IDENTIFICATION OF NOVEL SIGNALING PATHWAYS IN T CELLS MEDIATED BY PROTEIN KINASE C, CARMA1, MALT1 AND BCL10

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IDENTIFICATION OF NOVEL SIGNALING PATHWAYS IN T CELLS MEDIATED

BY PKC, CARMA1, MALT1 and BCL10

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Stimulation of T cells through the T cell receptor (TCR) and co-stimulatory molecules induces a diverse set of signaling events leading to T cell activation. Some characteristics of T cell activation are cellular proliferation, increase in cell size and changes in metabolic pathways. Our work has primarily focused on understanding the roles that PKC and Carma1, MALT1 and Bcl10 (the CBM complex) play in T cell activation, specifically, elucidating their requirement in distinct T cell signaling pathways. Here, I have shown a requirement for PKC, Carma1 and MALT1 in activation of the mTORC1 pathway. Inhibition of PKC impaired TCR-dependent S6 phosphorylation and that this requires Carma1 and the MEK/ERK pathways. In the absence of Carma1 or MALT1, we noted impaired TCR/CD28-induced stimulation of ribosomal protein S6, p70S6K and 4E-BP1 phosphorylation. Pharmacological inhibition of this pathway with a MALT1 protease inhibitor (z-VRPR-fmk) impaired T cell proliferation and activation-induced glycolysis, as seen by a decrease in oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). Thus, these findings present a novel mechanism for mTORC1 activation in T cells, through Carma1 and MALT1, leading to cellular proliferation and increased glycolysis. Surprisingly, Bcl10 was not required for S6 or S6K phosphorylation in T cells. We also demonstrate that MALT1 regulates stimulation dependent S6 phosphorylation in the ABC-DLBCL subtype. Here, we have identified novel signaling pathways whereby the proteins PKC, Carma1 and MALT1 activate the mTORC1 pathway. Our findings will provide new avenues for current therapies in dysregulated T cells.

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PREFACE

I would like to thank my mentor Dr. Lawrence Kane who has provided me the opportunity to develop as a scientist. I would like to thank all the members of my thesis committee- Dr. Sarah Gaffen, Dr. Yu Jiang, Dr. Binfeng Lu and Dr. Prabir Ray who have provided me assistance and advice over the last 5 years. I would also like to thank all the members of the Kane lab both past and present.

I would like to give a special thanks to my family- my parents and siblings who have encouraged and supported me through graduate school.

1.0   INTRODUCTION

1.1   TCR SIGNALING

1.1.1 T cell activation

Exposure to pathogen induces responses by both arms of the immune system- innate and adaptive (1). The innate immune system consists primarily of cells of the myeloid lineage- dendritic cells (DC), macrophages, basophils, neutrophils, mast cells and natural killer (NK) cells (of lymphoid lineage) (1). These cells are "first responders" and act quickly to prevent the deleterious effects of infection with a pathogen (1). On the other hand, cells of the adaptive immune system- T and B lymphocytes (T and B cells) are slower to react upon pathogen encounter and have a more specialized response (1, 2). Moreover, a key feature of these cells is their ability to "remember" previously encountered pathogens, respond accordingly and more rapidly upon re-challenge- a phenomenon termed immunological memory (1, 3). T cells recognize antigens presented by antigen presenting cells (APC) such as dendritic cells, after which they become activated and clonally expand (1). Antigens are recognized via specific and diverse T cell receptors (TCR) (1). These receptors induce a variety of signaling cascades leading to lymphocyte activation (4, 5).

T cells fall in to two main groups- CD4 and CD8 T cells- defined by their expression of the transmembrane CD4 or CD8 glycoproteins that associate with the T cell receptor (6). CD4 T cells recognize antigen presented by major histocompatibility complex II (MHC II) and differentiate into the major T helper subtypes, Th1, Th2, Th17, T regulatory cells (Treg) and T follicular helper (Tfh) cells (7, 8). These cells are able to assist other immune cells, releasing factors such as cytokines (e.g. IFN-γ, IL-4, IL-12, IL-2, IL-17, IL-23, IL-6, IL-10 and TGFβ), which aid in clearance of the pathogen or impair the immune response (in the case of Treg) (7, 8). CD8 T cells are cytotoxic T cells that directly respond to infected cells through release of cytolytic proteins, granzymes and perforin as well production of cytokines, eg. IFNγ (9).

In addition to stimulation through the TCR, T cells also require engagement of co-stimulatory receptors for activation (1). The most critical of which is the CD28 receptor, which binds the B7 ligand found on APCs (10, 11). Engagement of CD28 with its cognate ligand is required for T cell activation, survival, adhesion, differentiation and migration (1, 10-13). TCR stimulation in the absence of CD28 stimulation induces an anergic state, whereby T cells are hyporesponsive to antigenic challenge (1, 14). Stimulation CD28 induces activation of various signaling pathways, specifically the Akt, Ras/ Raf and "inside out" (integrin) signaling pathways (11, 12, 15).

1.1.2 Proximal T cell receptor signaling

Engagement of the T cell receptor with its cognate antigen and CD28 co-stimulation, triggers initial activation of the tyrosine kinase Lck in part through dephosphorylation of its inhibitory

phosphate group by CD45 (1). Lck is then able to phosphorylate tyrosines in the immunoreceptor tyrosine based motif (ITAM) found in the cytoplasmic region of the CD3 and ζ proteins as well as and zeta chain associated protein kinase of 70kDa (Zap70), for induction of further downstream signaling (16-18). Zap70 facilitates activation of a cluster of proteins through phosphorylation of the linker for activation of T cells (LAT), which serves as a central adaptor protein in the formation of the LAT 'signalosome' (19). Proteins that are bound to phosphorylated LAT include phospholipase C gamma (PLCγ), SH2 domain containing leukocyte protein of 76kDa (SLP-76), interleukin-2-inducible T cell kinase (Itk), Grb2 and Vav1 (20). Formation of this complex is critical for downstream signaling and subsequent activation of T cells (20) (Figure 1-1).

A significant feature of the formation of the LAT signalosome, is the generation of second messengers, specifically inositol triphosphate (IP_3) and diacylglycerol (DAG), which are necessary for potentiation of downstream signaling leading to activation of T cells (21-23). PLCγ cleaves phosphatidylinositol 4,5-biphosphate (PIP_2), generating IP_3 and DAG (24). These second messengers are then able to induce Ca^{2+} release and activate protein kinase C (PKC), both of which are required for activation of the essential transcription factors needed for T cell activation- nuclear factor of activated T cells (NFAT), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activation protein-1 (AP-1) (22). The LAT signalosome also facilitates "inside out" signaling, whereby integrins are activated and upregulated to enhance binding to the APC for sustained interaction between the T cell and APC, leading to continued signaling (22) (Figure 1-1).

1.1.3 Downstream TCR signaling

NFAT requires Ca^{2+} in order to be activated by its upstream regulators calmodulin and calcineurin (25). Generation of IP_3 leads to the release of Ca^{2+} from the endoplasmic reticulum, as well as an influx of extracellular Ca^{2+} through the CRAC channel, composed of STIM and Orai proteins (21). Calmodulin complexed with Ca^{2+} leads to the activation of the serine/threonine phosphatase calcineurin, which dephosphorylates NFAT. Upon dephosphorylation, NFAT translocates to the nucleus for transcription of specific genes (25). NFAT regulates transcription of IL-2, a cytokine essential for T cell proliferation as well as transcription of IL-4, IL-17 and IL-22 (25, 26). NFAT has also been shown to regulate transcription of the signature T regulatory (Treg) cell transcription factor Foxp3 (27, 28).

One of the primary pathways that activate NF-κB is through protein kinase C (PKC), Carma1, MALT1, Bcl10 (CBM) complex, leading to the activation of the IκB kinase (IKK) complex (29-31). PKC θ is activated by DAG and phosphorylates Carma1, which is then able to bind MALT1 and Bcl10, forming the CBM complex (32-35). TRAF6 also associates with this complex, and activates TAK1, leading to activation of the IKK complex, through its regulatory domain IKKγ (30). The catalytic domain IKKβ phosphorylates the inhibitory protein IκB allowing for translocation of the p50 and p65 subunits of NF-κB (36). Work by the Kane group has identified an alternate mechanism for activation of NF-κB with T cell receptor stimulation and CD28 costimulation through Akt and the CBM complex (37). NF-κB is necessary for expression of IL-2, CD25 (IL-2 receptor α chain) and other T cell survival genes necessary for T cell differentiation (36).

AP-1 consists of two subunits- Fos and Jun, which are regulated by activation via three mitogen-activated protein kinase (MAPK) pathways: extracellular signal-regulated kinase (ERK), Jun kinase (JNK) and protein of 38 kDa (p38) pathways (22, 38). Initial activation occurs through phosphorylation and activation of the TCR-proximal proteins, Ras/ Raf (for activation of the ERK pathway) and Ras/ Rac (for activation of the JNK and p38 pathways), which initiate a series of phosphorylation events leading to the activation of ERK, JNK, and p38 (22). Upon activation of these kinases, Fos and Jun are phosphorylated and translocate to the nucleus to regulate T cell transcription (39). Of note, ERK also potentiates activation of AP-1 through phosphorylation of Lck, preventing inhibition by SHP1 (40).

Activation of T cells upon receptor and co-receptor engagement requires a myriad of signaling events leading to transcriptional regulation of proteins essential for T cell survival, proliferation and differentiation. Immunologists are in the process of trying to tease out all the pathways leading to T cell activation and how they directly relate to regulation of the many known functions of T cells controlled by TCR and CD28 engagement. Our current knowledge has provided a solid foundation for understanding the complex nature of T cell signaling and will continue to for future discoveries.
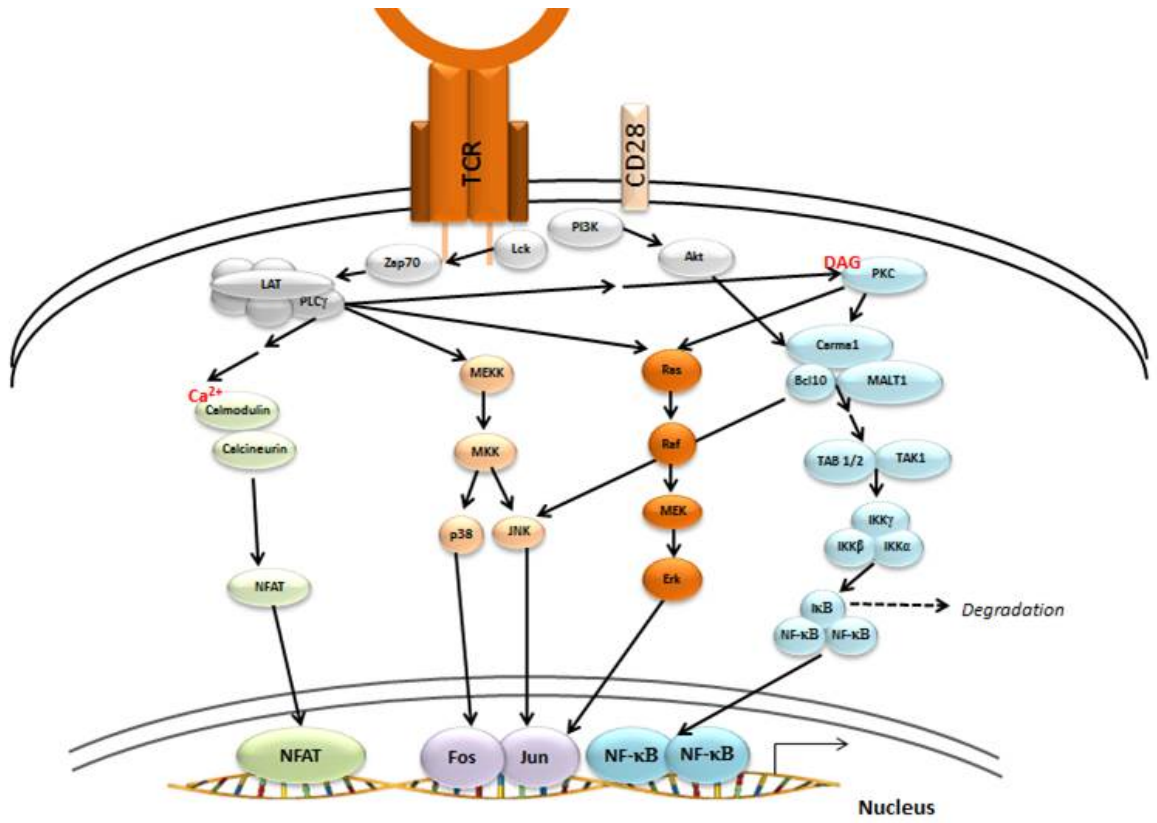
Figure 1-1 TCR Signaling

1.2   PROTEIN KINASE C

Protein kinase C (PKC) describes a group of proteins belonging to the Protein Kinase A, G and C (AGC) kinase family, which share sequence homology in their catalytic domains (41, 42). Most isoforms of PKC are ubiquitously expressed and mediate signaling close to the plasma membrane. The importance of the different PKC proteins has been identified through their roles in heart disease, cancer, neurological disorders and autoimmunity (41, 42). Understanding the mechanism through which PKCs are regulated and the pathways that lie downstream are crucial for understanding their roles in different diseases.

PKCs are grouped into three different classes- classical, novel and atypical. These categories are based on their structure as well as how they are regulated. The classical PKCs (PKCα, PKCβ$_I$, PKCβ$_{II}$, and PKCγ) require binding of calcium and diacylglyerol (DAG) to their regulatory C1 and C2 domains, which promotes activation of their kinase domains (41-43). Cleavage of PIP$_2$ by PLCγ generates DAG and IP$_3$, the latter of which induces release of cytoplasmic calcium by binding calcium transporters of the endoplasmic reticulum (ER) (23). The novel PKCs (PKCδ, PKCε, PKCθ, PKCη), like classical PKCs, require binding of diacylglycerol to be activated, but they do not require calcium (42). Of note, use of a synthetic analog of DAG, eg. phorbol-myristate- acetate (PMA), is also able to induce activation of

classical and novel PKC (44). Unlike the above discussed isoforms, atypical PKCs (PKCλ and PKCζ) do not require either DAG or calcium for their activation, and in some cases require phosphorylation to be activated (45).

1.2.1 PKC in T cell receptor signaling

Most classical and novel PKC isoforms are ubiquitously expressed and induce the aforementioned cellular responses (42). PKC θ is a significant signaling molecule linking proximal signaling events to transcriptional control of T cells leading to activation. PKC θ is required for TCR- and CD28- mediated activation of NF-κB and NFAT in T cells, through the CBM/ IKK complex and MAPK, respectively (29, 35). A unique feature of PKC θ is its localization to the so called "immunological synapse" upon engagement of the TCR with cognate antigen by peptide/MHC on the APC (46). This localization appears to be important for downstream function, specifically NF-κB activation and negative regulation of signaling in Tregs (46, 47).

A major function of PKC θ in mediating T cell activation is through regulation of the T cell cytoskeleton. PKC θ phosphorylates moesin and Wiskott–Aldrich syndrome protein (WASP) interacting protein (WIP) both of which regulate actin reorganization to potentially allow for sustained interaction of the T cell and APC, in the presence of antigen (48). PKC θ also phosphorylates Carma1 in its linker domain allowing for formation of the Carma1 signalosome, leading to activation of NF-κB (49). The restricted expression of PKCθ, as well as its differential

signaling capabilities compared with other PKC isoforms, makes it an interesting molecule to investigate in T cells.

1.2.2   Significance of PKC θ in T cell function

As expected PKC θ is not only required for activation of T cells but also for CD4 T cell differentiation (50). PKC θ has been shown by several groups to be required for Th2 and Th17 differentiation (50, 51). In the absence of PKCθ, mice displayed reduced Th2 cytokine expression in the Th2 mediated model of airway eosinophilia as well as reduced expression of the canonical Th2 transcription factor, GATA3 (52). PKC θ KO mice were also shown to develop a reduced clinical score of EAE, a Th17- mediated disease model (53). Interestingly, PKC θ does not seem to be a critical regulator of Th1 differentiation (54).

1.3   CARMA1, MALT1 AND BCL10

Signaling through the T cell receptor and co-stimulatory molecule CD28 drives a myriad of post-translational events, leading to subsequent activation of T cells. A defining characteristic of these events is the aggregation of proteins to potentiate downstream signaling, as discussed earlier. Of these, Carma1, Bcl10 and MALT1 (the CBM complex) have been shown to be necessary for

activation of the NF-κB and JNK pathways (32, 55, 56). Induction of both these pathways is required for activation, proliferation and maintenance of T cells (Figure 1-1).

Upon TCR and co-stimulatory (CD28) engagement, Carma1 recruits Bcl10 and MALT1, forming the Carma1 associated signalosome, which serves as a scaffold for other interacting proteins (36, 57). Specifically, formation of this complex allows for association of kinases, (eg. TAK1, IKK, Akt) and deubiquitinases (eg. CYLD and A20) (30, 31, 37, 58-61). These proteins all have catalytic activity and facilitate post-translational modifications necessary for activation of the IKK complex and eventual translocation of the p65 and p50 subunits of NF-κB to the nucleus (30, 31, 58, 59). Additionally, Carma1 and Bcl10 have been shown to associate with TAK1 and MKK7 to induce JNK2 activation and regulate steady-state levels of c-Jun protein expression, although the precise mechanism is still unclear (56).

Over the past twelve years, many articles have been published delineating the mechanisms through which Carma1, Bcl10 and MALT1 regulate activation of the NF-κB pathway downstream of the TCR. In contrast, the mechanism through which Carma1 and Bcl10 regulate JNK2 activation and c-Jun expression has yet to be fully defined. More importantly, additional TCR-induced signaling cascades that the CBM complex may regulate are yet to be identified. The following section will look at the individual components of the CBM complex-Carma1, Bcl10 and MALT1- their structure, function and relevance in immunity and disease states.

