3 Discussion

3.1 Ubiquitination of Malt1

This study intended to elucidate signaling processes, leading from T cell receptor engagement to activation of the transcription factor NF-κB. It was focused on the proteins Carma1, Bcl10 and Malt1, essential mediators of this pathway. The presented results demonstrate that Malt1 is modified by ubiquitination upon TCR engagement. Several lines of evidence were provided that TRAF6 serves as E3 ligase for Malt1 *in vitro* and *in vivo*, mediating assembly of K63-linked ubiquitin chains to multiple lysine residues in the C-terminus of Malt1. In addition, the functional significance of Malt1 ubiquitination for NF-κB and T cell activation was shown in reconstitution experiments with primary T cells in a close to physiological setting. Finally, recruitment of IKKγ to ubiquitinated Malt1 was identified as a crucial downstream function in T cell receptor signaling. Thus, ubiquitin chains attached to Malt provide the missing link between CBM and IKK complexes, thereby directing T cell receptor proximal signaling events to canonical NF-κB signaling upon T cell activation.

3.1.1 CBM complex formation and Malt1 ubiquitination

Upon stimulation of Jurkat T cells, the formation of a Carma1/Bcl10/Malt1 (CBM) complex through recruitment of pre-associated Bcl10/Malt1 to Carma1 could be observed in gelfiltration experiments. The size of this complex of more than 1500 kDa by far exceeds the expected molecular weight of a trimer (Fig 2.2). This could be due to oligomerization of Malt1 and/or Bcl10, which has been suggested to occur upon T cell activation and to be required for NF-κB activation [178, 183]. Alternatively, binding of additional proteins might also be responsible for the large size of the observed complex. Having determined that IKKγ is recruited to ubiquitinated Malt1, it is conceivable that association of the large cellular IKK complex mediates this shift in size. Both explanations are not mutually exclusive. Future experiments using IKKγ co-immunoprecipitation in combination with gelfitration will show whether Malt1-associated IKKγ and further IKK complex components can be found in the CBM complex fraction.

In the gelfiltration elution profiles it was observed that ubiquitinated Malt1 is mainly found in the CBM complex fraction (Fig 2.3). Mutation of the ubiquitin acceptor sites in Malt1 did not alter its stimulus-induced association with Carma1, indicating that Malt1 ubiquitination is not

required for CBM complex formation, but rather occurs in the CBM complex. This is in line with the observed kinetics of Malt1/TRAF6 interaction and suggests that Malt1 ubiquitination could be involved in downstream signal propagation.

Furthermore, Malt1 ubiquitination was shown to be transient (Fig 2.4). It occurs soon after stimulation and disappears after 50 min. The linkage of Malt1-attached ubiquitin chains was determined to occur through lysine 63 (K63) of ubiquitin, and no degradation of Malt1 was observed in the course of T cell stimulation. It is therefore reasonable to assume that a deubiquitinating enzyme exists, which removes ubiquitin chains from Malt1. Deubiquitinating enzymes (DUBs) represent important factors in ubiquitination-regulated processes. Two classes of these cysteine proteases have been assigned: ubiquitin-specific processing proteases (UBPs) and ubiquitin C-terminal hydrolases (UCHs). While UBPs remove and disassemble poly-ubiquitin chains, UCHs are mainly involved in cleaving ubiquitin precursors to generate active ubiquitin [220, 221]. Besides their housekeeping functions, some DUBs reveal substrate specificity and were shown to play crucial roles in signal transduction. The UBP CYLD (cylindromatosis) for example negatively regulates NF-κB signaling in response to TNFR stimulation by cleaving K63-linked poly-ubiquitin chains from TRAF2, TRAF6 and IKKγ [222, 223]. Because CYLD is induced by NF-κB in this context, this provides a novel autoregulatory feedback loop. Hence, it would be interesting to examine if a DUB exists which specifically removes ubiquitin chains from Malt1 and is thereby involved in terminating the TCR derived signal. This would further strengthen the physiological significance of regulatory ubiquitination in TCR induced signal transduction and add to the complex network of regulators.

3.1.2 TRAF6 function in Malt1 ubiquitination and TCR signaling

TRAF6 was found to induce Malt1 ubiquitination upon co-expression of the two proteins in cell culture and in *in vitro* ubiquitin ligation experiments. Furthermore, TRAF6 was shown to associate with Malt1 in a stimulus-dependent manner in T cells, coinciding with Malt1 ubiquitination and IκBα degradation. RNAi experiments demonstrated that TRAF6 is required for TCR-triggered Malt1 ubiquitination and NF-κB activation in Jurkat T cells. Importantly, it could be shown in the course of this thesis in a close to physiological setting, using reconstitution of Malt1 deficient CD4 positive T cells, that the TRAF6 binding sites in Malt1 [183] are required for PMA/ionomycin induced IκBα degradation and CD3/28 ligation

triggered production of interleukin-2 (IL-2), thus for NF-κB and T cell activation. Taken together, these results provide strong evidence for a physiological function of TRAF6 for antigen triggered NF-κB activation in T cells.

During the course of this thesis an involvement of TRAF6 in TCR signaling has also been suggested by two other independent groups based on RNAi studies [183, 190]. Additional support for this finding came from a study published during the writing of this thesis, where NF-κB activation by the IAP2-Malt1 fusion protein, found in a subset of MALT lymphomas (see 1.5.2.6.5), was investigated [224]. The IAP2-Malt1 fusion protein is thought to short cut regular antigen receptor signaling by intrinsic oligomerization mediated by the BIR domain of the IAP2 protein part. This publication demonstrates that NF-κB activation by IAP2-Malt1 fusion proteins critically depends on TRAF6. All fusion variants tested bind to TRAF6 and mutation of the known TRAF6 binding sites in Malt1 (2EA) impaired the ability of the fusion proteins to activate NF-κB in a reporter assay. Importantly, the authors demonstrate that IAP2-Malt1 fusion induced NF-κB activation is abrogated in TRAF6 deficient fibroblasts strengthening the notion of a crucial role for TRAF6 in Malt1 mediated signaling to NF-κB.

However, a recently published knock-out study has questioned the results obtained from cell culture [225]. This publication demonstrates that T cell specific deletion of TRAF6 leads to multiorgan inflammatory disease as a sign of spontaneous autoimmunity. TRAF6 deficient T cells reveal enhanced activity of the PI3 kinase/PKB pathway and are less responsive to suppression by regulatory T cells (Treg), which normally limit the activity of autoreactive T cells. It was further shown that PMA/ionomycin or CD3/28 induced NF-κB activation was not impaired in T cells lacking TRAF6. Thus, the authors suggest a so far unrecognized negative regulatory function of TRAF6 for T cell responses. However, the importance of regulatory K63-linked ubiquitination in TCR signaling is supported by the conditional deletion of Ubc13 in T cells, as thymocytes from these mice are severely defective in IKK/NF-κB activation [226]. Consequently, it remains an open matter of debate whether TRAF6 is involved in TCR-triggered signal propagation to NF-κB.

Obviously RNAi approaches can have consequences besides removing TRAF6 from the signaling cascade of interest. However, taken together evidence for a role of TRAF6 in TCR signaling accumulates and is strongly supported by the presented work of this thesis, showing stimulus-induced Malt1/TRAF6 interaction and significance of the TRAF6 binding sites in

Malt1 for TCR signaling. Thus, an explanation for the discrepancy between the presented results and the T cell phenotype of TRAF6 deficient mice could be, that other E3 ligases compensate for the positive role of TRAF6 in T cells of conditional knock-out mice, while its negative function is not affected by redundancy.

