G418-resistant ES cell clones by southern blotting.

Expression of the Uev1a gene in Jurkat T cells and RAW macrophages has been knocked down lentiviral delivery of shRNA. The results of these studies have suggested that Uev1a is necessary for NF-κB activation induced by antigen receptor engagement in T cells, and for LPS stimulation of macrophages. Therefore, Uev1a seems to be necessary for a diverse range of signals leading to NF-κB activation. Once a fully backcrossed Uev1a knockout is generated, we will focus our efforts on defining Uev1a’s functions in lymphocyte homeostasis and development, as well as in LPS signaling in macrophages. To further elucidate the mechanisms by which Uev1a acts, we are conducting biochemical purifications of stably-expressed, myc-tagged Uev1a from Jurkat cells stimulated with PMA/ionophore; RAW 264.7 macrophages stimulated with LPS; and HepG2 cells treated with TNFα. Thus far, we have identified a novel interactor of
Uev1a in TNF-treated HepG2 cells: a ubiquitin-specific protease called USP9x. USP9x is known to cleave lysine-63-dependent polyubiquitin linkages, and may represent a novel negative regulator of UBC13/Uev1a function (Vong et al., 2005).

Preliminary studies in breast and prostrate tumor samples suggest that Uev1a is overexpressed in these malignancies, and may also promote metastasis (W. Xiao, personal communication). We plan to explore the role of Uev1a in tumorigenesis using luciferase-expressing xenograft models. Tumour cells stably overexpressing Uev1a and luciferase will be introduced into nude mice. When these animals are injected with luciferin, these cells will glow and can be imaged in vivo using the Xenogen microscope system without sacrificing the animal. We have generated three cancer cell lines that stably express Uev1a, based on MDA-MB-231-luc and T47D-luc (two relatively non-invasive breast cancer cell lines), and on B16-F10-luc (a metastatic melanoma cell line).

Leucettamol A is a natural product derived from marine sponges that specifically inhibits the interaction between UBC13 and Uev1a without impinging on the interaction between UBC13 and mms2 (Tsukamoto et al., 2008). Therefore, Leucettamol A has the potential to specifically inhibit Uev1a-dependent NF-κB activation, and may have therapeutic utility. In collaboration with Yousef Al-Abed at the Feinstein Medical Institute in Manhasset, New York, we have synthesized Leucettamol A and 10 derivatives. We plan to test these compounds in vitro for their ability to inhibit NF-κB activation downstream of antigen receptors, Toll-like receptors, and TNFR. In addition, given the purported role of Uev1a in tumour promotion, we will test these compounds in in vivo models of tumorigenesis, including the xenograft system described above. The
results of these studies should yield insights into validity of targeting the Uev1a-NF-κB pathway for therapeutic purposes.
VIII. CONCLUSION

In the 23 years since the NF-κB family of transcription factors was first described, the field has exploded. A picture has emerged of a truly pleiotropic transcription factor that is activated by a diverse number of signaling pathways. These pathways are in turn initiated by many types of external stimuli and in many different cell types. Thus, despite the already dizzying amount of information available about NF-κB regulation, new advances in the field are a daily occurrence.

The work in this thesis was undertaken with the idea of finding out how NF-κB activation can be controlled to improve human health. Given that NF-κB activity is deregulated in many haematopoietic and solid malignancies, as well as in many autoimmune disorders, it is highly likely that inhibiting NF-κB in such patients would be beneficial. However, NF-κB plays a global role in cellular physiology, meaning that therapeutic intervention must be managed carefully to avoid undesirable side effects or toxicity (Baud and Karin, 2009). We believe that it may be possible to pharmacologically target specific pathways driving NF-κB activation in particular diseases, and that research into this type of therapeutic module is under-represented. Hence, the purpose of this thesis was to identify elements of NF-κB signalling pathways that might represent useful pharmacological targets. The results presented here suggest that targeting the proteolytic activity of MALT1 and/or disrupting the UBC13/Uev1a axis may be promising avenues for small molecule inhibition. Our hope is that our work leads to the development of a new class of drugs that has been rationally designed from biochemical and genetic studies of NF-κB signaling and improves the health of many future patients.
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