1.3.1 Carma1

1.3.1.1 Structure and Function

Caspase recruitment domain-containing membrane associated guanylate kinase (Carma1) or CARD11 is a member of the membrane associated guanylate kinase (MAGUK) group (62). A unique feature of Carma1 compared to Bcl10 and MALT1 is its pattern of expression, as it is predominantly found in lymphoid tissue, specifically T and B lymphocytes and Natural Killer (NK) cells (62). As such, it serves as a critical signaling molecule in the induction of the T and B cell response. At this point, Carma1 has been shown to mediate downstream signaling events solely by acting as a scaffold for binding of other proteins. There is no evidence showing kinase activity of Carma1 by the guanylate kinase domain (62-65). Here we will look at the different domains of Carma1 (Figure 1-2) and their known functions.

The caspase recruitment domain (CARD) is expressed in a number of proteins that regulate diverse of cellular processes (66). These "CARD" proteins are able to regulate activation of the NF-κB pathway (CARD11, CARD9, CARD14 and CARD8) and inflammasome activation (CARD5 and CARD12) (67).The significance of the CARD domain in Carma1 (CARD11) in T cells lies with its ability to associate with the CARD domain of Bcl10. Upon receptor engagement, Bcl10 is recruited in close proximity to the membrane where is interacts with Carma1 via their respective CARD domains (57). Carma1 and Bcl10, along with MALT1, are then able to aggregate with other proteins facilitating activation of NF-κB and JNK2 activation (65).

The linker domain lies between the coiled coil (CC) and the postsynaptic density protein [PSD95], Drosophila disc large tumor suppressor [DLG1] and zona occludans-1 protein [ZO1] (PDZ) domains (68). Carma1 is primarily regulated by phosphorylation of different residues in this region by PKCθ, IKKβ, Akt and CK1 (30, 31, 37, 58-61). Carma1 is phosphorylated by PKC θ with TCR stimulation, inducing a conformational change whereby Carma1 goes from a closed tertiary structure to an open one (49, 60). The open form of Carma1 is able to bind Bcl10, MALT1 and other associated proteins for further downstream signaling (69). Of note, deletion of the linker domain generates a constitutively 'active' Carma1, which is able to promote continued NF-κB activation without upstream cues (69).

Other domains of Carma1 include a coiled coil (CC), a PDZ, Src homology 3 (SH3) and a guanylate kinase (GUK) domain. These domains primarily function to facilitate association of Carma1 with other proteins (eg. CC to MALT1) and localization at the plasma membrane (GUK) (69). Of note, ubiquitination of Carma1 in the SH3 and GUK domains leads to degradation and subsequent termination of NF-κB and JNK activation, but requires initial Carma1 activation (open conformation) (70). This post translational modification is thought to be a negative feedback to prevent persistent downstream signaling with continued TCR stimulation.
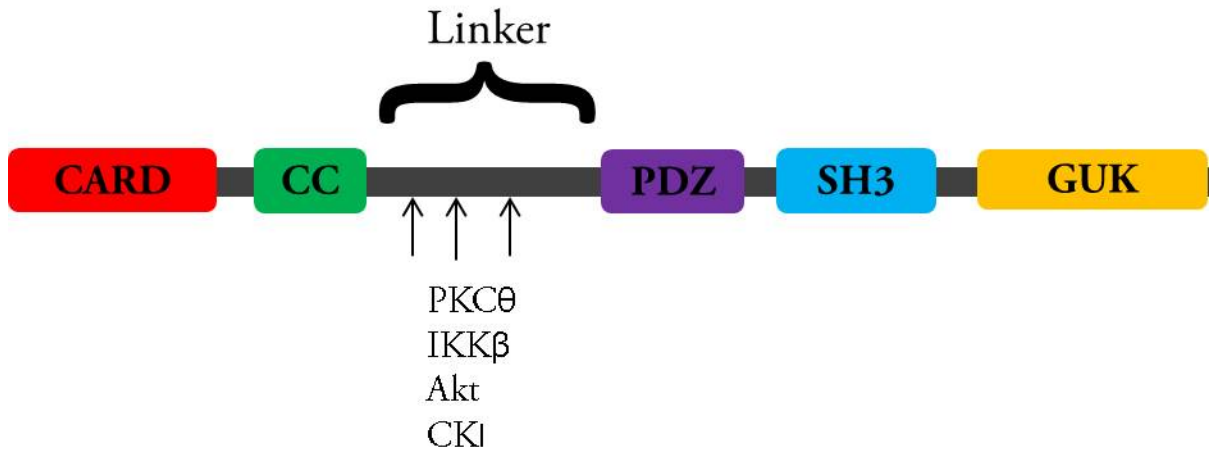
Figure 1-2 Domains of Carma1 and kinases that phosphorylate Carma1

1.3.1.2 Functional role for Carma1 & Significance

Carma1 regulation of NF-κB activation has been shown to require the TAK1 kinase, activation

of the IKK complex and subsequent degradation of the inhibitory subunit IkB, allowing nuclear

translocation of the p65 and p50 subunits of NF-κB (36). Our current understanding of the

mechanism of TCR-induced NF-κB activation through Carma1 has not been fully defined.

Regardless, it has been shown by several groups that in the absence of Carma1 T cells display

impaired stimulation-induced proliferation, cytokine expression and differentiation into different

T helper subtypes (65). This section will summarize Carma1's role in T cell activation,

differentiation and its clinical relevance (Figure 1-3).

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Naïve T cells from Carma1-deficient mice have normal thymic development. The consequences of Carma1 deficiency become apparent in the periphery, where T cells are unable to become fully activated, presumably due to impaired NF-κB and JNK activation. This impairment manifests as reduced T cell proliferation, cytokine expression and expression of the activation marker CD25 (IL-2Rα) (71). Carma1 has also been implicated in T cell survival, through recruitment to the membrane and increased expression of the anti-apoptotic protein Bcl-xL (72). Therefore, it is not surprising that Carma1 is also required for differentiation of particular T helper subtypes, which require TCR induced activation, co-stimulation and specific cytokines.

Carma1 regulates Th2 differentiation through modulation of JunB and GATA3 expression (65, 73). In the mouse model of asthma, the absence of Carma1 was shown to prevent disease onset and also play a critical role in the ability of effector and memory cells to become activated (74). Carma1 has also been shown to be necessary for differentiation of Th17 T cells, natural T regulatory cells (nTreg) and T follicular helper cells (Tfh) as assessed by expression of the signature transcription factors, RORγt, Foxp3 and Bcl-6, respectively (65, 68, 75).

Mutations in Carma1 have been implicated in various lymphomas (72, 76). Specifically, mutations within the coiled coil domain of Carma1 have been identified in human diffuse large B cell lymphoma (DLBCL) (77-79). Recently, it was shown that individuals with Carma1 deficiency have impaired induction of T cell activation, B cell activation and differentiation, leading to an immunodeficient phenotype (80)
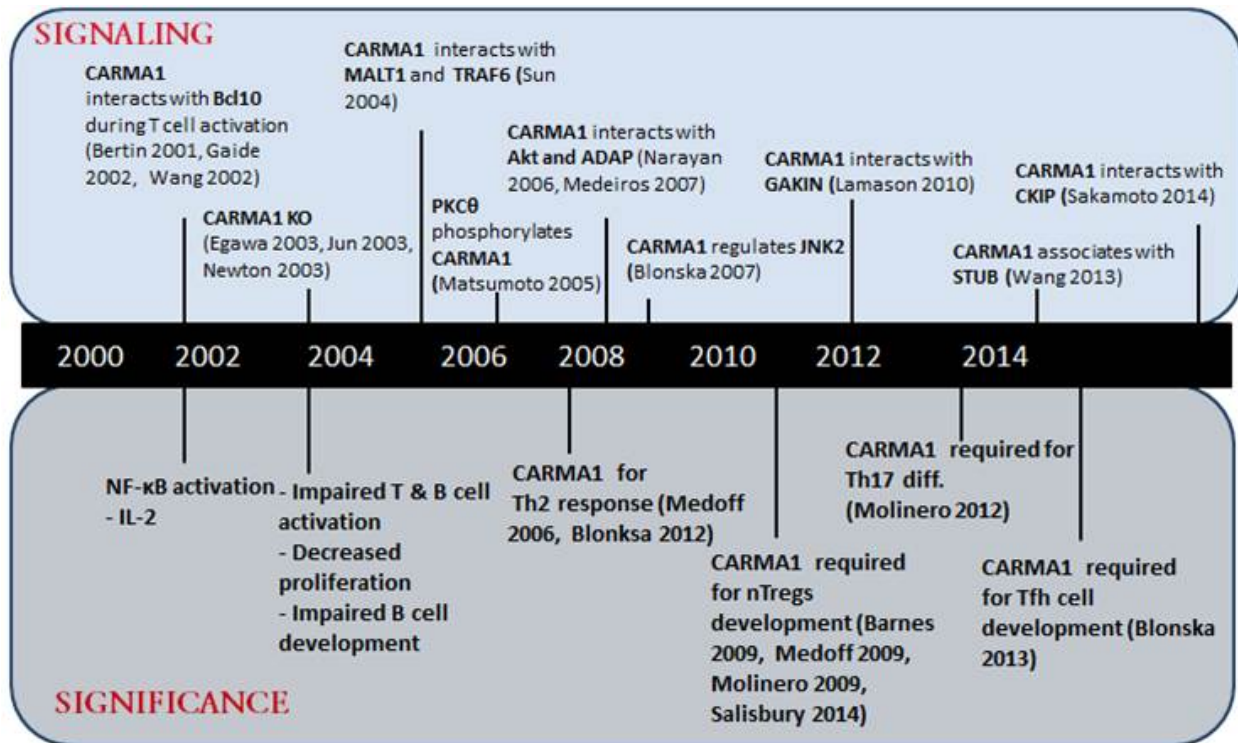
Figure 1-3 Hallmarks of Carma1 discoveries

1.3.2 Bcl10

1.3.2.1 Structure and Function

B cell lymphoma 10 (Bcl10) is a ubiquitously expressed protein in mammalian species (57). It was originally identified as a pro-apoptotic protein and later shown to be required for receptor-mediated NF-κB activation (33). Bcl10, like Carma1 and MALT1, plays a critical role in the axis

between receptor stimulation and downstream signaling, leading to activation of cells of the lymphoid and myeloid lineages (58). The following section will focus on Bcl10 structure and its functions in T lymphocytes.

Like Carma1, Bcl10 acts as a scaffolding protein to facilitate binding of other catalytically active proteins for activation of the canonical NF-κB pathway, JNK2 and stabilization of the AP-1 associated protein, c-Jun upon T cell receptor engagement (56, 66). Bcl10 consists of a CARD domain, which binds Carma1 upon TCR stimulation. Bcl10 constitutively associates with MALT1, which is also recruited to Carma1, to form the CBM complex. MALT1 associates with Bcl10 at its serine/threonine (S/T) rich domain (Figure 1-4) (69). As mentioned in the previous section, formation of this complex allows for binding of other proteins, eg. TRAF6, TAK1, IKKγ (58). Of note, Bcl10 associates with MALT1 and binds Card9 in monocytes (macrophages and DCs) upon receptor engagement (TLRs and Dectin receptors) to induce activation of the NF-κB and MAPK pathways (68). Through this, Bcl10 is able to positively regulate both the adaptive and innate immune response. Amongst the three proteins in the CBM complex, Bcl10 is most heavily regulated by post-translational modifications, and these modifications have all been shown to be stimulation dependent.

Interestingly, most Bcl10 modifications promote inhibition of the NF-κB or JNK pathway through degradation of Bcl10 or impairment of CBM complex formation. The E3 ligase, Itch, the proteins TAK1, IKKγ (NEMO), CRADD/RAIDD and p62, have all been implicated in destabilization of the Bcl10 protein, through ubiquitination, either direct or indirect (69, 81-85). Loss of Bcl10 expression significantly impaired NF-κB and/or JNK activation (34, 56). Of note, TCR stimulation is required to induce degradation of Bcl10 through the previously mentioned negative regulators (31).

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Conversely, phosphorylation by CAMKII, partial cleavage by MALT1, and association with the E3 ligase Mind Bomb-2 (MIB2) all appear to positively regulate T cell activation, through enhanced NF-κB or T cell adhesion leading, to sustained T cell receptor stimulation (MALT1 cleavage) (86-88).

1.3.2.2 Functional role for Bcl10 & Significance

Engagement of the T cell receptor and co-stimulation through CD28 initiates a cascade of proximal signaling events, leading to formation of the Carma1, Bcl10 and MALT1 complex (57, 58). Activation of NF-κB is necessary for T cell proliferation, survival and cytokine expression. Additionally, signaling through the Dectin, FcR and Toll-like receptors leads to assembly of the Card9, Bcl10 and MALT1 complex, which also promotes and NF-κB and MAPK activation and subsequent cytokine and chemokine expression (68).

Mice lacking Bcl10 (Bcl10 KO) show a similar phenotype to that of Carma1 KO mice in regards to impaired T cell and B cell activation, as well as impairment in formation of B cell germinal centers (57). Less is known in regards to the role Bcl10 plays during T cell differentiation, but it is predicted that loss of Bcl10 would also have a similar phenotype to that seen in the absence of Carma1. Clinically, mutations and overexpression of Bcl10 have been identified in MALT lymphomas, evoking continuous NF-κB activation and pro-survival signals of lymphoma cells (78, 81, 89, 90).
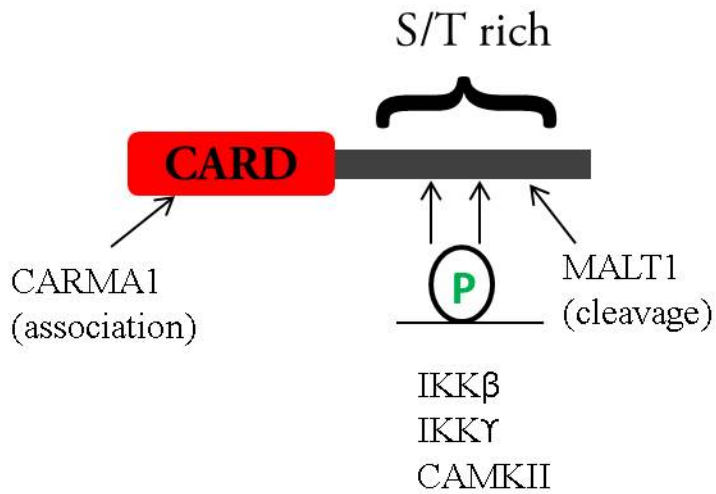
Figure 1-4: Domains of Bcl10 and associated proteins. P represents phosphorylation

1.3.3 MALT1

1.3.3.1 Structure and Function

Mucosa-associated lymphoid tissue translocation protein 1 (MALT1) was first identified in MALT lymphomas, where it is expressed as a fusion protein with cellular inhibitor of apoptosis 2 (cIAP2) (91). MALT1 serves as scaffolding protein upstream of the NF-κB and the JNK

pathways (66). Unlike the other two member of the CBM complex, however, MALT1 has also been shown to have catalytic activity, which contributes to downstream function (92). The following section will discuss the different domains of MALT1 (Figure 1-4) and their function as well as associated MALT1 proteins and the role of MALT1 paracaspase activity.

MALT1 contains a death domain (DD) and three immunoglobin (Ig) domains, two of which are required for constitutive association with Bcl10. The third Ig domain is located in the C-terminus of MALT1, becomes ubiquitinated upon stimulation and also facilitates association with IKKγ (36). TRAF6 also associates with MALT1 in a stimulation-dependent manner, promoting TRAF6-mediated ubiquitination of MALT1 in its C-terminus, allowing for binding of IKKγ (93); however the precise IKKγ binding site in MALT1 has yet to be fully determined. Association of TRAF6 is critical for further signaling through the CBM complex to induce activation of the NF-κB complex (30, 93). Upon association of TRAF6, the ubiquitylating enzymes ubiquitin-conjugated enzyme 13 (UBC13) and ubiquitin-conjugating enzyme E2 variant (UEV1A), TAB 1/2 and TAK1 are brought together. TAK1 phosphorylates the IKK complex, leading to subsequent nuclear translocation of the p50 and p65 subunits of NF-κB and gene transcription (94-96). Notably, MALT1 also facilitates binding of the de-ubiquitinases A20 and CYLD to TRAF6, which are necessary for removal of activation inducing ubiquitin and cessation of the NF-κB pathway upon receptor stimulation.