In this context Sun *et al.* have suggested a possible function of TRAF2 in TCR triggered activation of NF-κB based on RNAi experiments [183]. Although the mechanistic role of TRAF2 has not been closely investigated, simultaneous knock-down of TRAF6 and TRAF2 has been shown to result in stronger reduction of IKK activity and IL-2 production than knock-down of either E3 ligase alone. In the experiments performed for this thesis, no evidence was found that TRAF2 associates with Malt1 or induces Malt1 ubiquitination, which argues against a role of TRAF2 at this stage of the signaling pathway. However, a possible function of TRAF2 or other E3 ligases cannot be excluded *per se* considering that, in line with previous results, knock-down of TRAF6 in Jurkat T cells resulted in incomplete abrogation of Malt1 ubiquitination and NF-κB activation. It is thus interesting to note, that Sun *et al*. could reconstitute a minimal signaling pathway from Bcl10 to IκBα phosphorylation *in vitro* using recombinant Bcl10, Malt1, TRAF6, Ubc13, E1, ubiquitin, and TAK1 and IKK complex components. TRAF2, unlike TRAF6, could not activate the IKK complex in this cell free system. Hence it is possible that certain factors of the TRAF2 pathway were missing in that experimental setting, which could for example mediate an indirect recruitment of TRAF2. Thus, it could also be possible, that an additional factor is missing for TRAF2 to mediate Malt1 ubiquitination in the experiments of this thesis. Additionally, mice expressing a dominant-negative mutant of TRAF2 in T cells show defective IL-2 production upon stimulation by antigen presenting cells [227]. However, NF-κB activation has not been directly examined in that study and IL-2 production might be mediated by other transcription factors like NFAT or AP-1 [144].

Redundancy of TRAF proteins has already been observed in TNF receptor (TNFR) signaling. Although *ex vivo* studies had implied a role for TRAF2, TRAF5 and TRAF6 in NF-κB activation mediated by TNFR [228, 229], TRAF2 or TRAF5 deficient mice did not show defective TNF-induced NF-κB activitation [230, 231]. However, a combined knock-out of both TRAF2 and TRAF5 led to severely impaired NF-κB activity upon TNF stimulation [232]. Hence, it would be of great interest and importance to generate mice that lack TRAF6 and TRAF2 in T cells to finally judge the role of these E3 ligases in TCR signaling. The presented results strongly support a critical function of TRAF6 in Malt1 ubiquitination and TCR signaling. Nevertheless, future studies must determine, whether other E3 ligases

associate with the CBM complex that might have a redundant function with TRAF6 in TCR dependent NF-κB activation.

In their recent publication, Noels *et al.* also noticed the existence of two additional, overlapping TRAF6 interaction sites in Malt1 [224]. These sites (aa 311-316 and 314-319) are located just downstream of the second IgG-like domain in Malt1 and were shown to contribute to NF-κB activation induced by IAP2-Malt1 or Bcl10/Malt1 synergism in reporter assays. The respective sequence region is encoded by exon seven of Malt1, which only spans eleven amino acids. Interestingly, these eleven amino acids constitute the only difference between the two noted isoforms of Malt1 (A and B). No difference had been realized so far between the two Malt1 isoforms and both are employed by different groups working on antigen receptor signaling. In the presented work of this thesis the shorter isoform B was used in all experiments. Since ectopic expression of wt Malt1-B in Malt1 deficient T cells could restore IκBα degradation and IL-2 production, one can proceed on the assumption that this construct is fully functional. Noels *et al.* claim that inclusion of the second IgG domain and consequently the newly identified potential TRAF6 binding sites in IAP2-fusion proteins further enhanced NF-κB activation through intramolecular TRAF6 activation. In future experiments it would thus be interesting to examine the significance of this putative new TRAF6 interaction site and its influences on Malt1 ubiquitination and TCR signaling under more physiological conditions. If a so far unrecognized difference in the signaling properties of the two Malt1 isoforms for NF-κB activation existed, this would imply a putative influence of mRNA splicing on signaling processes. Hence, further studies should examine the expression patterns and properties of the Malt1 isoforms.

It is also important to note that Malt1 itself has been suggested to possess intrinsic E3 ligase activity and to directly mediate IKKγ ubiquitination [112]. Although it cannot be completely excluded that Malt1 is a TRAF6 dependent E3 ligase, in the presented experiments it was not observed that Malt1 is significantly auto-ubiquitinated after overexpression in cells or *in vitro* (Fig. 2.5 B and 2.8 B). Thus, Malt1 does not seem to confer sufficient E3 ligase activity, and it is rather unlikely that Malt1 itself performs its ubiquitination in a TRAF6 dependent manner. In line with these observations a separate study suggested that TRAF6 might be the E3 ligase that mediates Malt1 dependent IKKγ ubiquitination [233]. This is supported by a recent publication which demonstrates that TRAF6 can act as E3 ligase to directly ubiquitinate IKKγ *in vitro* [188].

The functional link between IKKγ ubiquitination and NF-κB activation in TCR signal transduction remained rather vague, because mutation of the potential acceptor lysine in IKKγ (K399R) only weakly impaired NF-κB activation in T cells. Furthermore, the kinetic of IKKγ ubiquitination upon T cell activation is delayed peaking after an hour or more indicating that IKKγ ubiquitination might be a secondary event [234]. However, the time course of Malt1 ubiquitination and the analysis of C-terminal Malt1 acceptor lysine mutants suggest that attachment of ubiquitin chains to Malt1 is a primary event to initialize IKK/NF-κB signaling in response to TCR/CD28 co-engagement.

3.1.3 IKKγ recruitment to the CBM complex

The role of Malt1 in TCR induced IKK activation seems to be similar to the function of RIP1 in TNFα signaling to NF-κB, where it has been shown that TNFα stimulation-induced RIP1 poly-ubiquitination, potentially catalyzed by the E3 ligase TRAF2, provides a platform for the recruitment of IKKs to the TNF receptor complex [109-111]. Furthermore, RIP1-attached ubiquitin chains were suggested to be required for recruitment of the TAK1/TAB2/TAB3 complex through the ubiquitin-binding domains of the TAB proteins. TAK1 is then thought to activate the IKK complex through T loop phosphorylation of IKKβ.