MALT1 contains a paracaspase or "caspase-like" domain, which was shown by the Beyaert and Thome groups in 2008 to have catalytic activity (95, 97). Similar to other caspases, human MALT1 contains two conserved residues Cys 464 and His 415. Mutation of Cys464 impaired TCR mediated NF-κB activation, revealing a potential requirement for MALT1 caspase activity in this pathway. Over the past six years, several substrates for MALT1 have been

identified (97). Cleavage of A20, CYLD and Rel-B have all been shown to be necessary for NF-κB activation, through inactivation of the aforementioned inhibitory proteins (98). MALT1 also cleaves Bcl10 near its C-terminus, which promotes adhesion of T cells to the β_1 ligand fibronectin (97). Other functional studies assessing the role of MALT1 catalytic activity have shown cleavage of Caspase 8 and activation of c-FLIP$_L$, leading to T cell proliferation, as well as cleavage and inactivation of the RNAse, Regnase, which normally suppresses effector T cell function (88, 99). A summary of MALT1 substrates and their functional significance in summarized in Table 1-1.
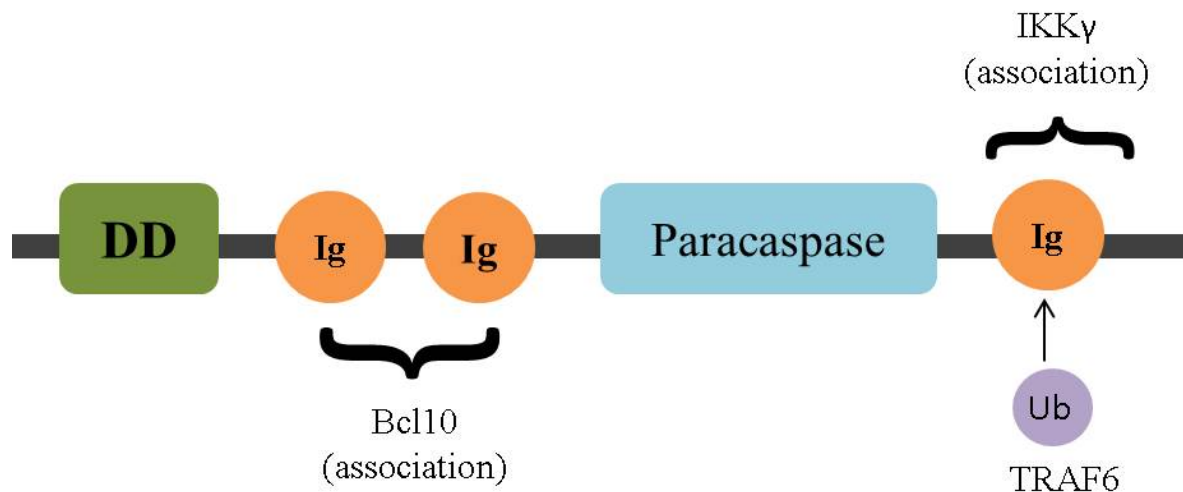
Figure 1-5 Domains of MALT1. Ub represent ubiquitination

Substrate	Functional Consequence of Cleavage
A20	Activation of NF-κB, through canonical NF-κB pathway
CYLD	Activation of NF-κB, through canonical NF-κB pathway
Rel-B	Activation of NF-κB, through canonical and non-canonical NF-κB pathways
Bcl10	Adhesion of T cells to the β_1 ligand fibronectin
Caspase 8	Activation of c-FLIP$_L$ and T cell proliferation
Regnase	Effector T cell function

Table 1-1 MALT1 substrates

1.3.3.2 MALT1 inhibitors

As demonstrated in many studies, an understanding of MALT1 catalytic activity, has enabled its manipulation in more sophisticated ways. Thus, the discovery that MALT1 is a functional paracaspase has led to design of inhibitors for this protein. The first and best defined inhibitor of MALT1 is z-VRPR-fmk, designed and originally synthesized by the Thome group (97). This irreversible peptide inhibitor was based on the substrate for the metacaspase found in *Arabidopsis thaliana* (97). Use of z-VRPR-fmk was proven to partially inhibit TCR-dependent NF-κB activation and IL-2 expression (97). This inhibitor was also shown to prevent MALT1 direct cleavage of Bcl10 and Rel-B (97, 100). Of note, z-VRPR-fmk had no effect on TCR-mediated activation of the JNK pathway through Carma1, Bcl10 and MALT1. Thus, MALT1 paracaspase activity has only been shown to regulate the NF-κB pathway to date.

Due to the nature of z-VRPR-fmk, a tetrapeptide conjugated to a ketone, a high concentration between 50μM and 150μM is required to see an effect on NF-κB activation, so it is a non-feasible drug in the clinical setting (97). As such, other groups have identified and generated alternative small molecule inhibitors of MALT1. The Krappman group identified a set of phenothiazine derivatives (mepazine, thioridazine and promazine) that serve as non-competitive MALT1 inhibitors. These compounds are traditional anti-psychotic drugs, but were also shown to inhibit NF-κB through MALT1 (101). The Melnick group generated a small molecule, irreversible, MALT1 inhibitor (MI-2) which is effective at nanomolar concentrations.

MI-2 was found to be effective in NF-κB inhibition both *in vitro* and *in vivo*, making it a potentially desirable drug for inhibition of MALT1 in vivo (102, 103).

1.3.3.3 Functional Role of MALT1 & Significance

Similar to Carma1 and Bcl10 KO mice, MALT1 KO displayed impaired activation of peripheral T lymphocytes and B lymphocytes (104). As expected, this phenotype can be attributed at least in part to the impairment of NF-κB activation in lymphocytes lacking MALT1 (104). Work assessing other functional consequences of the absence of MALT1 has shown a requirement for MALT1 in differentiation of Th17 cells, but not Th1 cells (96, 105). MALT1 is required effector Th17 cytokine expression and in the mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), MALT1 KO mice are resistant to disease, as compared with wild type mice (105). Additionally, other work assessing the role for MALT1 in a Th17 model revealed a requirement for MALT1 in the anti-fungal response, through the dectin receptors in dendritic cells (106). MALT1 is necessary for induction of Th17 polarizing cytokines by dendritic cells (106).

As described above, MALT1 was first identified for its role association with mucosa associated lymphoid tissue (MALT) lymphomas (91). Mutations in MALT1 have been well characterized in MALT lymphomas and have been shown to support lymphoma progression through aberrant activation of NF-κB, leading to expression of pro-survival signals (89, 91). Conversely, patients homozygous for a mutation of MALT1, resulting in no protein expression, suffer from combined immunodeficiency, including impaired NF-κB activation and IL-2 cytokine expression (107, 108). Thus, MALT1 is an essential immunoregulatory protein that is

necessary for eliciting an immune response, but overexpression/abnormal expression leads to development of hyper-activated lymphoid tissue and cancer.

MALT1 is also associated with development and progression of a subset of diffuse large B cell lymphomas (DLBCL) (109-111). DLBCL can be divided into two major sub types- germinal center B-cell like (GCB) and activated B-cell like (ABC) DBCL, based on their gene expression and identification of pathways critical for disease development (112, 113). Carma1, Bcl10 and MALT1 have all been implicated as critical regulators in ABC-DLBCL. Mutations in Carma1, leading to sustained NF-κB activation, have been identified in this disease (112, 113). Recently, the MALT1 inhibitor z-VRPR-fmk has been shown to be efficacious in limiting ABC-DLBCL growth *in vitro*, and that this was specific to ABC-DLBCL but not GCB- DLBCL cells (109, 114). The small molecule MALT1 inhibitor MI-2 has also been used in treatment of ABC-DLBCL in vivo. Treatment with this inhibitor in a mouse model of ABC-DLBCL inhibited tumor growth (102, 103).

1.4   MTOR SIGNALING

Mammalian or mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that was first identified by virtue of its inhibition by the immuno-suppressive drug rapamycin. This compound forms a complex with FK506-binding protein (FKBP12) to inhibit mTOR kinase activity (115-118). Through integration of multiple signals from growth factors, amino acids, antigenic stimulation and cytokines, mTOR serves as a critical regulator of cellular proliferation,

metabolism and growth (115, 118). For the past 15 years rapamycin has been used in the clinic as a potent immunosuppressant in the field of organ transplantation (118-120). Additionally, rapamycin is used in combination with other drugs for the treatment of some autoimmune diseases and cancers (118-123).

mTOR exists in two complexes in mammalian systems- mTORC1 and mTORC2. mTORC1 consists of mTOR, regulatory-associated protein of mTOR (RAPTOR), mammalian lethal with Sec13 protein 8 (mLST8), the proline-rich Akt substrate 40 kDa (PRAS40), and DEP domain- containing mTOR-interacting protein (DEPTOR) (118). mTORC2 consists of mLST8, DEPTOR, RAPTOR-independent companion of TOR (RICTOR), mSIN1 and protein observed with RICTOR (PROTOR) (118, 124, 125). The latter complex lies upstream of mTORC1 and directly regulates it, through phosphorylation of the protein kinase Akt at S473 (118). Signaling through Akt is one of the primary mechanisms through which mTORC1 is activated (118). However, much less is known about how mTORC2 is activated.

mTORC1 is activated by signals from growth factor receptors, antigen receptors (T and B cells) and amino acids, as well by the Wnt signaling pathway (126-129). Engagement of growth factor receptors induces activation of PI3K, which phosphorylates PIP_2 to generate PIP_3. This specific phosphatidylinositol activates the kinase phosphoinositol dependent kinase 1 (PDK-1), which phosphorylates Akt at T308 along with mTORC2 mediated phosphorylation (130, 131). Active Akt in turn phosphorylates and inactivates the tuberous sclerosis complex (TSC1/2) an inhibitor of the direct regulator of mTORC1, Rheb GTPase, leading to mTORC1 activation. Downstream of mTORC1 are two critical pathways: ribosomal protein p70 S6 kinase (p70S6K)/ ribosomal protein (S6) and eukaryotic translation initiation factor 4E-binding protein

(4E-BP1)/ eukaryotic translation initiation factor 4E (eIF-4E) pathways, necessary for cellular growth and proliferation (130, 131) (Figure 1-6).

Amino acids and ATP also serve as activators of mTORC1 through the small Ras related GTPases (Rag GTPases) and 5' adenosine monophosphate-activated kinase (AMPK), respectively (126, 128, 132). In the presence of amino acids, the Rag GTPases interact with RAPTOR in the mTORC1 complex, facilitating interaction with Rheb, which activates the mTOR kinase (128, 132). In conditions of low energy (reduced ATP levels), AMPK is activated, which activates the inhibitory complex TSC1/2 leading to inhibition of mTOR (128, 132). Other upstream regulators of the mTOR pathway are Ras/Raf, which activate the MEK/ERK pathway inducing inhibition of TSC1/2 (133). Alternately, activation of the MAPK pathway also induces activation of ribosomal protein p90 S6K, a pathway parallel to mTORC1 that phosphorylates and activates S6 (134) (Figure 1-6). Lastly, signaling through cytokine receptor IL-2 has been shown to induce activation of the downstream targets of mTORC1, S6K and S6 (129, 135, 136). Recently, stimulation through the TNF receptor was also shown to induce mTORC1 activation through activation of IKK and inhibition of TSC1/2 (137).

Since mTORC1 serves as a central regulator for external signals to coordinate the induction of relevant survival responses, its downstream targets- S6 and eIF-4E- are paramount. The ribosomal protein S6 is a member of the 40S ribosomal subunit, which is required for translation of 5' terminal oligopyrimidine (5' TOP) mRNAs, as well as for cellular growth and proliferation (138, 139). mTORC1 catalyzes phosphorylation of S6K at T389, a site that is necessary for S6K activation (140). Activated S6K is then able to phosphorylate S6 at sites S235/236 (human) and S240/244 (mouse), leading to S6 phosphorylation and function in the 40S

ribosomal subunit (139). S6 is phosphorylated at multiple sites, however the primary sites, 235/236 and 240/244, have been shown to be sufficient for S6 activity (139).

The eIF4E-binding proteins (4E-BPs) are a set of translational repressors that inhibits the formation of the eIF4F complex, by binding to eIF4E (141). There are three 4E-BPs: 4E-BP1, 4E-BP2 and 4E-BP3 (142). EIF4E is the rate limiting step of the eIF4F complex formation; other members of this complex are eIF4A (ATP-dependent helicase) and eIF4G (scaffold protein) (142). Stimulation by growth factors, cytokines and the TCR induces phosphorylation of 4E-BP1 through mTORC1 (142, 143). Hyperphosphorylation of 4E-BP1 prevents association with eIF4E, allowing for complex formation which facilitates translation of 5' capped mRNAs (144). 4E-BP1 is phosphorylated at sites T37, T46, T70 and T65 (145).

1.4.1   mTOR signaling in T cells

Engagement of the T cell receptor by MHC and peptide induces a cascade of signaling events leading to activation mTORC2 and mTORC1 (119, 146). As with other receptor-ligand interactions, stimulation of the T cell receptor, along with the co-receptor CD28, induces activation of PI3K and Akt leading to mTORC1 activation (119, 146). The Cantrell group recently proposed an additional mechanism for mTORC1 activation in T cells. They showed that activation of the mTORC1 downstream targets, S6K, S6 and 4E-BP1 does not absolutely require PI3K/Akt, as inhibitors of these kinases have a modest effect on phosphorylation of these

mTORC1 targets (147). Interestingly, PDK1 was shown to be necessary for activation of S6K, S6 and 4E-BP1, since in the absence of PDK1 there was little to no phosphorylation of these substrates (147). These findings corroborated earlier work showing that Akt is dispensable for glycolytic function in activated CD8 T Cells, but PDK is not (147-149). However, the precise mechanism of PDK regulation of mTORC1 and its downstream targets is still not fully understood.

Signaling through the IL-2 receptor has also been shown activate mTORC1 T cells. IL-2 is a critical cytokine, necessary for T cell survival and proliferation (129, 135). Signaling through the IL-2 receptor induces activation of PI3K and Akt, leading to mTORC1 activation and downstream function (129, 135). Stimulation of the IL-2 receptor induces association of the mTOR protein with Survivin and Aurora B; activation of this pathway regulates G_1 to S cell cycle entry (150).

1.4.2   mTOR Function in T cells and Significance

As with other cell types, mTOR primarily serves as a regulator of T cell proliferation, growth and metabolism (119, 150). However, mTORC1 has additional vital functions in T cells, specifically the regulation of T cell metabolism as well as the differentiation of both CD4 and CD8 T cell subsets (120, 130).

Activation of naïve T cells requires a large energy supply for increased cell size, proliferation and effector function, and thus, an increase in metabolic output of T cells (131). A major regulator of this increase in metabolic output is mTORC1, which promotes increased oxidative phosphorylation and a switch to glycolysis (131, 151). mTORC1 regulation of glycolysis helps shift T cells from an anabolic state to a catabolic one, through expression of glycolytic proteins and glucose and amino acid transporters. Of note, this metabolic switch is a characteristic of effector T cells (excluding T regulatory cells), and memory T cells revert to a more catabolic state (131, 151).

mTORC1 also regulates further T cell differentiation. Inhibition of mTORC1 promotes differentiation of memory CD8 T cells, identifying a new target for development of vaccines in viral infections (152). mTORC1 has also been shown to regulate differentiation of Treg (130). Inhibition or activation of PI3K/Akt or mTORC1 was shown to increase or impair differentiation of T regulatory cells, respectively (153, 154). This additional function of mTORC1 opens a variety of possibilities for control of autoimmune diseases by mTOR modulation.

As mentioned previously, use of rapamycin is efficacious in immunosuppression for prevention of transplant rejection (155). Inhibition of mTORC1 has also been shown as a method for treatment in lymphomas in combination with other therapies (122, 156, 157). mTORC1 is a central regulator of T cell responses and as such has and will continue to be an attractive target in the clinical setting.
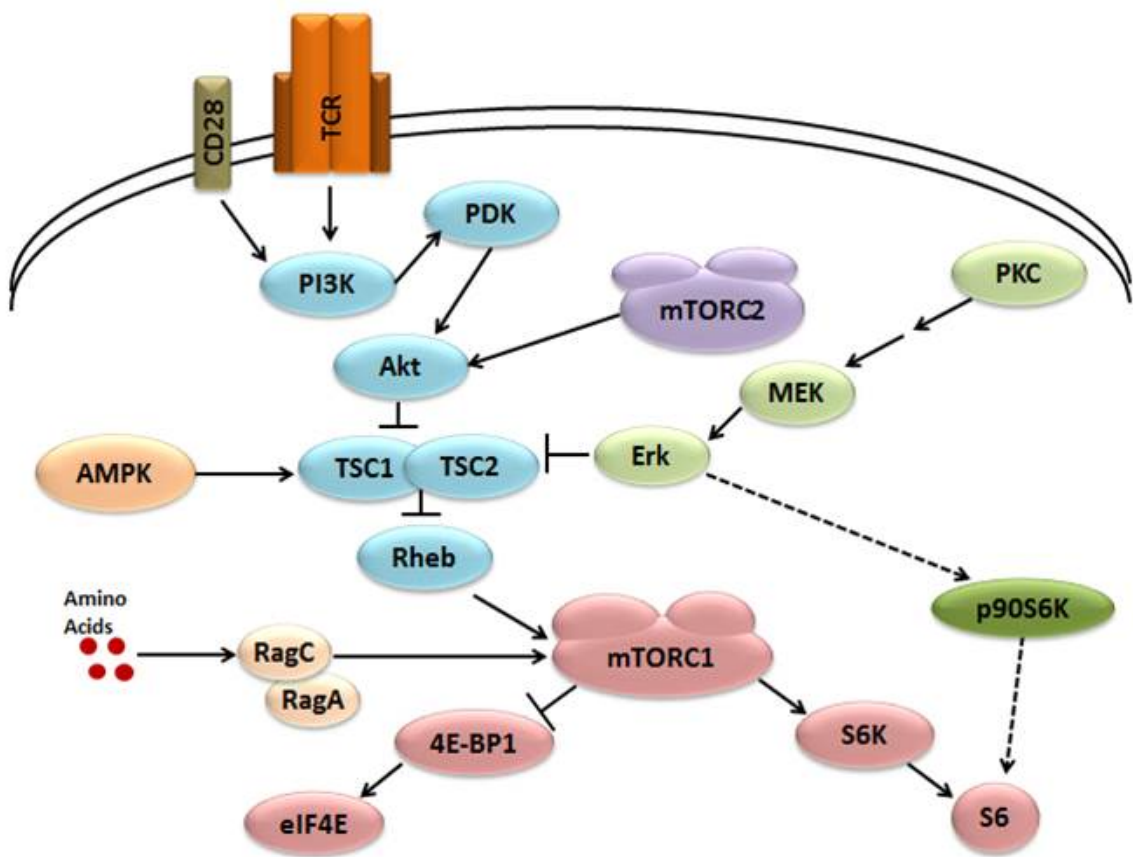
Figure 1-6 mTOR signaling in T cells

1.5   STATEMENT OF THE PROBLEM

Stimulation through the TCR/CD3 complex and the co-stimulatory molecule CD28 induces a cascade of signaling events leading to T cell activation. Our current understanding of the players and mechanisms through which this processes occurs is substantial. However, it is clear based on the continued pace of new discoveries regarding TCR signaling, that there is still a long way to go for us to fully understand all signaling events involved in T cell activation.

PKC, Carma1, Bcl10 and MALT1 (the latter three comprising the "CBM" complex) are key players in T cell activation, primarily through their activation of NF-κB and JNK pathways (56, 58). These proteins are required for activation of peripheral T cells upon antigen encounter, as well as for differentiation of CD4 T helper subtypes. PKC θ, Carma1 and MALT1 regulate differentiation of Th2, Th17 and natural Treg (nTreg) development (54, 74, 75, 106, 158, 159). These characteristics make them critical for adaptive immunity. Thus, understanding the molecular mechanisms that drive T cell activation and differentiation through PKC, Carma1, Bcl10 and MALT1 is crucial for development and improvement of therapies in the areas of infection, cancer and autoimmunity. Based on the fundamental roles that PKC, Carma1, Bcl10 and MALT1 play in T cell activation and differentiation we hypothesized that these proteins regulate other cellular pathways beyond the NF-κB and the JNK pathways.

We found that in the absence of Carma1, TCR and CD28 stimulation-dependent S6 phosphorylation was impaired, suggesting that the CBM complex may regulate the mTORC1 complex. With this finding, we plan to further investigate the requirement for Carma1, Bcl10 and MALT1 in receptor mediated activation of S6 and its upstream regulators S6K and mTORC1, in T cells lacking Carma1, Bcl10 or MALT1. Additionally, we will also assess the requirement for

PKC in activation of S6 in a TCR/CD28 dependent manner. To understand the functional

relevance of this alternative pathway of receptor-mediated activation of mTORC1, we also

intend to assess the ability of T cells to proliferate and modulate their metabolic output upon

activation in the presence of a MALT1 inhibitor. The results of these studies may identify one or

more novel signaling pathways leading to the activation of mTORC1 in T cells, thus providing

alternate sources for targeted therapy regulating mTORC1 activation. Also, characterization of

additional pathways that PKC, Carma1, MALT1 and Bcl10 regulate may provide new insight

into their relevance for T cell activation, T cell differentiation and lymphoma progression,

separate from the NF-κB and JNK pathways, which are currently thought to be their only modes

of action.

2.0   PROTEIN KINASE C REGULATES RIBOSOMAL PROTEIN S6

PHOSPHORYLATION IN T CELLS

2.1   INTRODUCTION

Proximal T cell receptor signaling events, along with stimulation through the co-receptor CD28, lead to production of the secondary messengers IP_3 and DAG (16). Release of DAG is crucial for the downstream activation of protein kinase C (PKC) (29). This kinase induces T cell activation in part through phosphorylation and activation of Carma1, leading to NF-κB activation. In addition, PKC mediates activation of the MEK/ERK pathway through phosphorylation of its upstream regulator Raf, promoting activation of AP-1 (29, 160-162).

PKC phosphorylates the inhibitory linker domain of Carma1, shifting it from a closed conformation to an open one, allowing for association of the proteins MALT1 and Bcl10 and the formation of the CBM complex (60). Formation of this complex allows for activation of the IKK complex and nuclear translocation of NF-κB (see Chapter 1.3) (60). However, less is known about PKC regulation of other pathways necessary for T cell activation, proliferation and maintenance.

Stimulation with phorbol esters (analogues of DAG) induces activation of the mTORC1 pathway through ERK, presumably through initial activation of PKC (163-166). The MEK/ERK pathway has dual roles in the activation of mTORC1 and its downstream target S6, through

inactivation of the upstream inhibitory complex TSC1/2, as well as direct phosphorylation of S6 by p90S6K (163-166). We hypothesize that stimulation of T cells through the TCR and CD28, leading to DAG production and PKC activation, might induce similar effects in T cells, i.e. activation of mTORC1 and phosphorylation of its downstream target ribosomal protein S6.

Our data show that, upon TCR and CD28 stimulation, PKC is able to induce S6 phosphorylation, and that this in part mediated by the MEK/ERK pathway. We also identified an otherwise unappreciated role for Carma1 in induction of S6 phosphorylation, whereby PKC requires Carma1 for S6 phosphorylation in T cells.

2.2   METHODS AND MATERIALS

Mice

C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Experimental protocols were approved by members of Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh.

Cell Separation

CD4+ T cells were isolated from spleen and lymph nodes and were separated by MACS beads from Miltenyi Biotech (Auburn, CA) according to the manufacturer's protocol. The purity of the cell population was >90%.

Antibodies and Reagents

Phospho-specific antibodies protein kinase C theta (T538), Alexa Flour 647 conjugated S6 (S235/236) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibody (phycoerythrin (PE)-conjugated) to pERK (202/204) was obtained from BD Biosciences (San Jose, CA). Stimulating antibodies, mouse anti-human CD28, hamster anti-mouse CD3 and CD28, and phospho–specific antibody to Akt (S473) were obtained from Life Technology (Grand Island, NY). HRP-conjugated Protein A was from GE Healthcare (Piscataway, NJ). Antibody specific to the Jurkat TCR (C305), purified from C305.2 hybridomas was from ATCC (Manassas, VA). Stimulating antibody, Affini-Pure Rabbit Anti-Syrian Hamster IgG and secondary antibody, donkey anti-rabbit horse radish peroxidase, were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Akt Inhibitor VII (Akti1/2), U0126 and Rottlerin were obtained from Calbiochem (Billerica, MA). Bisindolylmaleimide VIII acetate was obtained from Enzo Life Sciences (Farmingdale, NY).

Flow Cytometry

Flow cytometry staining was performed as previously described (167). In short, cells were starved in 1% bovine serum albumin (BSA), in PBS for 1 hr at 37°C; for indicated experiments cells were also pretreated with inhibitor during starvation period. After stimulation cells were fixed with 1.5% paraformaldehyde for ten minutes at r.t. and permeablized with cold methanol on ice for fifteen minutes. Cell were then washed three times with buffer (PBS, 1% BSA) and

stained with phospho specific antibodies at room temperature for up to 30 minutes. Samples were processed on BD LSRII instrument and data were analyzed using FlowJo software.

Western Blotting

Cells were lysed with NP-40 lysis buffer (1% NP-40, 1mM EDTA, 20mM TrisHCl, 150mM NaCl), containing protease inhibitors (aprotinin, pepstatin, 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF) and leupeptin) and phosphatase inhibitors (sodium fluoride, beta-glycerophosphate and sodium orthovandate). Proteins were resolved on 8-10% SDS-PAGE gels, transferred to PVDF membrane and blocked in 4% BSA. Membranes were then probed with specified primary antibodies, followed by HRP-conjugated secondary antibodies. Proteins were detected using the SuperSignal West Pico ECL substrate from Thermo Scientific and imaged on a Kodak Image Station.

2.3   RESULTS

2.3.1   PKC inhibitor bisindolylmaleimide inhibits PKC θ activation

PKC has been shown in various systems to regulate activation of ribosomal protein S6 (166, 168, 169). To understand if PKC also regulates S6 phosphorylation upon stimulation through the TCR and co-stimulatory molecule CD28, we used the pan-PKC inhibitor bisindolylmaleimide VII (BIM) (170). However, due to the promiscuity of some inhibitors we first wanted to assess its ability to inhibit another AGC kinase, Akt. The PI3K/Akt pathway, leading to mTORC1 activation and S6 phosphorylation, is well- characterized at this point (118). To ensure that treatment with the PKC inhibitor BIM would inhibit PKC, and not Akt, we assessed Akt activation through phosphorylation at S473 (Fig. 2-1A). We noted that treatment with BIM did not inhibit Akt phosphorylation, but a previously reported Akt inhibitor (Akti1/2) did (171) (Fig. 2-1A). We also pretreated Jurkat T cells with the novel PKC inhibitor rottlerin, as PKC θ (a novel PKC) is an upstream regulator of T cell signaling events and then assessed phosphorylation of Akt (41) (Fig. 2.1B). However, we noted that rottlerin treatment inhibited Akt phosphorylation (Fig. 2-1B). As such, we proceeded with BIM for our PKC inhibitor in assessment of PKC's role in activation with TCR and CD28 co-stimulation.
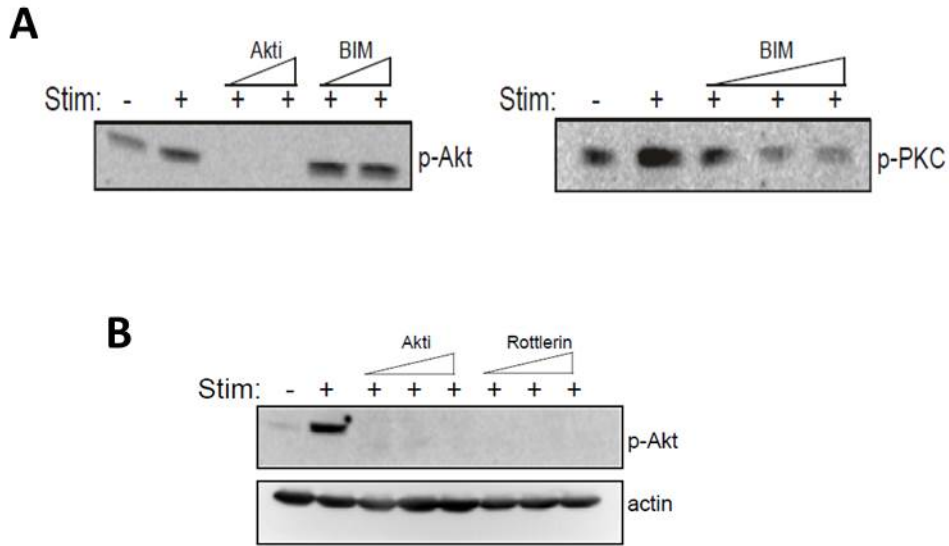
Figure 2-1 BIM inhibits PKC θ phosphorylation in T cells. (A) Jurkat T cells were pretreated with varying concentrations of Akti and/or bisindolylmaleimde VII for 1 hour, stimulated with anti-TCR/anti CD28 for 15 minutes and Akt (*left*) and PKC θ (*right*) phosphorylation assessed by immunoblotting. (B) Jurkat T cells were pretreated with Akti or rottlerin for 1 hr, stimulated and Akt phosphorylation assessed.

2.3.2 PKC is required for inducible S6 phosphorylation in T cells

After confirming the efficacy of the PKC inhibitor BIM, (Fig. 2-1A) we then assessed the potential requirement for PKC in S6 phosphorylation upon TCR and CD28 stimulation of T cells. We used phosphorylation of S6 at sites S235/236 as our read-out for S6 phosphorylation and function (139). We pre-treated Jurkat T cells with BIM, stimulated with anti-TCR/CD28 Abs, and assessed S6 phosphorylation by flow cytometry. We observed significant inhibition of S6 phosphorylation in T cells pretreated with BIM, suggesting that PKC is indeed required for TCR/CD28-dependent S6 phosphorylation (Fig 2-2A). We also pre-treated cells with the Akt inhibitor and observed inhibition of inducible S6 phosphorylation, but not to the same extent as with BIM (Fig. 2-2A). These results were not surprising to us, as the Cantrell group have previously provided evidence for alternative regulation of mTORC1, apart from Akt, downstream of the TCR (147). To confirm the requirement of PKC in TCR/CD28 mediated regulation of S6 phosphorylation in T cells, we also assessed S6 phosphorylation in primary CD4+ T cells pretreated with BIM. Similar to results obtained with Jurkat T cells, we noted diminished S6 phosphorylation when PKC was inhibited (Fig 2-2B). Our data show that PKC is required for TCR/CD28 stimulation-dependent S6 phosphorylation in T cells.
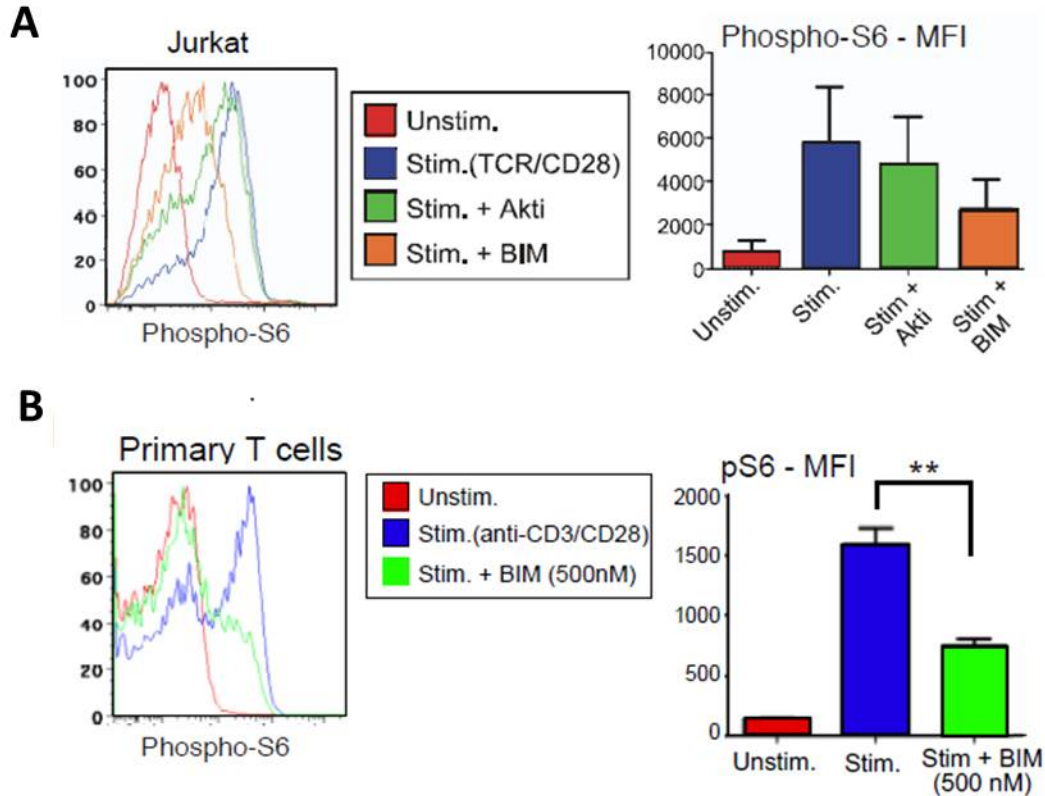
Figure 2-2 PKC regulates S6 phosphorylation in T cells. (A) Jurkat T cells were pretreated with Akti or BIM for 1 hr, stimulated for 15 minutes and S6 phosphorylation assessed. (B) Primary CD4 T cells were pretreated with BIM for 1 hr, stimulated for 30 minutes and S6 phosphorylation assessed. All values represent mean + S.E.M of three independent experiments (Jurkat T cells) or duplicates of a single experiment (primary cells). Data are representative of three independent experiments.

2.3.3 PKC regulates S6 phosphorylation in T cells through Carma1

Engagement of the TCR and co-stimulatory molecule CD28 induces signaling events leading to PKC activation, particularly the novel isoform PKC θ. Upon activation of PKC θ, Carma1 is phosphorylated, allowing for its association with Bcl10 and MALT1 and subsequent induction of NF-κB (50, 58). As we noted a requirement for PKC in TCR/CD28-mediated activation of S6 (Fig. 2.2), we hypothesized that PKC may be regulating S6 through Carma1. To this end, we assessed the ability of T cells to induce S6 phosphorylation (+/- PKC inhibition) in the absence or presence of Carma1. Parental Jurkat T cells and Carma1-deficient (JPM 50.6) Jurkat T cells were pretreated with varying concentrations of BIM and S6 phosphorylation was assessed after stimulation through the TCR and CD28 (Fig. 2-3). Treatment of Jurkat T cells with BIM at concentrations as low as 25nM inhibited inducible S6 phosphorylation, and this trend continued up to 500 nM BIM (Fig. 2-3). However, pretreatment of Carma1-deficient JPM 50.6 cells with BIM did not inhibit inducible S6 phosphorylation, even at higher concentrations of BIM (Fig 2-3). JPM 50.6 cells show impaired TCR/CD28 inducible S6 phosphorylation compared to parental Jurkat T cells and BIM treatment does not further inhibit the residual S6 phosphorylation in JPM 50.6 cells. Thus, our data show that PKC regulates S6 phosphorylation through Carma1 upon TCR/ CD28 stimulation.
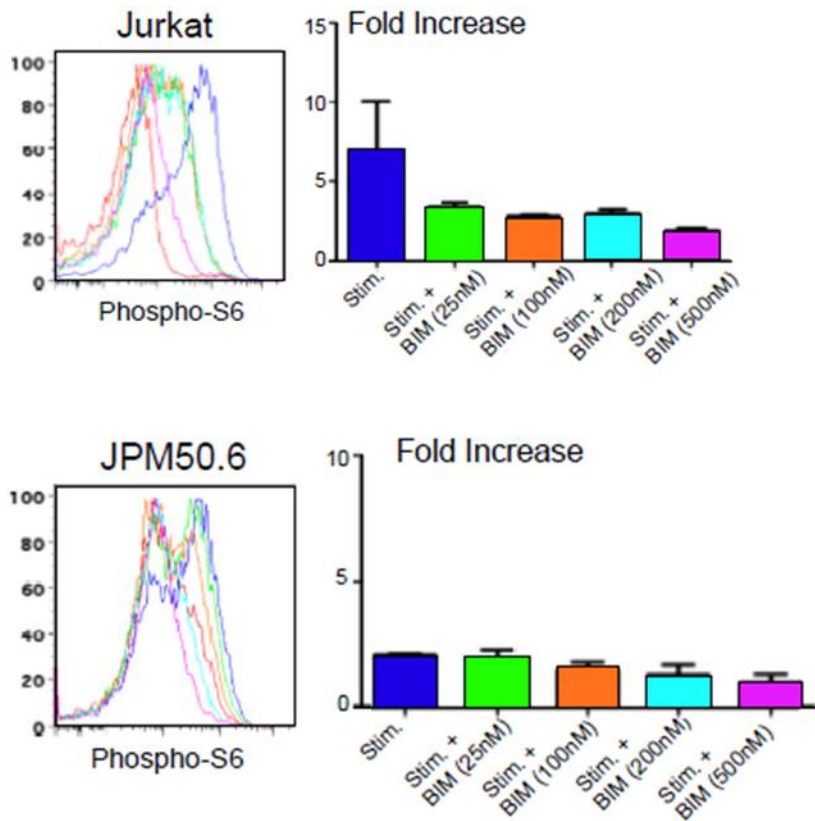
Figure 2-3 Carma1 is required for PKC regulation of S6 phosphorylation in T cells. Jurkats (*top*) or JPM 50.6 (*bottom*) cells were pretreated with varying concentrations of BIM for 1 hr, stimulated for 15 minutes and S6 phosphorylation assessed. All values represent mean + S.E.M of duplicates of a single experiment.