Analogous to RIP1 ubiquitination in TNF signaling, IKKγ recruitment was assigned as a crucial function for TRAF6 dependent Malt1 ubiquitination in TCR signaling to NF-κB. First, IKKγ was shown to interact with Malt1 in a stimulus-dependent manner. As seen in CoIPs from Jurkat T cells, IKKγ mainly associates with ubiquitinated Malt1 and no association of the two proteins could be observed in unstimulated cells (Fig. 2.20). Furthermore, interaction depends on an intact ubiquitin-binding domain of IKKγ, demonstrating that ubiquitin-attachment to Malt1 represents a crucial prerequisite for IKK complex recruitment (Fig. 2.21 and 2.22 A). In contrast, recombinant StrepIKKγ could bind unmodified Malt1 in the performed pull-down assays. This discrepancy is probably due to the different experimental conditions. A low affinity of IKKγ to Malt1 might be detected in the pull-down assay, where a large amount of recombinant protein is present as potential binding partner for Malt1. This affinity, however, might not be sufficient to mediate association of unmodified Malt1 with IKKγ under physiological conditions. Analogous observations have been made for the interaction of ubiquitinated RIP1 and IKKγ. Although yeast two hybrid assays and co-immunoprecipitations of the overexpressed proteins demonstrated an interaction between RIP1 and IKKγ [110, 235], association of the two proteins is induced upon TNFα stimulation

and critically depends on the ubiquitin-binding motif in IKKγ under physiological conditions [109, 110]. Thus, the different findings for Malt1/IKKγ interaction inside cells and in pull-down assays are in line with the current hypothesis that a low affinity of IKKγ to the binding partner must exist to refer substrate specificity to the ubiquitin-mediated interaction. If IKKγ unselectively bound to ubiquitin chains, it would be sequestered by many ubiquitin conjugated proteins in the cell. An increased affinity to ubiquitinated Malt1 compared to unmodified Malt1 provides the possibility for regulation of this interaction. Furthermore, the inhibitory effect of IKKγ mutations on Malt1 association is less pronounced in the pull-down experiment compared to CoIPs. This might be due to the experimental setting as described above. Furthermore, endogenous IKKγ present in the cell lysate of Jurkat cells might dimerize with the StrepIKKγ proteins [236], and thus indirectly recruit ubiquitinated Malt1 to the IKKγ mutant proteins, thereby weakening the investigated effect.

Mutation of its ubiquitin-binding motif rendered IKKγ unable to restore NF-κB activation in IKKγ deficient cells upon T cell stimulation. This clearly shows that sensing of ubiquitin chains by IKKγ is crucial for TCR triggered signaling to NF-κB. In this context it should also be noted that the recently identified ubiquitin-binding domain of IKKγ (NUB) overlaps with the so-called minimal oligomerization domain (MOD). The MOD has been shown to represent the minimal region necessary for dimerization and has been mapped to stretch from aa 246 to 365. Overexpression of the MOD inhibits IKK activation in cells [236]. Hence, it remains to be clarified whether impairment of IKK activity by MOD overexpression or mutation is mediated by affected ubiquitin binding, oligomerization or both and how these two processes might depend on each other.

Most of the studies on TRAF6 dependent IKK activation were based on cell-free systems, thus, although supporting a novel ubiquitin-dependent TRAF6 pathway, leaving the role of TRAF6 auto-ubiquitination for induction of NF-κB activity *in vivo* an open question [101, 187]. A recent study has now provided evidence that regulated K63-linked TRAF6 auto-ubiquitination at lysine 124 (K124) represents a prerequisite for IKK activation in response to interleukin-1 [188]. This might represent a general feature of TRAF6 mediated signal propagation, what would imply that IKKγ could in part be recruited to auto-ubiquitinated TRAF6 in response to T cell activation. However, no TRAF6 auto-ubiquitination could be detected during TCR signaling in the course of this thesis (data not shown), strongly suggesting that IKKγ directly associates with ubiquitinated Malt1.

Taken together, the presented results concerning IKKγ recruitment to Malt1 provide evidence that stimulus-induced regulatory Malt1 ubiquitination represents the so far unknown mechanistic link between the CBM and IKK complexes.

3.1.4 Multiple lysines as ubiquitin acceptor sites

Through consecutive N-terminal deletion of Malt1, the very C-terminal part was determined to represent the target region for TRAF6 induced ubiquitination (aa 613-813, Fig. 2.7 A). No mechanistic function had been assigned to the C-terminus of Malt1 so far. A caspase-like domain is located between aa 330 and 560, but no catalytical activity or other function could be shown for this domain. However, a crucial involvement of the C-terminal part in signal propagation has been reported. IAP2-Malt1 fusion proteins, which only comprise the Malt1 C-terminus, are capable of activating NF-κB, which strengthens this notion [178, 179, 181, 211]. Furthermore artificially induced oligomerization or membrane association of the Malt1 C-terminus was shown to be sufficient for NF-κB activation [178, 183, 205]. In this context, it seems reasonable that the ubiquitin acceptor lysines, which are required for downstream signal propagation from Malt1 to the IKK complex, are located in the Malt1 C-terminus.

Mapping of ubiquitination sites is problematic, because ubiquitination seems to be rather promiscuous, and mutation of primary site(s) can lead to ubiquitination of other lysines in the protein. This has hindered analysis of the physiological significance of many ubiquitination processes. Consequently, it was rather unusual that a single point mutation in RIP1 (K377) abrogated TNFα induced RIP1 ubiquitination [110, 111]. However, mutation of K377 in RIP1 impaired its inducible interaction with TNF receptor complexes, indicating that lack of ubiquitination could be caused by disturbed recruitment rather than mutation of the substrate attachment site. In contrast, multiple C-terminal lysines have been mapped as acceptor sites for Malt1 ubiquitination (Fig. 2.15 C, 2.16 B and 2.17 A), suggesting that any lysine within an acceptable distance seems to be suitable. This is in agreement with observations that RING E3 ligases often do not precisely position the ubiquitin chain to specific acceptor lysines [22]. Nevertheless, considering that mapping has been performed in overexpression experiments, one cannot completely exclude that only a subset of these lysines represents the primary target(s) under physiological conditions. However, the observed effects on the stimulus-induced ubiquitination in Jurkat cells and IκBα degradation or IL-2 production in primary T cells are more pronounced when eleven lysines instead of six lysines were mutated (Fig.

2.17A, Fig. 2.18 and 2.19). This suggests that all of the analyzed lysines can be involved in stimulus-induced Malt1 ubiquitination.

Exchanging amino acids in a protein might also affect its overall structure and not only remove a site of posttranslational modification. Therefore, all mutants were carefully examined for their binding properties to all known interaction partners in overexpression experiments and upon stimulation of Jurkat T cells. It was most important to prove unaltered affinity of the Malt1 lysine mutants to TRAF6, because the TRAF6 binding sites are located in close proximity. Since the C-terminal lysine mutants of Malt1 associate with Bcl10 and TRAF6, it is highly probable that no gross structural alterations have been evoked by lysine exchange. Furthermore, all mutants integrate into the CBM complex, demonstrating that upstream signaling is not defective. Thus, all mutants were rigorously tested for their known functions, and the data strongly suggest that the observed signaling defects are due to removal of ubiquitination acceptor sites.

Mapping of the acceptor lysines for TRAF6 and T cell activation-induced Malt1 ubiquitination allowed experiments, aiming to elucidate its functional role. Because no Malt1 deficient T cell lines exist, the experiments had to be performed in primary T cells from Malt1 deficient mice. Reconstitution through retroviral infection only led to a small cell population expressing significant amounts of ectopical Malt1 proteins. Therefore, single cell experiments like intracellular immunofluorescence staining combined with FACS analysis had to be performed. This experimental setup enabled investigation of the examined Malt1 mutants in a close to physiological setting. Analysis of IκBα degradation and IL-2 production in T cells of wildtype animals and T cells from Malt1 deficient mice reconstituted with Malt1 wt demonstrated a more effective rescue of IL-2 production than of IκBα degradation. (Fig. 2.13, 2.14, 2.18 and 2.19). An explanation could be that already a slight induction of IκBα degradation might be sufficient to induce an NF-κB response and thus IL-2 production that is comparable to wildtype cells. Nevertheless, the relative effects of lysine mutant constructs Malt1 6R and 11R on both read-outs were comparable and revealed a substantial decrease of IκBα degradation and IL-2 production (to approximately 50-60% and 30%, respectively). These results thus demonstrate that the C-terminal lysines in Malt1 are required for IκBα degradation and IL-2 production, providing evidence for a critical physiological function of Malt1 ubiquitination in NF-κB and T cell activation.