2.3.4 MEK/ERK pathway is required for stimulation dependent S6 phosphorylation in T cells

Upon TCR and CD28 crosslinking, PKC also activates the MEK/ERK pathway through the kinase Raf, which lies upstream of MEK and ERK (166). PKC activates mTORC1 by inactivation of the inhibitory complex TSC 1/2, through ERK (161, 166). However these previous studies all assessed the ability of mTORC1 to become activated after stimulation with PMA (164-166) or with cytokine stimulation (172). Our data show that TCR and CD28 induction of S6 phosphorylation requires PKC and Carma1. We next wanted to understand if regulation of S6 phosphorylation after TCR/CD28-mediated T cell activation required the PKC downstream target ERK. First, we assessed the effects of the MEK inhibitor U0126 on Jurkat T cells. Thus, U0126 inhibited TCR-inducible ERK phosphorylation (Fig. 2-4A). We then assessed S6 phosphorylation after U0126 treatment, and found it inhibited S6 phosphorylation, in a concentration-dependent manner (Fig.2-4B). We also assessed regulation of S6 phosphorylation by the MEK/ERK pathway in primary CD4+ T cells stimulated with anti-CD3/CD28 Ab's. Similar to the result in Jurkat T cells, U0126 inhibited S6 phosphorylation in primary T cells (Fig. 2-4B). These findings provide another mechanism through which PKC regulates S6 phosphorylation in T cells, namely through the MEK/ERK pathway.
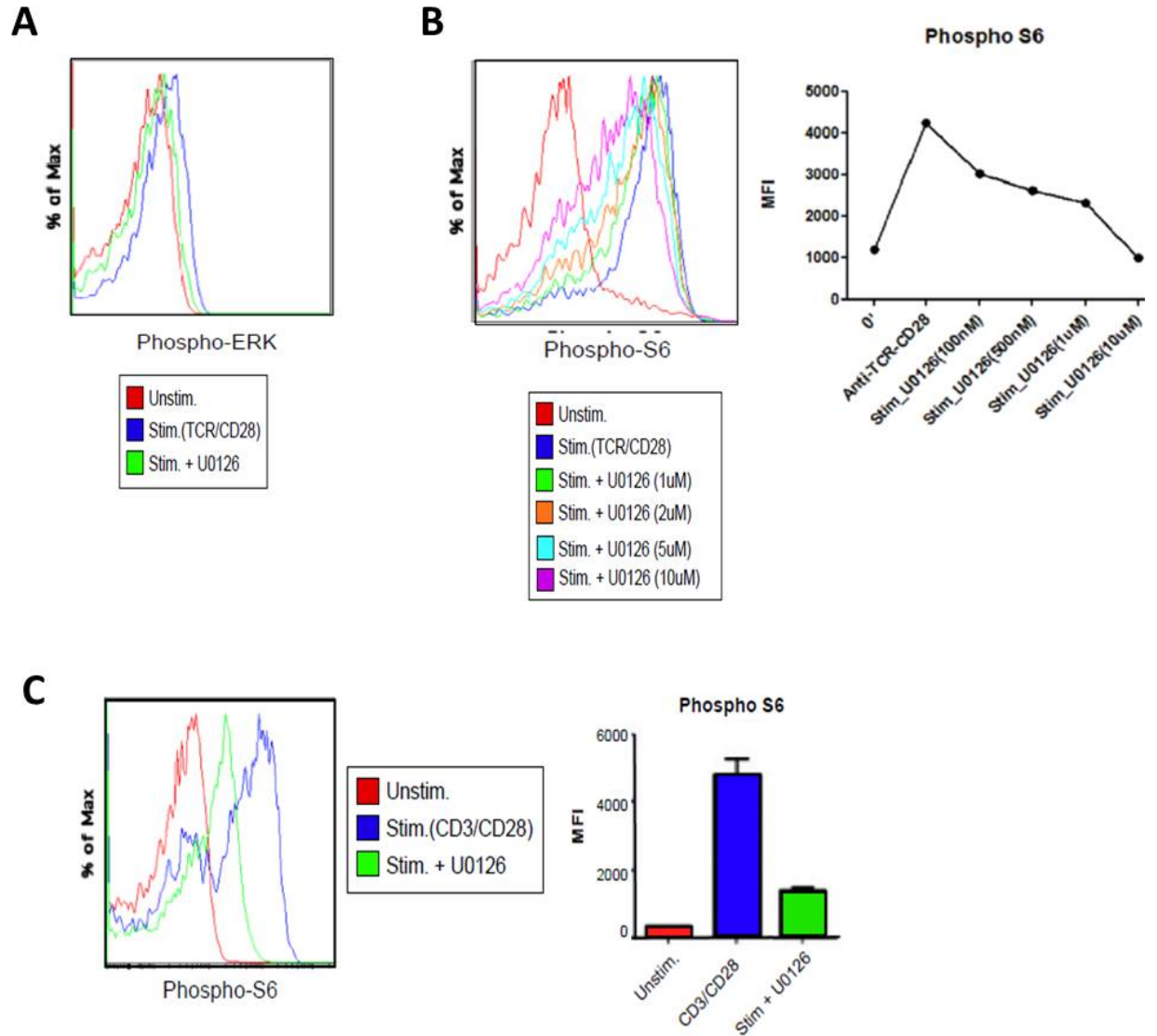
Figure 2-4 MEK/ERK pathway is required for stimulation dependent S6 phosphorylation in T cells. (A) ERK phosphorylation in Jurkats pretreated with U0126 (1uM) for 1 hour. (B) Jurkats were pretreated with varying concentrations of U0126, stimulated with anti-TCR/CD28 for 15 minutes and S6 phosphorylation assessed. (B) CD4 T cells were pretreated with U0126 (10uM) for 1 hour, stimulated for 30 minutes and S6 phosphorylation was assessed. All values represent mean + S.E.M of duplicates of a single experiment (C). Data are representative of two independent experiments.

2.4   DISCUSSION

Here, we show that induction of ribosomal protein S6 phosphorylation downstream of the TCR and co-stimulatory molecule CD28 requires PKC, acting through Carma1 and the MEK/ERK pathway (Figure 2-5). Previous work outlining S6 phosphorylation upon TCR and CD28 stimulation showed a requirement for PI3K/Akt activation, leading to mTORC1 and S6 phosphorylation, as the primary pathway (146). However, work published by the Cantrell group has identified alternate activators of the mTOR pathway, leading to differential responses in T cells (147, 173). Here, we have identified another mechanism downstream of the TCR leading to S6 phosphorylation. We propose that engagement of the TCR and CD28, which induces production of the second messenger DAG, activates PKC, leading to S6 phosphorylation through both Carma1and the MEK/ERK pathway.

Our experiments focused on a broad range of PKC isoforms, using the pan-PKC inhibitor BIM. Next, we would like to identify the specific PKC isoform(s) which may potentially regulate this pathway, as BIM targets both classical and novel PKC isoforms (see Chapter 1.2). The isoform PKC θ is a critical signaling molecule in T cells leading to NF-κB activation and so we hypothesize that this isoform may also play a significant role in the induction of S6 phosphorylation. We initially tried using the novel PKC inhibitor rottlerin, to more specifically establish a requirement for PKC θ; however, we noted that this inhibitor was non-specific and

had additional kinase targets, namely, Akt (Fig. 2-1B). Thus, we were unable to narrow the search for the PKC isoform. For future work, delineating the isoforms involved in S6 phosphorylation with stimulation, we would like to take genetic approaches, specifically knocking down PKC θ expression in T cells and to also use PKC θ KO T cells.

The requirement for Carma1 in PKC regulation of S6 phosphorylation in T cells does indicate that PKC θ is playing a role, since this PKC isoform has been shown to positively regulate Carma1 and downstream signaling (49, 60). We are interested in the mechanism through which PKC and Carma1 regulates S6, i.e. the potential requirements for Bcl10 and MALT1 (see Chapter 3). An interesting facet of this pathway involving Carma1 is that it is predominantly found in lymphocytes, so PKC regulation of S6 through Carma1 is mostly limited to lymphocytes (57). Thus, there seem to be additional mechanisms of S6 regulation in T cells (and potentially B cells), compared with other cell types.

We have also identified a role for the MEK/ERK pathway in the regulation of S6 phosphorylation in T cells stimulated through TCR/CD28. We propose two mechanisms whereby ERK phosphorylates the inhibitory TSC1/2 complex leading to mTORC1 activation, as well as association with p90S6K, which directly phosphorylates and activates S6 (164-166, 168). Additional experiments assessing TSC 1/2 phosphorylation and p90S6K activity will need to be performed to assess the precise mechanistic connections between the MEK/ERK and S6 pathways in T cells.

Through this work, we suggest additional novel regulators of S6 phosphorylation in TCR/CD28-stimulated T cells, namely PKC, Carma1 and MEK/ERK. We envision that PKC regulates S6 phosphorylation through Carma1 and MEK/ERK, although the actual mechanism needs to be teased out. We propose the presence of intermediary molecules between Carma1 and

the MEK/ERK pathway, since activation of the MEK/ERK pathway is unaffected by the absence

of Carma1 (174). We also do not rule out the possibility that these mechanisms may be working

through parallel pathways. Our findings contribute to the growing body of knowledge of S6 and

mTORC1 regulation, specifically in T cell
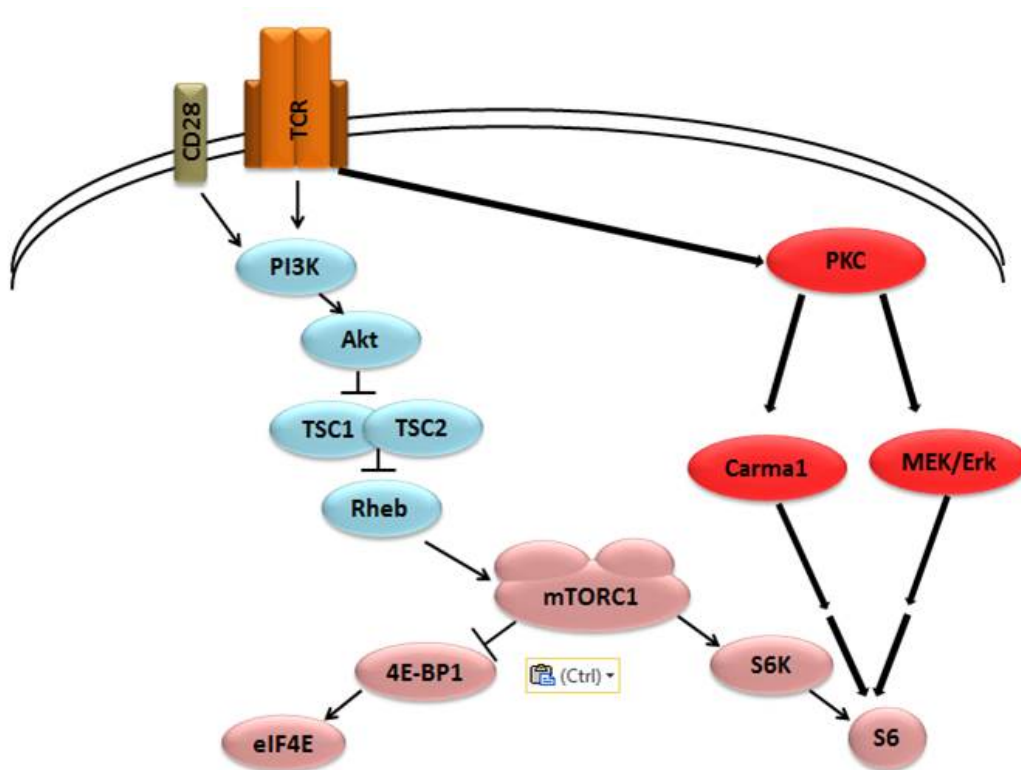
Figure 2-5 Model for TCR-dependent PKC regulation of S6 phosphorylation

3.0 A CARMA1/MALT1-DEPENDENT, BCL10 INDEPENDENT, PATHWAY REGULATES ANTIGEN RECEPTOR-MEDIATED MTOR SIGNALING IN T CELLS

3.1 INTRODUCTION

Upon antigenic stimulation, naïve T cells rapidly proliferate, produce cytokines and migrate from lymphoid organs, after which they mediate diverse effector functions in tissues. Dysregulation of T cell signaling events has been associated with autoimmune diseases and lymphomas, so dissection of the mechanisms leading to T cell activation may lead to more efficacious therapies. Signaling events initiated by antigen, growth factor and cytokine receptors lead to the activation of phosphatidylinositol 3-kinase (PI3K), Akt and the mechanistic target of rapamycin (mTOR), to regulate cellular growth and proliferation (175, 176). The 70 kD ribosomal protein S6 kinase (p70S6K), which directly phosphorylates ribosomal protein S6, is a key effector of mTOR (139). S6 itself is a critical regulator of protein translation, as it is necessary for ribosome biogenesis, and is thus an indirect regulator of cellular proliferation (139). Another important substrate of mTOR is the translational inhibitor eIF4E-binding protein 1 (4E-BP1), phosphorylation of which releases its inhibition of the translation of certain mRNA's (118) (Figure 1-6).

In T cells, engagement of the TCR and the co-stimulatory receptor CD28 induces activation of PI3K and Akt, which can lead to mTOR, p70S6K and S6 phosphorylation. Investigation into the specific roles of S6K and S6 in activation of T cells has revealed a

requirement for these proteins in cellular proliferation. Notably, heterozygous expression of S6 has been shown to limit T cell proliferation, although not cellular size or early activation events, after stimulation through the TCR (177). The serine/threonine kinase Akt is a central modulator of T cell signaling pathways controlling metabolism, growth, movement, and activation (149, 178, 179). However, recent studies have suggested that S6 phosphorylation downstream of the TCR and CD28 is not strictly dependent on Akt (180, 181).

Carma1 is an adaptor protein, predominantly found in lymphocytes, that interacts with B cell lymphoma 10 (Bcl10) and mucosa associated lymphoid tissue lymphoma translocation protein 1 (MALT1) upon antigen receptor stimulation, forming the "CBM" complex. This group of proteins is necessary for optimal activation of the NF-κB and c-Jun kinase (JNK) signaling pathways (58, 66). In addition, both Carma1 and MALT1 can act as tumor-promoting proteins in diffuse large B cell lymphoma (DLBCL) (77, 109, 110). Recent studies on the molecular mechanisms by which MALT1 regulates T cell activation have revealed it as a novel "paracaspase" (92, 95, 97, 182). Thus, inhibition of MALT1 catalytic activity with the selective inhibitor z-VRPR-fmk leads to partially impaired activation of NF-κB (95, 110).

Here we demonstrate a novel role for Carma1 and MALT1 in the activation of T cells, specifically through a signaling pathway leading to mTORC1 activation. Loss of Carma1 or MALT1 impaired TCR/CD28-mediated phosphorylation of S6, as well as another downstream target of mTOR, 4E-BP1. Surprisingly, by contrast, loss of the Carma1- and MALT1-associated protein Bcl10 had no discernable effect on mTORC1 activation. Furthermore, the MALT1 inhibitor z-VRPR-fmk inhibited both phosphorylation of S6 and proliferation of primary CD4+ T cells. Inhibition of MALT1 activity also impaired the ability of activated T cells to increase their metabolic output, which is largely regulated by the mTOR pathway (183-185). Thus, our

studies have revealed the existence of a previously unappreciated connection between Carma1, MALT1 and mTORC1, leading to enhanced T cell proliferation and metabolism.

3.2   METHODS AND MATERIALS

Mice

C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Carma1 KO spleens were a kind gift from Dr. X. Lin (MD Anderson, Texas). Bcl10 KO spleens were a kind gift from Dr. L. McAllister-Lucas (University of Pittsburgh). Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh.

Cell Separation

CD4+ T cells were isolated from spleen and lymph nodes and separated by MACS beads from Miltenyi Biotech (Auburn, CA) according to the manufacturer's protocol. The purity of the cell population was >90%.

Antibodies and Reagents

Phospho-specific antibodies to ribosomal protein S6 (S235/236 and S240/244), p70 ribosomal protein S6K (T389), mammalian target of rapamycin (mTOR) (S2448), tuberous sclerosis complex 2 (TSC2), 4E-BP1 (Thr70), phospho Akt substrate and Alexa Flour 647-conjugated S6 (S235/236 and S240/244) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies to ribosomal protein S6, 4E-BP1 and mTOR were also obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies targeting phosphorylated extra cellular signal regulated kinase (ERK) 1/2 (T202/Y204) were from BD Biosciences (San Diego, CA). MALT1, p70 S6K, Bcl10 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Carma1 antibody was obtained from ProSci Incorporated (Poway, CA). β-actin antibody was from Sigma (St. Louis, MO). Mouse anti-human CD28, hamster anti-mouse CD3 and CD28, PE-conjugated anti-CD69 were obtained from Life Technologies (Grand Island, NY). FITC conjugated anti-mouse CD71 and PE-conjugated anti-mouse CD98 were obtained from BioLegend (San Diego, CA). Protein A-HRP was from GE Healthcare (Piscataway, NJ). Antibody specific to the Jurkat TCR (C305), purified from the C305.2 hybridoma, was from ATCC (Manassas, VA). Biotin conjugated anti-mouse-CD3, -CD4 and -CD28 were obtained from BD Biosciences (San Jose, CA). Unconjugated anti-CD3/CD28 antibodies, rabbit anti-Syrian hamster IgG and secondary antibody, donkey anti-rabbit-HRP, were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). MALT1 inhibitor z-VRPR-fmk was obtained from A.G. Scientific (San Diego, CA). Recombinant human TNF-α was from R&D Systems (Minneapolis, MN). Phorbol-12-myristate-13-acetate (PMA), ionomycin, rapamycin and IKK inhibitor II (wedelolactone) were from Calbiochem/EMD (Billerica, MA). MEM amino acid solution was from Hyclone (Logan, Utah).