The importance of TRAF6 binding sites was investigated in the same experimental setting. In contrast to lysine mutants Malt1 6R and 11R, the effect of TRAF6 binding site mutation on

IκBα degradation and IL-2 production differed in severity. While IκBα degradation was almost not detectable after 20 min of PMA/ionomycin stimulation (Fig. 2.13), IL-2 production was only diminished by 50 % after 3h of CD3/28 receptor co-ligation (Fig. 2.14). This could be due to the fact that the 2EA mutation only impairs, but not completely abolishes Malt1/TRAF6 interaction, thus only substantially delaying Malt1 ubiquitination. Therefore, a strong negative effect is observed upon short PMA/ionomycin (20min), but not after longer stimulation (3h, CD3/28), where residual Malt1/TRAF6 interaction might overcome the inhibitory effect of the mutation. To clarify this point, different stimulation time points would have to be included in the analysis. This would allow examining if IκBα degradation can be observed in Malt1 2EA reconstituted T cells upon prolonged PMA/ionomycin stimulation, and how IL-2 production is influenced by shorter CD3/28 stimulation. Nevertheless, both read-outs clearly show a strongly impaired reconstitution ability of the 2EA mutant compared to wt Malt1, which provides evidence for a functional role of TRAF6 binding sites in TCR triggered NF-κB and T cell activation. Although it cannot be completely excluded that other unknown E3 ligases might be able to interact with the same sites in Malt1 (see also chapter 3.1.2), a crucial involvement of TRAF6 in TCR signaling represents the logical conclusion.

3.1.5 A new model for CBM mediated IKK/NF-κB activation

The stimulus-dependent recruitment of Bcl10/Malt1 to Carma1 upon its PKCθ mediated phosphorylation has been shown to be essential for TCR/CD28 co-ligation induced NF-κB activation [152]. However, the molecular mechanisms downstream of CBM complex formation leading to IKK complex activation have not been fully understood. Phosphorylation of Bcl10 has been suggested to be involved, however several recent studies including our own investigations have assigned a negative regulatory role to this process [180, 196, 197]. In addition, Bcl10 ubiquitination and subsequent degradation have been demonstrated as mechanisms to terminate signaling, thus preventing an excessive T cell response [199 and references therein]. However, no posttranslational modifications of Malt1 had been identified so far. With this thesis evidence for stimulus-induced, transient regulatory ubiquitination of Malt1 is provided. Based on the presented results, showing the physiological significance and function of Malt1 ubiquitination in T cells, a new model for CBM mediated IKK activation in response to TCR/CD28 co-ligation can be suggested (Fig. 3.1): TCR/CD28 co-engagement and a series of receptor-proximal events lead to the activation of PKCθ [149]. Phosphorylation of the linker region of Carma1 by PKCθ then enables recruitment of pre-

associated Bcl10/Malt1 to Carma1 and thus CBM complex formation [152, 153]. The ubiquitin-ligase TRAF6 is then recruited to the C-terminus of Malt1 and mediates attachment of K63-linked ubiquitin chains to lysine residues in the vicinity. IKKγ associates with ubiquitin chains on Malt1 through its ubiquitin-binding domain and thereby recruits the IKK complex. It remains to be resolved if binding of IKKγ to Malt1 can induce proximity dependent auto-activation of IKK complexes. Another conceivable scenario is activation of the IKK complex through recruitment of TAK1/TAB2/TAB3. Both mechanisms are not mutually exclusive. Although the necessity for TAK1 in TCR signaling has not been completely resolved (see 1.5.2.6.3), it would be interesting to determine in future experiments if the TAK1/TAB2/TAB3 complex is also recruited to ubiquitinated Malt1. To what extent and how IKKγ ubiquitination is involved in IKK activity also awaits further studies.

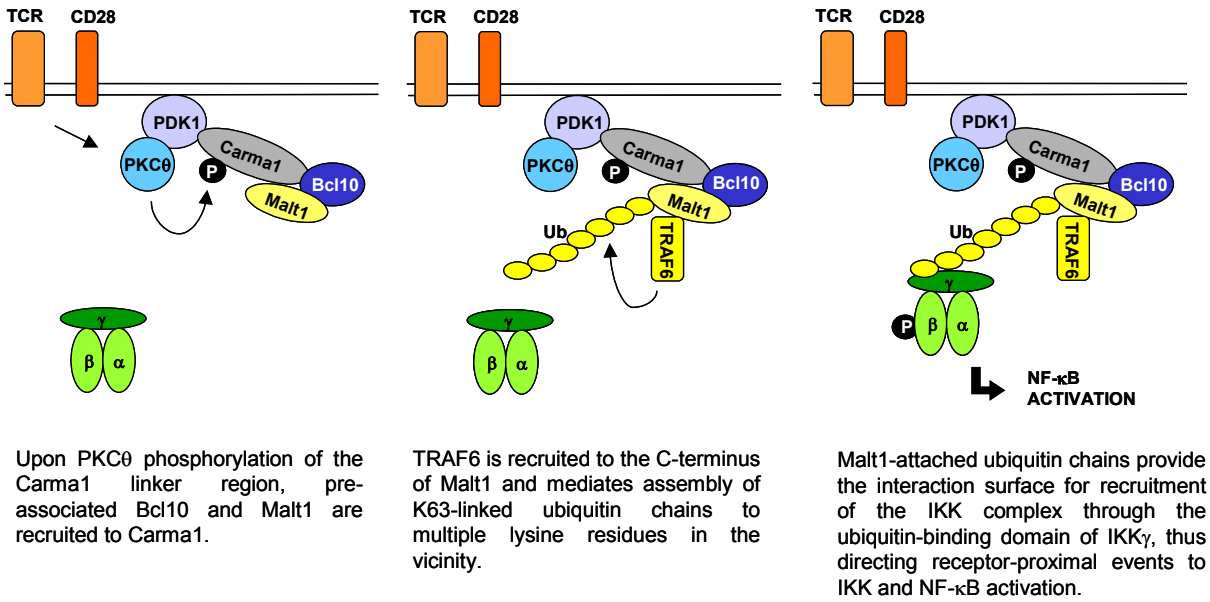

Figure 3.1: Representation of the suggested model for IKK activation in T cells. Upon TCR/CD28 co-ligation, PDK1 recruits PKCθ as well as Carma1 to the immunological synapse. PKCθ phosphorylates Carma1, which enables association of Bcl10/Malt1 with Carma1. TRAF6 associates with the C-terminus of Malt1 and mediates attachment of K63-linked ubiquitin chains to Malt1. IKKγ is recruited to Malt1 through interaction of its ubiquitin-binding domain with ubiquitin chains on Malt1. Subsequent activation of the IKK complex probably involves TAK1 dependent IKKβ phosphorylation.

It becomes apparent that a complex network of proteins and protein modifications including regulatory ubiquitination is needed to tightly regulate TCR signaling. Only the sum of many different activating and deactivating regulatory mechanisms enables a proper T cells response that can be adjusted to different conditions.

3.1.6 Implications for other signaling pathways

In contrast to Carma1 (CARD11), which is only found in cells of the lymphoid lineage, Bcl10 and Malt1 are expressed in many different tissues [237]. Recent studies have now shown that the function of Bcl10 and Malt1 is not restricted to B and T cells (Fig. 3.2). In mast cells, the Bcl10/Malt1 module was demonstrated to mediate Fcε receptor (FcεR) signaling to NF-κB [238, 239]. The Fcε receptor represents a high affinity receptor for the Fc portion of IgE and regulates mast cell degranulation and production of inflammatory cytokines. Other studies have provided compelling evidence that Bcl10 and Malt1 also exert functions downstream of G protein-coupled receptors (GPCR) on the route to NF-κB activation [237, 240, 241, 242]. Klemm *et al.*, Wang *et al.* and McAllister-Lucas *et al*. could show that Bcl10/Malt1 and their recruitment to the Carma1 (CARD11) homolog Carma3 (CARD10) are crucial for signal transduction downstream of lysophosphatidic acid (LPA), endothelin-1 (ET-1) and angiotensinII (AngII) induced GPCR receptors [237, 241, 242]. Since NF-κB activation upon LPA stimulation was also shown to depend on PKC activity [243, 244], it is possible that analogous processes mediate CARD/Bcl10/Malt1 complex formation in response to antigen receptors and GPCRs. GPCR-induced NF-κB activation is abrogated in TRAF6 deficient MEFs, suggesting an involvement of TRAF6 in these pathways [245]. In addition, Dectin-1 mediated antifungal immunity in dendritic cells is mediated by Bcl10/Malt1 recruitment to the CARD domain containing protein CARD9 [246].

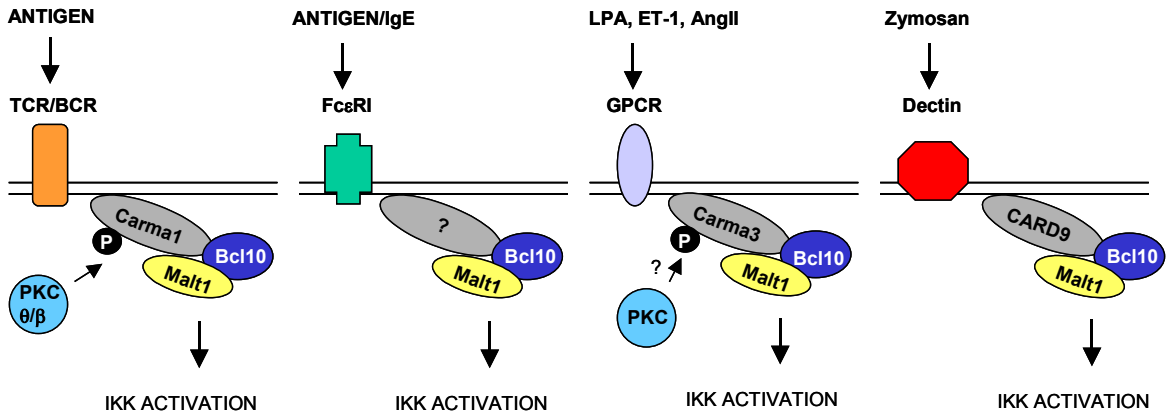

Figure 3.2: Representation of different Bcl10/Malt1 dependent pathways. The Bcl10/Malt1 signaling module was shown to link several receptors to IKK and NF-κB activation by interaction with different CARD domain containing adaptors. In lymphocytes, TCR or BCR signaling depends on Carma1 phosphorylation through PKCθ or PKCβ, respectively. Since GPCR mediated IKK activation also requires PKC activity a similar mechanism seems possible for this pathway. Upon FCεR signaling in mast cells, Bcl10/Malt1 associate with a unknown adaptor protein, while recognition of zymosan by Dectin-1 in dendritic cells leads to recruitment of Bcl10/Malt1 to CARD9. LPA, lysophosphatidic acid; ET-1, endothelin-1; AngII, angiotensinII.

Taken together, these results reveal that receptors of adaptive and innate immune cells use different CARD domain containing adaptors to recruit and activate the Bcl10/Malt1 signaling module. Thus, Bcl10/Malt1 mediated signaling is crucial for a broad range of pathways besides the TCR and BCR signaling cascades (Fig. 3.2). Consequently, Malt1 ubiquitination could represent a conserved mechanism to direct receptor proximal events to IKK activation in different pathways.

3.1.7 Conclusions and perspectives

Understanding the functional role of regulatory ubiquitination in signal transduction is a field of active cell biological research. With this study, Malt1 has been identified as a new target for regulatory ubiquitination and the pivotal role of Malt1 ubiquitination in TCR signaling provides strong evidence for this basic concept. Furthermore, Malt1 was revealed as a new substrate for the E3 ubiquitin ligase TRAF6. A critical function for TRAF6 in TCR triggered NF-κB activation is strongly supported by the presented results. Ubiquitin chains attached to Malt1 were shown to function as interaction surface for recruitment of IKKγ, thus providing the connection between CBM and IKK complexes. It was an open issue, how Malt1 can communicate with the IKK complex. Malt1 ubiquitination was now shown to represent the missing link and to direct receptor proximal signals to IKK/NF-κB activation.

This mechanistic insight into signal propagation downstream of the CBM complex represents an important advance in our understanding of TCR signaling. Since T cells exert various crucial functions in orchestrating the immune responses, a detailed knowledge of TCR signaling is necessary for our understanding how alterations in immune regulation result in autoimmune diseases. Furthermore, gaining insight into the complex signaling networks provides the basis for designing therapeutic agents targeted at interfering with aberrant T and B cell responses and for manipulating the immune system to counteract disease states like infection, autoimmunity and cancer. An understanding of the exact role of Malt1 is of importance as Malt1 represents a crucial factor in the development and progression of MALT lymphomas. Furthermore, Malt1 was now shown to be involved in several other pathways. Thus, Malt1 ubiquitination could represent a conserved mechanism under various physiological and pathological conditions to direct activating signals to the IKK complex.

Future studies have to prove if C-terminal acceptor lysines are also required for GPCR, Dectin-1- or FcεRI induced NF-κB activation and for Malt1 or IAP2-Malt1 mediated constitutive NF-κB activity in Malt1 lymphomas and diffuse large B cell lymphomas

(DLBCLs). Furthermore, the exact mechanism of IKK activation after recruitment to the CBM complex has not yet been investigated in detail, but a role of TAK1 seems conceivable. A closer investigation of IKKγ ubiquitination and its impact on TCR signaling would provide a substantial advance in our understanding of the role of regulatory ubiquitination in this signaling process and help elucidating the complex regulation mechanisms that are required for proper T cell responses.

3.2 Sumoylation of Bcl10

The presented results provide evidence that Bcl10 can be modified by sumoylation. Lysine 110 (K110) of Bcl10 serves as specific acceptor site for SUMO attachment. Even though a small amount of Bcl10 seems to be constantly modified by SUMO, this modification does not change in response to T cell activation. Bcl10 sumoylation is enhanced by induction of nuclear localization, and fusion of SUMO to Bcl10 targets Bcl10 to the nucleus. Unexpectedly, SUMO fusion also enhanced the NF-κB activation potential of Bcl10 in reporter assays.