Cells

Jurkat T cells, Jurkat T cell mutants (JPM 50.6, MALT1shRNA, Bcl10shRNA, Carma1shRNA cells, Control shRNA cells and JPM+ Carma1 cells) and a fast-growing variant of the mouse T cell clone D10 were maintained as previously described (15, 167, 171).

Flow cytometry

Flow cytometry staining was performed as previously described (167). In short, cells were starved in 1% bovine serum albumin (BSA), in PBS for 1 hr at 37°C; for indicated experiments cells were also pretreated with inhibitor during starvation period. After stimulation cells were fixed with 1.5% paraformaldehyde for ten minutes at r.t. and permeablized with cold methanol on ice for fifteen minutes. Cell were then washed three times with buffer (PBS, 1% BSA) and stained with phospho specific antibodies at room temperature for up to 30 minutes. Samples were processed on BD LSRII instrument and data analyzed using FlowJo software.

Luciferase Assays

Jurkat T cells were transfected with 15 ug of NF-κB luciferase reporter vector by electroporation, cultured in complete medium (RPMI, 5% BGS and penicillin/ streptomycin) for 16-20 hrs. Cells were pre-treated with inhibitor before stimulation with anti-TCR and anti-CD28, PMA and ionomycin or rhTNFα, for six hours. Luciferase assays were performed as previously described (15).

Western Blotting and Immunoprecipitation

Cells were lysed with NP-40 lysis buffer (1%NP-40, 1mM EDTA, 20mM TrisHCl, 150mM NaCl) or with RIPA buffer (1% NP-40, 150 mM NaCl, 25 mM Tris-HCl, sodium deoxycholate, SDS) for immune-precipitation (IP) experiments and kinase assays and protease (aprotinin, pepstatin, 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF) and leupeptin) and phosphatase inhibitors (sodium fluoride, beta-glycerophosphate and sodium orthovandate) also added. Proteins were resolved on 8-10% SDS-PAGE gels, transferred to PVDF membrane and blocked in 4% BSA. Membranes were then probed with specified primary antibodies, followed by HRP-conjugated secondary antibodies. Proteins were detected using the SuperSignal West Pico ECL substrate (Thermo Scientific) and imaged on a Kodak Image Station. β-actin was used as a loading control for western blot experiments.

In vitro Kinase Assay

S6K was IP'd from stimulated Jurkat T cells and IPs were washed with kinase buffer (50mM Hepes, 200mM NaCl, 1% NP-40 and 1mM EDTA). Beads were incubated for 30 mins at 30°C in kinase reaction mix (kinase buffer, GST-S6, and γ-^{32}P-ATP), and reactions were stopped with 2x Laemmli sample buffer (Bio-Rad, Hercules, CA).

Lentiviral Transduction and Stable Cell Line

HEK 293T cells were co-transfected with Carma1 shRNA human pLKO.1 lentiviral vector or Carma1 in pLEX vector, psPAX2 packaging plasmid and pMO2.G envelope plasmid using Effectene reagent (Qiagen, Valencia, CA). Viral supernatant was collected 48 hours later and used for generation of the stable cell line. Jurkat T cells were cultured with viral supernatant and

complete medium at a 1:1 ratio and replaced with complete medium 24 hrs later. For control shRNA cells, Jurkat T cells were transiently transfected with shRNA and placed in selecting media two days later. Seeding and selection of positive clones using puromycin (Carma1shRNA and JPM + Carma1) or G418 (control shRNA) was as previously described (186).

MTT Assay

Primary T cell proliferation was assessed using the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Madison, Wisconsin). CD4+ T cells were stimulated with anti-CD3 and anti-CD28 for indicated times, cells were collected and incubated with MTS/PMS solution for 24 hrs at 37°C in a humidified, 5% CO_2 atmosphere. Absorbance was recorded at 490nm on an ELISA plate reader.

Seahorse analysis of T cell respiration

Naïve CD4+ T cells were isolated from C57BL/6 mice, pretreated with z-VRPR-fmk or rapamycin, where indicated and stimulated for 24 hours in a XF24 cell culture microplate (Seahorse Bioscience). Cells were then analyzed using the Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience) as previously described (187).

3.3 RESULTS

3.3.1 Carma1 regulates phosphorylation of ribosomal protein S6 in T cells

Our group previously showed that Akt and Carma1 function cooperatively during T cell activation, specifically via the transcription factor NF-κB (37). To understand other possible downstream effectors of Akt and Carma1, we compared phosphorylation of potential Akt substrates between the parental human Jurkat T lymphoma cells, and a variant lacking Carma1 - JPM 50.6 (174). Jurkat T cells, JPM 50.6 cells and JPM 50.6 cells re-expressing Carma1 were stimulated with anti-TCR and anti-CD28 antibodies. Employing a phospho-Akt substrate antibody for western blotting, we detected a prominent band of about 30 kD (note arrow) with differing patterns of phosphorylation between parental, mutant and reconstituted Jurkat T cells (Fig. 3-1A). Importantly, Akt activation itself is normal in JPM 50.6 cells, as shown by us and others (37, 174). However, Akt falls within the large class of AGC kinases and as such shares at least partial substrate specificity (RxRxxS/T) with these kinases, notably the protein kinase p70S6K (R/KxRxxS/T) (188). Based on the mobility of the band noted in Fig. 3-1A, we hypothesized that it may be the ribosomal protein S6, a prominent substrate for p70S6K and a downstream effector of the mTOR pathway that is activated by growth factor or T cell receptor signaling (115). Thus, we directly assessed activation of S6 induced by the T cell receptor and co-stimulatory molecules through phosphorylation at sites S235/236. We confirmed our suspicion by flow cytometry with a phospho-specific antibody, which revealed impaired induction of S6 phosphorylation in Carma1-deficient JPM 50.6 cells, compared with parental Jurkat T cells (Fig. 3-1B). Next, we examined phosphorylation of S6 at an additional cluster of

phosphorylated residues - S240/244 - another established readout for S6 phosphorylation (176). Again, we noted decreased S6 phosphorylation at these sites in JPM 50.6 cells, compared with parental Jurkat T cells (Fig. 3-1C). We sometimes observed increased basal S6 phosphorylation in the JPM 50.6 cells (Fig. 3-1B). To determine whether other pathways of S6 phosphorylation were normal in JPM 50.6 cells, we assessed amino acid-dependent stimulation after starvation (189), and found that this treatment elicited a similar response in Jurkat T cells and JPM 50.6 cells, while TCR/CD28-mediated S6 phosphorylation was still impaired (Fig. 3-1D). Thus, the cellular response to amino acid levels, which also results in S6K activation and S6 phosphorylation, does not require Carma1. We also assessed total S6 protein expression in Jurkat T cells and JPM50.6 cells and found similar levels of the protein between the cell lines, as expected (Fig. 3-1E). These data suggest that regulation of S6 by Carma1 may be relatively receptor- or cell type-specific.

JPM 50.6 cells were originally derived by chemical mutagenesis of Jurkat T cells (174). To further validate the results obtained with these cells, we specifically knocked down Carma1 expression in parental Jurkat T cells. We then analyzed phosphorylation of S6 after anti-TCR/CD28 antibody stimulation of parental Jurkat T cells and Carma1 knock-down cells, ("Carma1shRNA," in which we achieved over 75% knock-down of Carma1 protein). Similar to results obtained with JPM 50.6 cells, we found much weaker induction of S6 phosphorylation in Carma1 knock-down cells, compared with Jurkat T cells knocked down with control shRNA (Fig. 3-1F). To further confirm our findings with primary T cells, we assessed S6 phosphorylation after PMA/ionomycin stimulation of naive T cells from Carma1 KO and WT mice (44). Consistent with the results obtained with cell lines described above, we observed impaired induction of S6 phosphorylation in the Carma1 KO T cells (Fig. 3-1G). Taken together,

these data reveal the existence of a previously unrecognized role for Carma1 in TCR signaling

events that regulate phosphorylation of ribosomal protein S6.
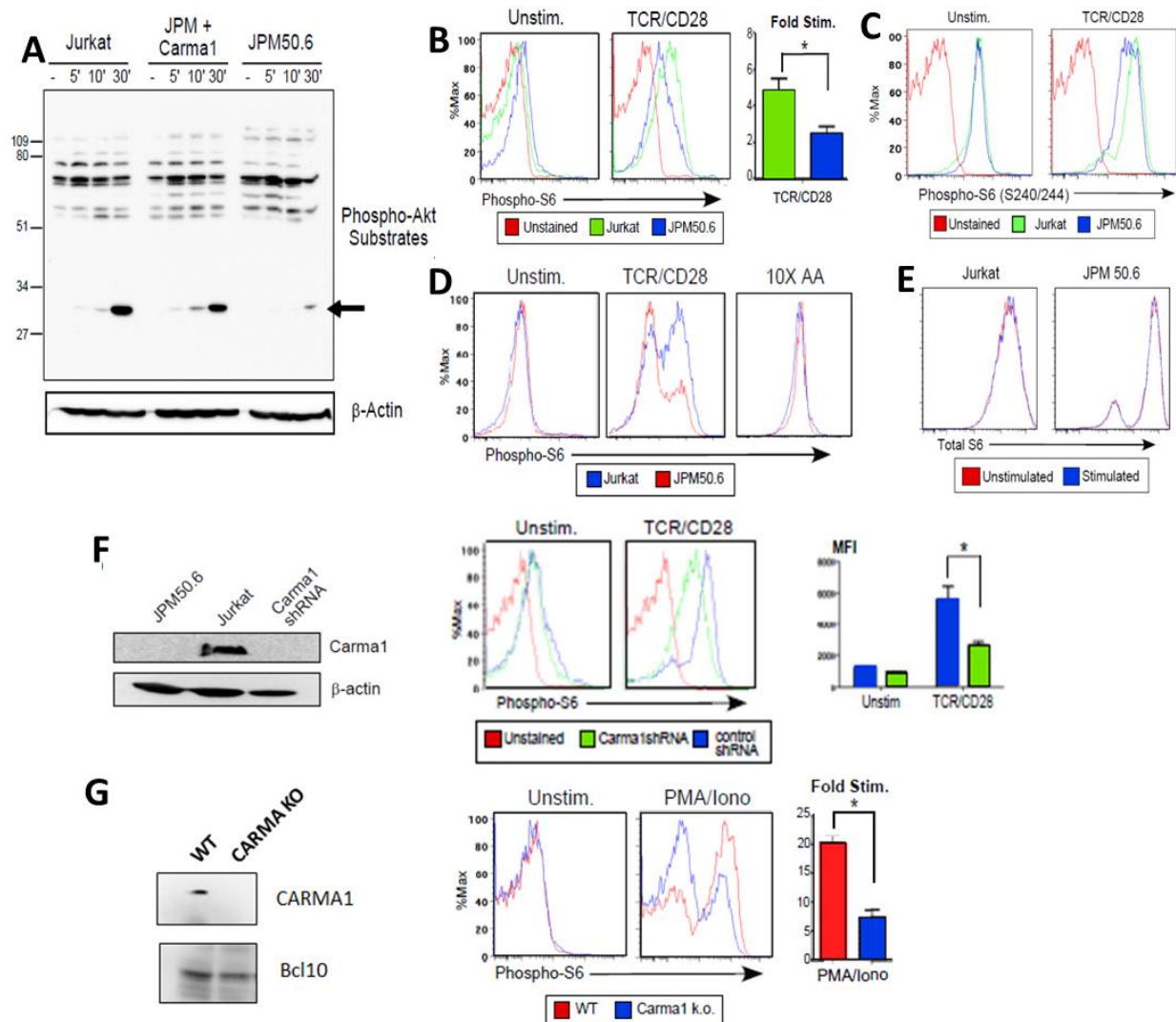
Figure 3-1 Carma1 is required for S6 phosphorylation in T cells. (A) Western blot of Jurkat T cells, JPM 50.6 cells reconstituted with Carma1 (JPM + Carma1) and JPM 50.6 cells stimulated for the indicated times and probed with phospho-Akt substrate antibody. (B-D) Flow cytometry histograms of Jurkat T cells and JPM 50.6 cells stimulated with anti-TCR/CD28 (B and C) or with amino acids (D) and stained with Alexa-647 conjugated anti-phospho-S6 (S235/236) (B and D) or phospho-S6 (S240/244) (C), fold induction (relative to un-stimulated). (E) Total S6 phosphorylation in Jurkat T cells and JPM 50.6 cells. (F and G) Western blot (*left*) and flow cytometry histograms (*right*) of Control shRNA cells and Carma1shRNA (F) or WT or Carma1 KO primary CD4+ T cells (G) stimulated with TCR/CD28 (F) or PMA/ionomycin (G). Values represent mean +/- S.E.M of three independent experiments. Carma1 KO data are representative of duplicates from a single experiment. The unpaired Student's t-test was used to determine significance: *p < 0.05; **p <0.01. Data are representative of three independent experiments.

3.3.2   Carma1 regulation of p70S6K activation and 4E-BP1 phosphorylation

Our results showing a requirement for Carma1 in S6 phosphorylation led us to assess activation of the upstream regulators of S6, i.e. TSC and mTOR, after TCR and CD28 stimulation (Fig. 3-2A and B). Our experiments suggested that inducible phosphorylation of TSC2 and mTOR in Jurkat T cells and JPM 50.6 cells was similar, although we could only ever achieve modest, if any, inducible phosphorylation of mTOR or TSC2 by stimulation with anti-TCR/CD28 antibodies (Fig. 3-2A and B). To further define the role of Carma1 in mediating phosphorylation of S6 and associated pathways, we assessed activity of the kinase immediately upstream of S6 - p70S6K (S6K). After TCR and CD28 engagement, this kinase becomes activated, leading to phosphorylation of S6 and increased ribosome biogenesis (115). We directly assessed S6K phosphorylation in stimulated JPM 50.6 cells and Jurkat T cells expressing Carma1, and also noted impaired S6K phosphorylation i   the JPM 50.6 cells (Fig. 3-2C). Additionally, we performed *in vitro* kinase assays with S6K isolated from Jurkat T cells and JPM 50.6 cells, using GST-S6 as a substrate. Thus, we observed an increase in S6K activity after stimulation of parental Jurkat T cells, but not Carma1-deficient JPM 50.6 cells (Fig. 3-2D).

Since S6K activity can be regulated by mTOR (115), we investigated this pathway in more detail. We then sought to determine if other downstream regulators of mTOR, e.g. 4E-BP1, are regulated by Carma1. Investigation of this pathway in JPM 50.6 cells revealed that these cells do not express the protein 4E-BP1, or the mRNA encoding this protein (Fig. 3-2E) and (data not shown), so we employed the Carma1 knock-down cells described above to determine a possible role for Carma1 in regulation of 4E-BP1. Thus, we observed substantially reduced TCR/CD28-

inducible 4E-BP1 phosphorylation in the Carma1 deficient Jurkat T cells, despite apparently normal expression levels of 4E-BP1 itself (Fig. 3-2F). These results indicate that Carma1 also plays a role in regulation of mTOR activation, upstream of S6K, S6 and 4E-BP1.
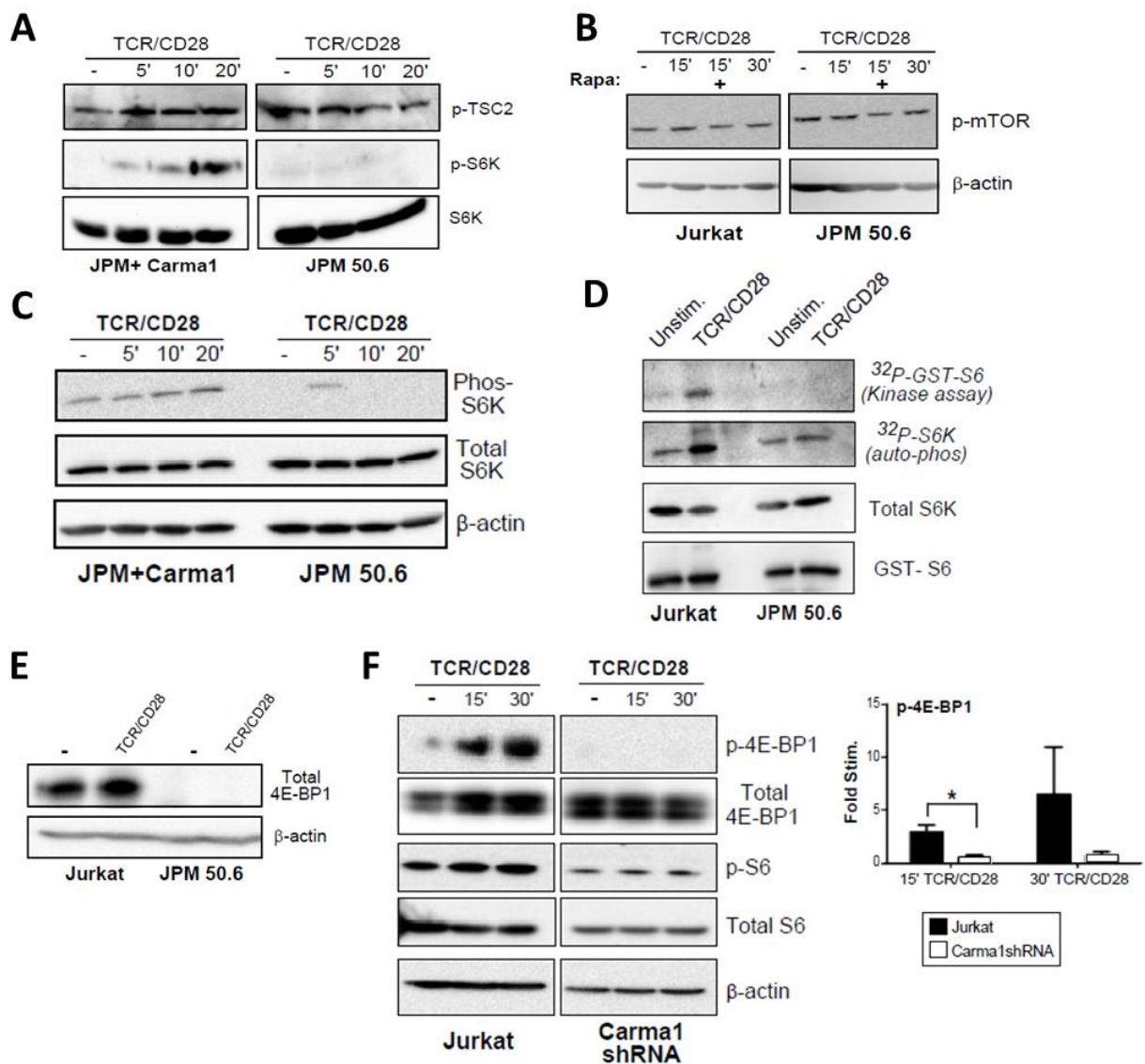
Figure 3-2 Carma1 is required for mTORC1 activation in T cells. (A) TSC2 phosphorylation in JPM+Carma1 and JPM 50.6 cells stimulated with anti-TCR/CD28 for indicated times. (B) mTOR phosphorylation in Jurkat T cells and JPM 50.6 cells stimulated with anti-TCR/CD28, where indicated cells were pretreated with rapamycin (50nM). (C) S6K phosphorylation in JPM+Carma1 and JPM 50.6 cells stimulated with anti-TCR/CD28. (D) *In vitro* kinase assay with S6K IP'd from stimulated Jurkat T cells or JPM 50.6 cells, using GST-S6 as an exogenous substrate (top panels); S6K auto-phosphorylation is also shown (second row from top). Lower rows: western blot of total S6K and GST-S6. (E) 4E-BP1 expression in Jurkat T cells and JPM 50.6 cells (F) Jurkat T cells and Carma1shRNA cells were stimulated with anti-TCR/CD28 and immunoblotted for phospho 4E-BP1 or phospho S6 (*top*), graph of densitometry of fold induction of normalized values (phospho 4E-BP1, normalized to total 4E-BP1 expression) (*bottom*).