3.2.1 Bcl10 sumoylation and nucleocytoplasmic translocation

A yeast two hybrid assay, performed in the laboratory by Elmar Wegener, had suggested an interaction of the N-terminus of Bcl10 with the sumoylation E2 enzyme Ubc9 and SUMO1. Overexpression of Bcl10, Ubc9 and SUMO1 in HEK293 cells then clearly showed that Bcl10 can be modified by SUMO attachment. Sumoylation of Bcl10 occurs specifically at lysine 110, which represents one of two predicted sumoylation consensus sites [31], indicating that this motif is accessible in the folded protein inside cells (Fig. 2.26). Ectopic expression of SUMO in Jurkat cells also demonstrated that a small fraction of endogenous modified Bcl10 can be detected in T cells. However, stimulation of Jurkat T cells with PMA/ionomycin did not effect sumoylation of Bcl10, suggesting that this modification is not involved in signal propagation from the TCR to NF-κB. Hence the question arose, under which conditions Bcl10 sumoylation could occur and what its effects might be.

Including the signaling deficient Bcl10 mutant L41Q, which partially localizes to the nucleus, in the described overexpression experiments unexpectedly revealed an enhanced modification of the mutant protein. Further analysis then led to the finding that artificially induced nuclear

localization of wt Bcl10 promotes its sumoylation, suggesting that Bcl10 sumoylation might represent a nuclear event. Nuclear localization has been reported to be a prerequisite for sumoylation of a variety of substrates and proteins of the cellular sumoylation machinery are to a large extent localized in the nucleus, but are also found at nuclear pore complex filaments in the cytosol [32, 33, 34]. In accordance, overexpressed MycUbc9 and MycSUMO were found in both nucleus and cytoplasm (data not shown). However, immunofluorescence experiments showed that wt Bcl10 resides in the cytosol. It was thus examined if Bcl10, despite its steady-state cytosolic localization, can shuttle between cytosol and nucleus so that a nuclear pool of Bcl10 could be prone to sumoylation. For that purpose, cells expressing Bcl10 were treated with leptomycin B (LMB), a potent inhibitor of nuclear export. No effect of LMB treatment on Bcl10 subcellular localization was observed, suggesting that Bcl10 is not exported from the nucleus in a NES-dependent manner to mediate its cytosolic localization. Although it cannot be completely excluded that nuclear export of Bcl10 is mediated by a different, NES-independent mechanism, this strongly indicates that Bcl10 does not shuttle between cytoplasm and nucleus. In contrast to Bcl10, Malt1 was found to shuttle between cytosol and nucleus as monitored by its nuclear accumulation after LMB application, indicating the existence of NES sequences in Malt1. Taken together, these results show that nuclear localization promotes Bcl10 sumoylation, but suggest that there is no substantial shuttling of Bcl10 under normal conditions, which could enable nuclear sumoylation of Bcl10.

Despite often representing a nuclear event, sumoylation can also occur in the cytosol and was shown to subsequently mediate nuclear import and retention of the respective substrates [32, 33]. Hence, experiments addressing a possible influence of sumoylation on Bcl10 localization were performed. Probably due to the very small proportion of Bcl10 that became sumoylated upon co-expression of Ubc9/SUMO, no differences in Bcl10 localization could be observed in this experimental setting (data not shown). Thus, a Bcl10-SUMO fusion construct was generated as experimental tool to observe a putative effect of sumoylation on Bcl10 localization. Since for a lot of substrates the fraction of sumoylated protein was found to be small (see Introduction 1.1.3, [33]), fusion of SUMO to the protein of interest has been employed before by others to investigate putative effects of SUMO modification. SUMO fusion was for example used to examine the function of IKKγ sumoylation for NF-κB activation upon DNA damage [73]. Here, IKKγ-SUMO fusion resulted in nuclear accumulation, indicating that sumoylation could explain the nuclear localization of IKKγ

observed upon DNA damage. In IKKγ, the zink finger (ZF) is required for sumoylation of the protein, and SUMO fusion to IKKγ was shown to compensate for NF-κB signaling defects caused by zinc finger deficiency, thus justifying the use of SUMO fusion as an experimental tool in this case.

C-terminal SUMO fusion to Bcl10 resulted in its nuclear import and retention, indicating that covalent modification by SUMO could influence the subcellular localization of Bcl10. Although SUMO fusion does not properly represent Bcl10 sumoylation at K110, this suggests that either cytosolic sumoylation of Bcl10 could mediate its nuclear accumulation or that nuclear sumoylation could lead to its retention in the nucleus (Fig. 3.3).

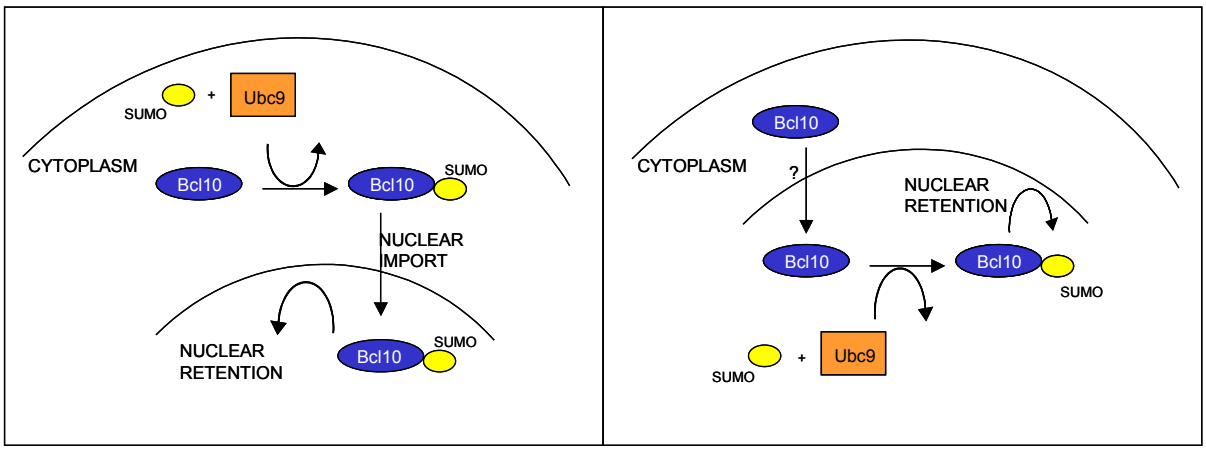

Figure 3.3: Possible scenarios for the interplay of Bcl10 sumoylation and localization. The presented results for Bcl10 sumoylation imply two possible scenarios. Cytosolic Bcl10 sumoylation could mediate Bcl10 translocation to the nucleus and nuclear retention (left panel). Alternatively, other unknown mechanisms could mediate nuclear import, and sumoylation of nuclear Bcl10 could result in its retention in the nuclear compartment (right panel).

However, mutation of the SUMO acceptor lysine in Bcl10 L41Q did not affect the partially nuclear localization of this protein, suggesting that other mechanisms besides sumoylation must exist that can target Bcl10 to the nucleus. How interference with CARD domain function (L41Q) mediates nuclear localization remains to be resolved.

It is interesting to note that nuclear localization of Bcl10 has been observed in a variety of MALT lymphomas (see 1.5.2.6.5), although Bcl10 was found to be cytosolic in marginal zone B cells, which are thought to represent the normal counterpart of MALT lymphoma cells [209, 247]. What causes nuclear localization of Bcl10 under these pathological conditions remains elusive. Thus, Bcl10 sumoylation could represent a possible mechanism to either induce nuclear localization or to mediate nuclear retention in MALT lymphomas.

Furthermore, it was shown here that Bcl10 seems to mediate the subcellular localization of Malt1. Bcl10 co-expression inhibited Malt1 nuclear accumulation upon LMB treatment, suggesting that Bcl10 predominantly influences localization of the Bcl10/Malt1 complex (Fig. 2.25). In line with this, Bcl10-SUMO fusion not only resulted in nuclear localization of Bcl10, but also led to nuclear accumulation of Bcl10/Malt1 complexes (Fig. 2.27). Thus, it is conceivable that sumoylation of Bcl10 could also influence the localization of associated Malt1. However, future experiments are needed to confirm sumoylation at K110 in Malt1-complexed Bcl10. Interestingly, gelfiltration analysis of Jurkat T cell lysates has suggested the existence of a Malt1-unassociated Bcl10 pool (Fig. 2.2), indicating the possibility of Malt1 independent functions of Bcl10.

3.2.2 Enhanced NF-κB activation by Bcl10-SUMO

Although the existing literature demonstrates a correlation between nuclear localization of Bcl10 and the malignancy of MALT lymphomas, it remains unclear how nuclear Bcl10 exerts its oncogenic role [208, 209, 247]. MALT lymphomas critically depend on constitutive NF-κB activity. Therefore, the effect of different Bcl10 constructs on NF-κB-dependent transcription was examined in reporter assays. Induced nuclear localization (NLS-Bcl10) was shown to reduce the potential of Bcl10 to activate NF-κB, suggesting that aberrant nuclear localization of Bcl10 does not directly lead to enhanced NF-κB activity. This is in line with results provided by Nakagawa *et al.*, which demonstrate that NF-κB activation by Bcl10/Malt1 synergism is reduced after treatment of the cells with LMB [219]. Interestingly, it could be shown in this thesis that SUMO fusion to Bcl10 significantly enhances NF-κB activation compared to wt Bcl10 in reporter assays. Thus, it is tempting to speculate that sumoylation of nuclear Bcl10 would increase its potential to activate NF-κB and maybe drive malignancy. Since NLS-Bcl10 did not provoke NF-κB activity in reporter assays, it seems that under normal conditions nuclear localization alone is not sufficient to induce substantial SUMO modification for affecting NF-κB activation in HEK293 cells. However, one could speculate that sumoylation might be stronger in other cell types or that so far unrecognized mechanisms might enhance sumoylation under pathological conditions.

NF-κB activation by Bcl10-SUMO fusion was shown to depend on IKKβ activity and IκBα degradation, since expression of respective dominant-negative constructs abrogated induced NF-κB activation. How Bcl10-SUMO could engage IKKβ to trigger the canonical NF-κB pathway awaits further elucidation. One could also assume a scenario where sumoylated

Bcl10 enhances basal NF-κB activation by an unknown mechanism. In this context it is interesting, that in DNA damage signaling nuclear sumoylation and ubiquitination of IKKγ finally result in activation of cytosolic IKK complexes, suggesting a so far uncharacterized interplay between nuclear and cytoplasmic events.

3.2.3 A working model for nuclear localization of Bcl10 in MALT lymphomas

During the course of this thesis, another study provided first suggestions on molecular mechanisms, which might govern nuclear Bcl10 localization in MALT lymphomas [219]. The results provided by Nakagawa *et al.* are based on their observation that overexpressed Bcl10 is localized in both cytoplasm and nucleus. In agreement with this thesis, the authors showed that Malt1 shuttles between nucleus and cytoplasm, while LMB treatment did not effect Bcl10 localization. Subsequently, two regions in Malt1 required for its nuclear export (NES1 aa 329-380 and NES2 aa 461-562) were identified. Furthermore, the authors reported that upon co-expression of Bcl10 with Malt1 (aa 1-380) and LMB treatment, both proteins accumulated in the nucleus, suggesting that under normal circumstances Malt1 exports Bcl10 out of the nucleus. Co-expression of an IAP2-Malt1 fusion protein did not lead to nuclear export of Bcl10, and the authors hypothesize that this is due to lack of interaction between IAP2-Malt1 and Bcl10. Based on their findings Nakagawa and colleagues proposed the following model for regulation of subcellular Bcl10 localization under normal and pathological conditions (Fig 3.4): In a normal lymphocyte Bcl10 is found in the cytoplasm due to export from the nucleus by Malt1. In MALT lymphoma cases bearing t(11;18) translocations, Malt1 protein levels are reduced, because an IAP2-Malt1 fusion protein is expressed from the translocated allele. Since IAP2-Malt1 does not export Bcl10 from the nucleus, Bcl10 partially resides in the nucleus. In cases of Malt1 lymphomas with t(1;14) translocations, which lead to Bcl10 overexpression, the relative Malt1 protein level is not sufficient to effectively export Bcl10, which thus remains nuclear.

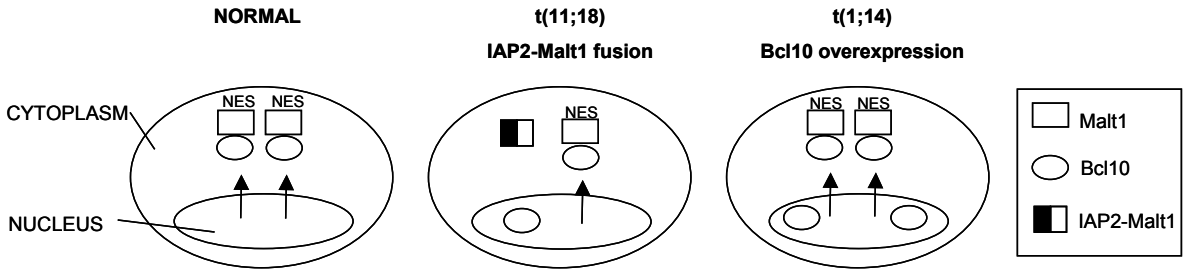

Figure 3.4: Model suggested by Nakagawa and colleagues for mechanisms influencing subcellular Bcl10 localization under normal conditions and in Malt1 lymphomas - adopted from Nakagawa *et al.* [219]. The presented model suggests that Bcl10 localization is mediated by its interaction properties with Malt1, which under normal circumstances mediates nuclear export and thus cytoplasmic localization of Bcl10.

However, some of the results provided by Nakagawa *et al.* are questionable, thus rendering the suggested model problematic. First of all, the observed partial nuclear localization of Bcl10 under normal conditions is in contrast to evidence provided by others [200, 209 and references therein] and the presented findings of this thesis, which demonstrate Bcl10 to be exclusively cytosolic. The proposed mechanism, in which Malt1 mediates Bcl10 localization, is directly questioned by this thesis, where Bcl10 was shown to critically determine localization of Bcl10/Malt1 complexes. Furthermore, the Bcl10 L41Q mutant was demonstrated to partially localize to the nucleus in this thesis. Bcl10 L41Q however still interacts with Malt1, and Malt1 co-expression does not influence its nuclear localization (data not shown). Thus, an influence of Malt1 on Bcl10 localization seems unlikely. Next, different IAP2-Malt1 fusion variants exist in Malt1 lymphomas, which contain no, one or two IgG-like domains dependent on the translocation breakpoint. Several IAP2-Malt1 fusion proteins have been shown to bind to Bcl10 [179], and according to another study, the IAP2-Malt1 fusion construct used by Nakagawa *et al*. should also retain its ability to interact with Bcl10 [204]. Consequently, this construct should be able to mediate nuclear export of Bcl10. Since Nakagawa *et al.* did not provide any evidence for abrogated Bcl10/IAP2-Malt1 interaction, this severely questions the drawn conclusions. Besides, a requirement of Bcl10 for IAP2-Malt1 fusion induced NF-κB activation remains controversial [224 and references therein], so that a Bcl10 independent mechanism for IAP2-Malt1 induced NF-κB activation has to be taken into account. Thus, further studies are mandatory to more closely investigate the effects of different IAP2-Malt1 proteins on Bcl10 localization. Importantly, although nuclear localization correlates well with t(1;14) and t(11;18) translocations, nuclear staining of Bcl10 has also been reported for about 20-50% of MALT lymphomas without these translocations [207, 210, 219, 248]. Taken together, this shows that other so far unrecognized mechanisms

must influence Bcl10 localization, and that the processes that mediate nuclear localization of Bcl10 in MALT lymphomas remain poorly described.