3.3.3   Role of MALT1 in TCR/CD28-mediated mTOR signaling

Activation of NF-κB by Carma1 is mediated by at least two other proteins - Bcl10 and MALT1 (31). After TCR and CD28 engagement, these proteins associate to form the Carma1, Bcl10 and MALT1 (CBM) complex, in which they function to activate downstream pathways, including IKK activation (31). Thus, we first sought to determine if MALT1 also plays a role in activation of S6, by using T cell lines with greatly reduced expression of MALT1 (190) (Fig. 3-3A). Our results assessing differences in mTOR phosphorylation in Jurkat T cells and MALT1shRNA cells were similar to our findings in JPM 50.6 cells (Fig. 3-2B), whereby we noted little difference between parental Jurkat T cells and MALT1 knockdown cells (Fig. 3-3B). Consistent with the findings in JPM 50.6 and Carma1shRNA cells discussed above, we observed impaired phosphorylation of S6 after TCR and CD28 stimulation of MALT1shRNA cells (Fig. 3-3C). Thus, these results suggest that MALT1, like Carma1, is required for optimal phosphorylation of S6 downstream of TCR/CD28 signaling. We also assessed 4E-BP1 phosphorylation in MALT1shRNA cells (Fig. 3-3D). Similar to the phenotype observed in Carma1shRNA cells, there was greatly reduced 4E-BP1 phosphorylation in cells with reduced MALT1 expression. We also assessed S6K phosphorylation and noted little TCR/CD28-dependent phosphorylation in MALT1shRNA cells compared with Jurkat T cells (Fig. 3-3E). These data reveal a requirement for MALT1 in inducible phosphorylation of the mTOR targets S6K, S6 and 4E-BP1, downstream of TCR and CD28.

To further delineate the mechanism of MALT1-dependent S6K activation, we investigated whether these proteins might be present together in a biochemical complex. We IP'd MALT1 from T cell lysates and probed for the presence of S6K. Anti-MALT1 IP from the

MALT1shRNA cells served as a negative control. Thus, we observed an association between MALT1 and S6K in Jurkat T cells (Fig. 3-3F). We were also able to detect mTOR in a complex with MALT1 (Fig. 3-3F), although it is still unclear whether any of these interactions is direct. Interestingly, however, formation of this complex appears to occur independently of TCR signaling, at least in Jurkat T cells.
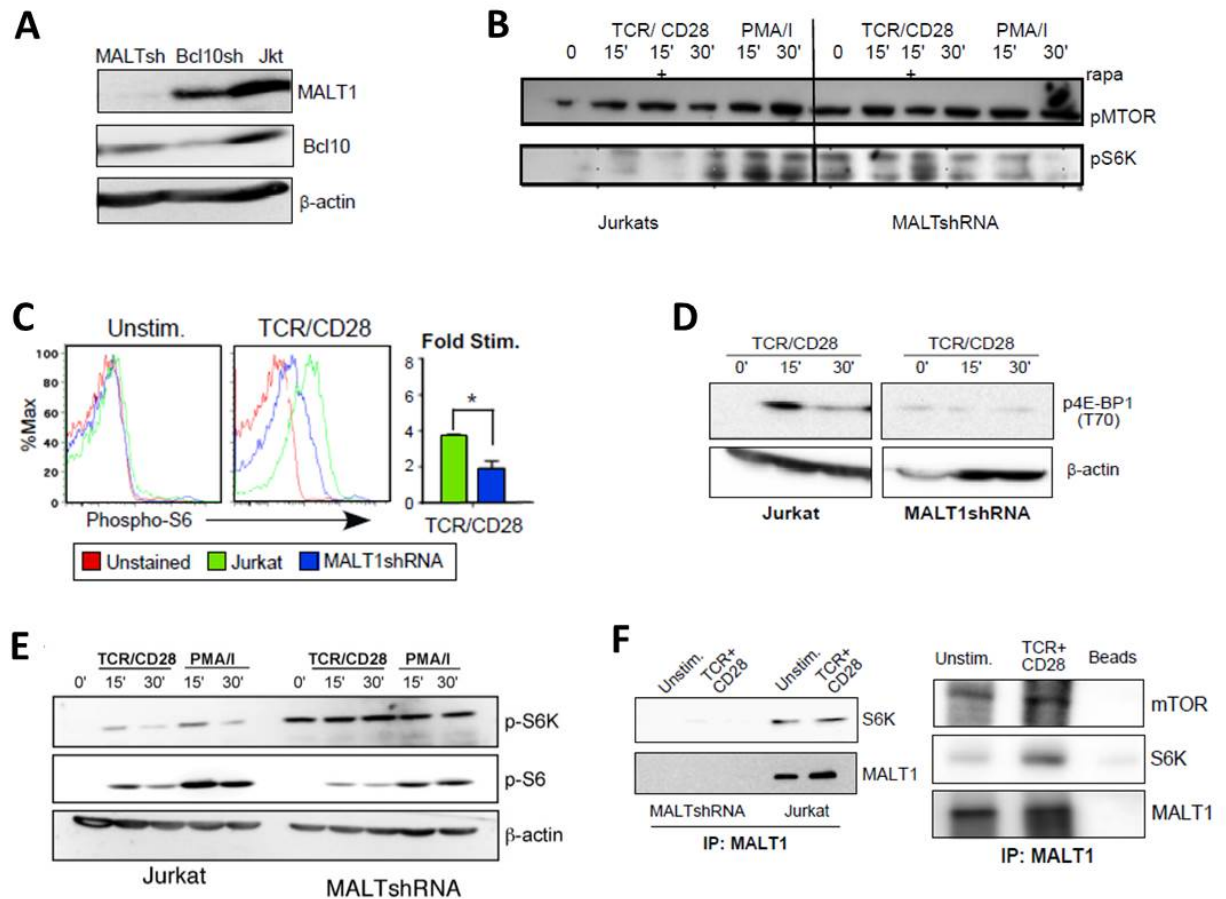
Figure 3-3 MALT1 is required for mTORC1 activation in T cells. (A) Western blot of parental Jurkat T cells, MALT1- and Bcl10-knockdown cells showing MALT1 and Bcl10 expression. (B) mTOR phosphorylation in Jurkat T cells and MALTshRNA stimulated with anti-TCR/CD28; cells were pretreated with rapamycin where indicated. (C) Jurkat T cells and MALTshRNA cells were stimulated for 15 minutes with anti-TCR/CD28 antibodies and S6 phosphorylation (S235/236) was assessed by flow cytometry. (D and E) 4E-BP1 (D) and S6K (E) phosphorylation in Jurkat T cells and MALTshRNA cells stimulated with anti-TCR/CD28 and/or PMA/ionomycin (E). (F) MALT1 was immune-precipitated from Jurkat T cells and S6K (*left*) and mTOR (*right*) association assessed by western blotting. All values represent mean +/- S.E.M of three independent experiments. The unpaired Student's t-test was used to determine statistical significance: *p < 0.05; **p <0.01. Data are representative of three independent experiments.

3.3.4 Role of Bcl10 in TCR/CD28-mediated mTOR signaling

Since our data revealed a requirement for Carma1 and MALT1 in activation of the mTORC1 pathway, we next assessed a potential role for the third member of the CBM complex, Bcl10. Thus, we sought to determine if Bcl10 also plays a role in activation of S6, by using T cell lines with greatly reduced expression of Bcl10 (190) (Fig. 3-3A). We were surprised to observe apparently normal S6 phosphorylation in Bcl10shRNA cells (Fig. 3-4A). To confirm these results, we stimulated CD4+ T cells isolated from WT or Bcl10 KO mice and found that both sets of cells were able to induce S6 phosphorylation at comparable levels (Fig. 3-4B and C). We also assessed S6K phosphorylation in WT or Bcl10 KO cells with CD3 and CD28 or PMA/ionomycin stimulation and noted similar S6K phosphorylation in both sets of cells (Fig. 3-4D). Thus, these results suggest that while Carma1 and MALT1 are required for optimal phosphorylation of S6 downstream of TCR/CD28 signaling, Bcl10 does not play as critical a role in this pathway. The fact that S6 phosphorylation is normal in Jurkat T cells lacking Bcl10 also suggests that the impairment in S6 phosphorylation in Carma1-deficient T cells is not merely a result of dysregulated NF-κB activity. Nonetheless, we confirmed this more directly by assessing the effect of a small molecule inhibitor of IKK on S6 phosphorylation. This compound had no effect on CD3/CD28-mediated S6 phosphorylation in primary T cells (Fig. 3-4E), indicating that this previously unappreciated pathway is indeed independent of IKK signaling.
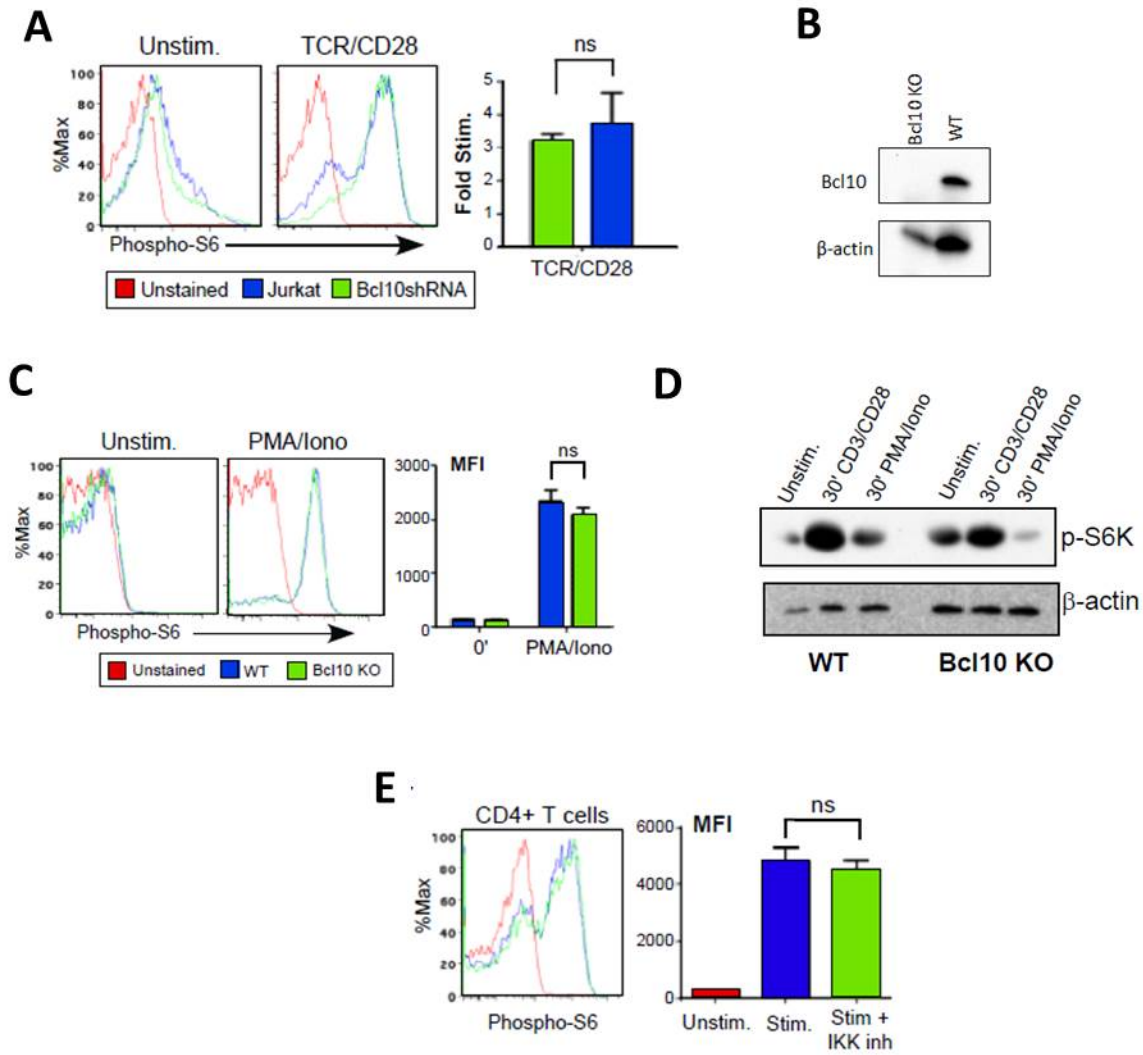
Figure 3-4 Bcl10 is not required for S6 phosphorylation in T cells. (A) Parental Jurkat T cells and Bcl10 knockdown cells were stimulated for 15 minutes with anti-TCR/CD28 antibodies, and S6 phosphorylation (S235/236) was assessed by flow cytometry. (B) Bcl10 expression in WT or Bcl10 KO primary cells. (C) Phosphorylation of S6 in PMA/ionomycin-stimulated WT or Bcl10 KO primary CD4+ T cells. (D) Western blot of stimulated WT of Bcl10 KO primary CD4+ T cells and probed with a phospho-S6K antibody. (E) Primary CD4+ T cells were pre-treated with the IKK-2 inhibitor, then stimulated for 30 minutes and S6 phosphorylation was assessed. All values represent mean +/- S.E.M of three independent experiments. For primary cell experiment values represent mean +/- S.E.M. of triplicates (C) or duplicates (E) of a single experiment. The unpaired Student's t-test was used to determine statistical significance: *p < 0.05; **p <0.01. Data are representative of three independent experiments, except for panel D, which was performed once.