The results presented here, showing that Bcl10 is sumoylated and that Bcl10-SUMO fusion leads to nuclear accumulation of Bcl10, thus provide a biochemical basis for a conceivable mechanism that could mediate nuclear localization of Bcl10.

Besides the possibility that cytosolic sumoylation could mediate nuclear import of Bcl10, unaffected nuclear localization of Bcl10 L41Q despite mutation of the SUMO acceptor lysine clearly revealed, that other mechanisms must exist that can target Bcl10 to the nucleus. Although the existing stainings for Malt1 are rather weak, recently provided evidence demonstrates a cytosolic localization of Malt1 in MALT lymphomas, which had until then only been suggested based on overexpression of Malt1 and IAP2-Malt1 fusion proteins in cell culture [249]. Thus, although Bcl10-SUMO fusion was shown to be able to target associated Malt1 to the nucleus, it is highly probable that under pathological conditions Bcl10 exerts its oncogenic role in the nucleus independent of Malt1. This is in line with the finding that a Malt1-unassociated Bcl10 pool exists inside cells.

Based on the presented results of this thesis, a new working model for the regulation and role of nuclear Bcl10 localization in MALT lymphomas can thus be suggested (Fig. 3.5). Taken together, it seems most probable, that a so far uncharacterized mechanism apart from sumolyation mediates the nuclear localization of Bcl10 in a Malt1-independent manner. Nuclear Bcl10 could then become modified by SUMO attachment, resulting in its nuclear retention. According to the presented NF-κB reporter assays, sumoylated Bcl10 might influence NF-κB activity in Malt1 lymphomas with nuclear Bcl10 localization. However, nuclear localization alone was not able to induce sufficient sumoylation to affect NF-κB activity in HEK293 cells. One could speculate that some other effects might enhance sumoylation in different cell types or under pathological conditions. Consequently, future studies are needed to clarify, if sumoylated Bcl10 is present under pathological conditions in MALT lymphomas and by which mechanism sumoylation of Bcl10 could influence NF-κB activity.

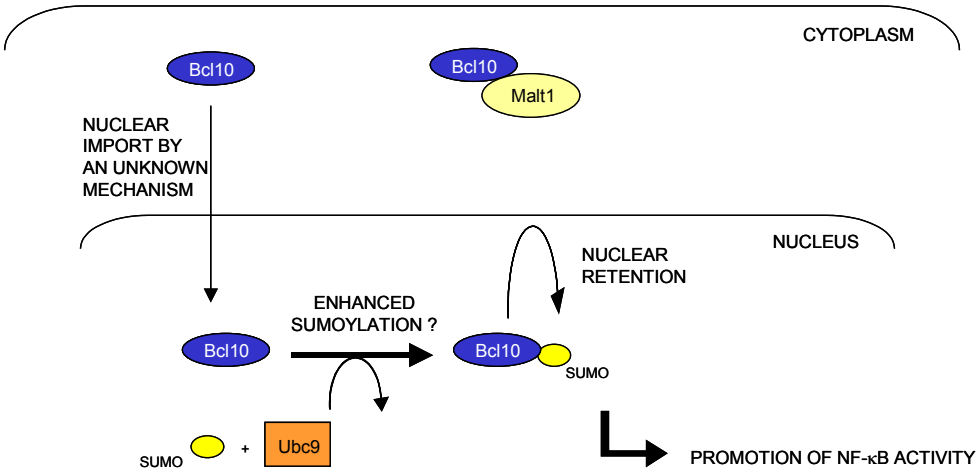

Figure 3.5: Suggested working model for regulation and effects of the aberrant nuclear localization of Bcl10 in MALT lymphomas. Based on the experiments presented in this thesis, it seems unlikely that Malt1 mediates cytosolic localization of Bcl10. The data suggest, that nuclear localization of Bcl10 is mediated independently of Malt1 and that, despite the ability of Bcl10 to regulate the localization of Bcl10/Malt1 complexes, an Malt1-unassociated Bcl10 pool might translocate to the nucleus in MALT lymphomas. Enhanced sumoylation of nuclear Bcl10 might then lead to its nuclear retention and promote NF-κB activity by a so far uncharacterized mechanism.

3.2.4 Conclusions and perspectives

The presented results show that Bcl10 can be posttranslationally modified by sumoylation at lysine 110, demonstrating that this consensus site for sumoylation in Bcl10 is accessible in the folded cellular protein. Bcl10 sumoylation can be directly mediated by the E2 enzyme Ubc9, however future studies need to clarify, if an E3 ligase exists, that can support Bcl10 sumoylation. Evidence is provided for an interplay of Bcl10 sumoylation and subcellular localization, which implies that sumoylation could represent a so far unrecognized mechanisms to regulate nuclear localization of Bcl10 (see Fig 3.3). Because nuclear localization of Bcl10 is observed in many cases of Malt1 lymphomas and is thought to be associated with advanced forms, which are resistant to *H.pylori* eradication, this is an interesting field for future research. Although all evidence indicates that Bcl10 exerts its oncogenic role in the nucleus, the underlying molecular mechanisms remain elusive [200, 208, 250]. In this context, it might be of special interest that SUMO fusion to Bcl10 was shown to result in enhanced NF-κB activating potential. Further investigation is needed to clarify the molecular mechanisms underlying this phenomenon.

The presented data provide an interesting biochemical basis for further experiments aiming to elucidate the role of Bcl10 subcellular localization and suggest an alternative model for regulation and effects of nuclear localization of Bcl10 in MALT lymphomas. However, they do not prove a physiological function for sumoylation and it awaits further investigation,

whether Bcl10 becomes sumoylated under pathological conditions. Due to the extremely restricted amounts of lymphoma material, an analysis of Bcl10 sumoylation in MALT lymphomas awaits the development of novel *in situ* techniques for the identification in biopsy samples. Furthermore, it would be of great advantage to establish a mouse model for MALT lymphomas to enable biochemical analysis of this protein modification.

In the course of this thesis, ubiquitin (-like) modifications of Bcl10 and Malt1, two essential mediators of NF-κB activation in lymphocytes, were investigated. For the regulatory ubiquitination of Malt1, a detailed model for its crucial function in IKK activation upon T cell receptor stimulation could be developed. The basic results for Bcl10 sumoylation hint at an interesting new field of research, emanating from the complex interplay of protein modification and localization. The presented findings thus reflect the manifold facets of the regulation of signaling processes by ubiquitin (-like) protein modifications.