3.3.5　MALT1 catalytic activity regulates S6 phosphorylation

Selective inhibition of MALT1 paracaspase activity with the compound z-VRPR-fmk partially inhibits activation of NF-κB, and retards the growth of activated B cell-like diffuse large B cell lymphoma (ABC DLBCL) cell lines (97, 100, 110). Consistent with earlier studies, we confirmed that this inhibitor partially prevented activation of an NF-κB reporter by TCR/CD28 stimulation, but not by TNF (Fig. 3-5A). In addition, z-VRPR-fmk had no effect on ERK phosphorylation (Fig. 3-5B). However, pre-treatment of Jurkat T cells with z-VRPR-fmk impaired mTOR activation, as revealed by decreased S6 phosphorylation (Fig. 3-5D). These results were recapitulated in primary murine CD4+ T cells, in which pre-treatment with z-VRPR-fmk significantly inhibited CD3/CD28-mediated phosphorylation of S6 (Fig. 3-5E). To ensure the specificity of this inhibitor we assessed S6 phosphorylation in Jurkat T cells lacking MALT1 (Fig. 3-3A) and did not see any impairment of S6 phosphorylation in these MALT1 deficient T cells with z-VRPR-fmk treatment (Fig. 3-5C). We also assessed S6K phosphorylation in primary murine CD4+ T cells with z-VRPR-fmk treatment for up to three days and noted impaired S6K phosphorylation compared with untreated cells (Fig. 3-5F). These results reveal a specific role for the catalytic activity of MALT1 in regulation of the mTOR pathway in T cells.
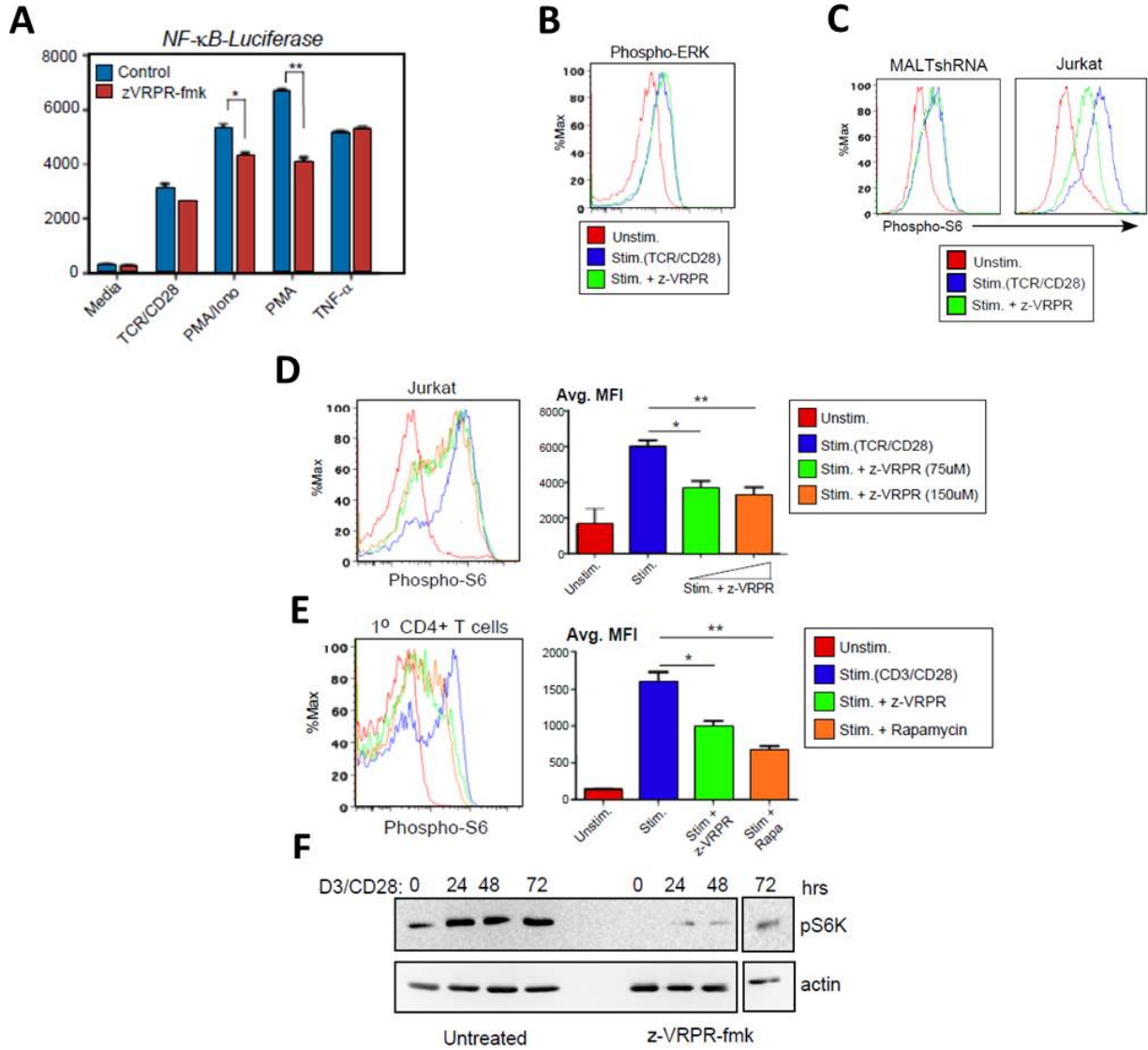
Figure 3-5 MALT1 catalytic activity regulates S6 phosphorylation. (A) NF-κB luciferase reporter assay of Jurkat T cells pre-treated with z-VRPR-fmk (75 uM) and stimulated with anti-TCR/CD28, PMA/ionomycin or rhTNF-α for six hours. (B) Jurkat T cells were pre-treated with z-VRPR-fmk, stimulated and stained with Alexa-647 conjugated phospho-ERK antibody. (C) S6 phosphorylation in Jurkat T cells and MALTshRNA cells pretreated with z-VRPR-fmk (75uM) and stimulated. (D and E) S6 phosphorylation (S235/236) in Jurkat T cells (C) or primary murine CD4+ T cells (D) pre-treated with z-VRPR-fmk before stimulation with anti-TCR/CD28 (Jurkat T cells) or anti-CD3/CD28 (CD4+ T cells) antibodies. (F) S6K phosphorylation in stimulated CD4+ T cells untreated or pretreated with z-VRPR-fmk. Values represent the mean +/- S.E.M of three independent experiments. The unpaired Student's t-test was used to determine statistical significance: * p< 0.05; **p <0.01. Data are representative of three independent experiments. For CD4+ T cells, data are representative of triplicates of a single experiment.

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3.3.6 MALT1 catalytic activity regulates proliferation, but not early activation events, in T cells

Previous studies on the functions of Carma1, Bcl10 and MALT1 in T cell activation have predominantly focused on regulation of the transcription factor NF-κB (31, 58, 94). As our data indicated a requirement for Carma1 and MALT1 in optimal mTOR activation upon T cell stimulation, we next sought to investigate the functional consequences of this novel pathway. Heterozygous expression of S6 has been shown to impair proliferation, but not the increase in cell size ("blasting"), that follows T cell activation (177). Based on the role of MALT1 in S6 phosphorylation that we have uncovered, we hypothesized that treatment of naïve T cells with the MALT1 inhibitor might phenocopy the effects of reduced S6 expression on T cell proliferation. We pre-treated naïve CD4+ T cells with z-VRPR-fmk and stimulated with anti-CD3 and anti-CD28 antibodies for up to 72 hours and assessed cell number, cell size and activation markers. As expected, there was an initial drop in T cell numbers, due to cell death induced by the strong polyclonal anti-CD3 stimulation (Fig. 3-6A). While this trend eventually reversed by 72 hours in the vehicle-treated T cells, due to T cell proliferation, this reversal was not observed in cells treated with z-VRPR-fmk. We also assessed the effects of z-VRPR-fmk on T cell proliferation more directly with an MTT assay. This was done in the presence of exogenous IL-2 to ensure that the reduction in cell numbers was not due to reduced autocrine production of IL-2, as might be expected due to partial inhibition of NF-κB by z-VRPR-fmk. Thus, z-VRPR-fmk impaired T cell proliferation by primary CD4+ T cells (Fig. 3-6B), even in the presence of exogenous IL-2. Because IL-2 can also activate S6 via the mTOR pathway (191), this suggests the existence of a non-redundant TCR-CD28-dependent pathway that requires

MALT1 catalytic activity. Consistent with previous findings on S6 (177), we saw that the decreased number of z-VRPR-fmk-treated cells was not reflected in altered T cell blasting. Thus, cell size, as measured by forward light scattering, was similar between vehicle and inhibitor treated T cells (Fig. 3-6C). Also of note, initial activation of MALT1 inhibitor-treated T cells was comparable to cells treated with vehicle control, as determined by expression of the early activation marker CD69 (Fig. 3-6D). Thus, we have not only shown a requirement for Carma1 and MALT1 in phosphorylation of S6 and its upstream regulator S6K, but also that this pathway controls a known downstream functional target of S6 in T cells, namely cellular proliferation, while being dispensable for early activation events.
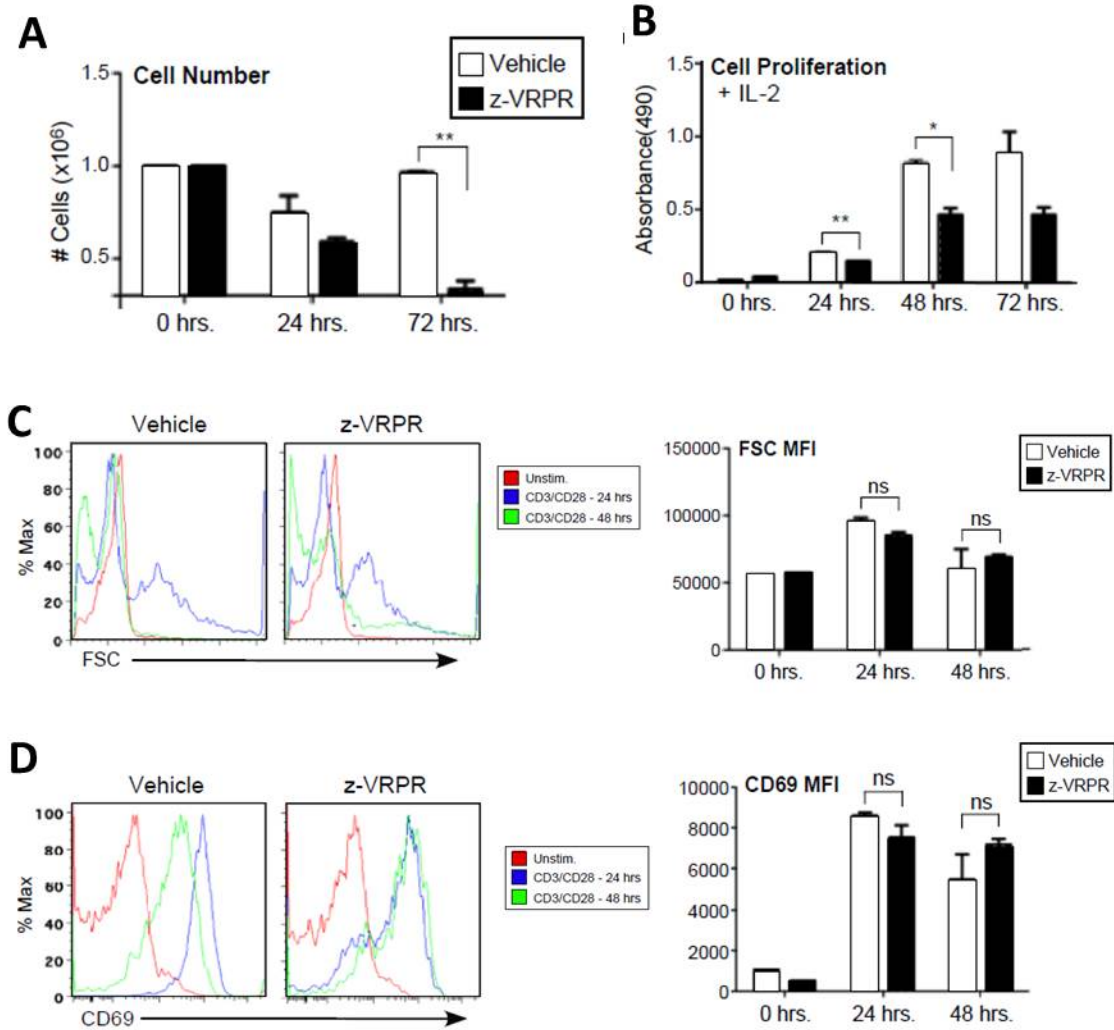
Figure 3-6 MALT1 regulates T cell proliferation but not early activation events. (A) Enumeration of primary murine CD4+ T cells, pre-treated with vehicle or z-VRPR-fmk, and stimulated with anti-CD3/CD28 over the course of three days. (B) MTT Assay of stimulated CD4+ T cells pre-treated with vehicle or inhibitor in the presence of exogenous IL-2. (C) Size (forward scatter) of CD4+ T cells pre-treated with or without inhibitor. (D) Expression of CD69 on CD4+ T cells stimulated with anti-CD3/CD28 over three days. (C-D) Left - representative flow cytometry; right – combined data from duplicate points from a single experiment, representative of three that were performed. Values represent mean +/- S.E.M; n=2. The unpaired Student's t-test was used for statistical analysis: * p< 0.05; **p <0.01.

3.3.7 MALT1 regulates the metabolic switch in activated T cells

Naïve T cells are relatively quiescent with respect to cellular metabolism. However, upon activation through the TCR, T cells undergo a dramatic transcriptional and post-transcriptional reprogramming (192). The mTOR pathway is a critical regulator of this increase in cellular metabolism when T cells shift from a quiescent to activated state (7, 131, 184). As our data revealed a role for MALT1 in regulation of the mTOR pathway, we further assessed the functional consequences of this effect. To this end, we assessed oxidative metabolism in CD4+ T cells stimulated overnight in the presence of the MALT1 inhibitor, z-VRPR-fmk, or the mTORC1 inhibitor rapamycin. As expected, we observed a significant increase in oxygen consumption in stimulated T cells compared with naïve T cells. Treatment with z-VRPR-fmk or rapamycin inhibited the shift to increased metabolic activity (Fig. 3-7A). This effect was apparent both in the basal state and after treatment with FCCP, which induces maximal oxygen consumption (Fig. 3-7A). We also assessed the role of MALT1 in the upregulation of aerobic glycolysis (ECAR). As expected, stimulation of naïve T cells dramatically increased glycolysis (Fig. 3-7B). z-VRPR-fmk partially inhibited glycolysis in stimulated T cells, though not to the extent of rapamycin (Fig. 3-7B). Upregulation of CD98 (an amino acid transporter) and CD71 (transferrin receptor) in activated T cells have also been described as mTOR-dependent events (193, 194). However, we noted no difference in the upregulation of these markers in z-VRPR-fmk treated T cells (Fig. 3-7D). Importantly, and consistent with data discussed above, we noted similar CD69 expression under all conditions, indicative of the efficiency of early TCR-mediated activation, regardless of MALT1 inhibitor treatment (Fig. 3-7C).
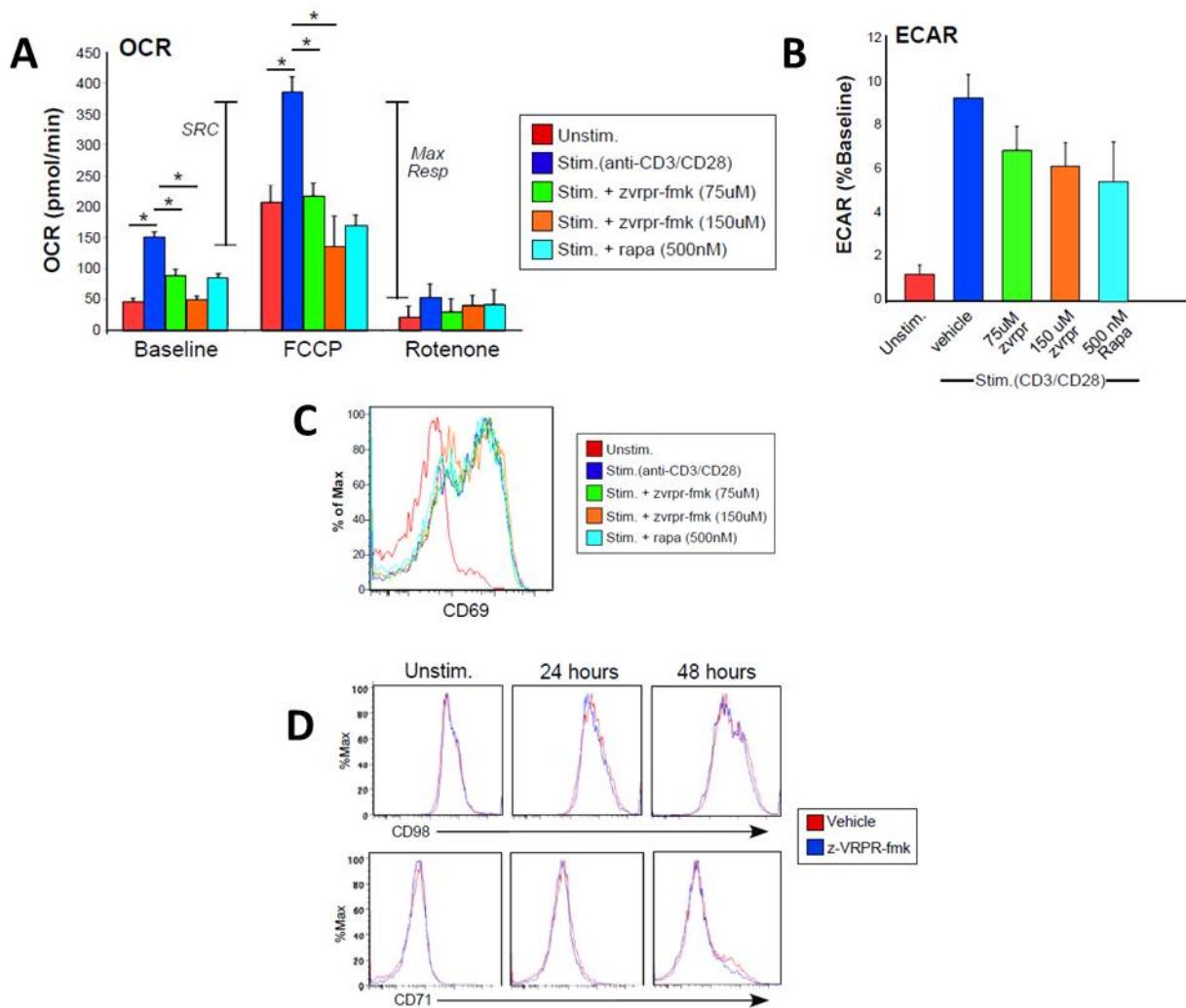
Figure 3-7 MALT1 regulates the metabolic switch in activated T cells. Naïve T cells were isolated from C57Bl/6 mice, pretreated with the indicated inhibitors and stimulated in vitro with anti-CD3/CD28 antibodies. After 24hrs cells, were analyzed on a Seahorse XF24 analyzer. The oxygen consumption rate (OCR) (A) and extracellular acidification rate (ECAR) (B) were determined. (C) Equivalent activation of the cells was confirmed by flow cytometry analysis for CD69 after conclusion of the Seahorse analysis. (D) CD98 (*top*) and CD71 (*bottom*) expression in CD4 T cells pretreated with inhibitor and stimulated with antiCD3/CD28 for indicated times.

3.4 DISCUSSION

It was previously thought that TCR and CD28-dependent S6 phosphorylation proceeds exclusively through Akt, the TSC1/TSC2 complex, mTOR and S6K (130). However, recent studies have introduced additional complexity to this simple linear model, specifically with regard to the Akt requirement, as shown recently in CD8+ T cells (149). Here we reveal a previously unappreciated role for the proteins Carma1 and MALT1 in signaling downstream of mTOR, resulting in phosphorylation of ribosomal protein S6 and the translation regulator 4E-BP1, during T cell activation (Fig. 3-8). While we have uncovered novel roles for Carma1 and MALT1, surprisingly we found that Bcl10 is dispensable for S6 phosphorylation in T cells. Although most studies indicate that Carma1 and Bcl10 act in concert to induce T cell activation, Carma1 has been shown to disassociate from Bcl10 and bind other proteins (e.g. GAKIN) upon TCR engagement (195).

Our results demonstrate that Carma1 and MALT1 proteins also regulate the phosphorylation of another target of mTOR signaling - 4E-BP1. Thus, we present a model whereby Carma1 and MALT1 regulate mTORC1 activation and its major downstream targets S6K and 4E-BP1 (Fig. 3-8). We found that MALT1 associates with both p70S6K and mTOR, consistent with its apparent role in regulating this signaling pathway in T cells, although it is not clear if either of these interactions is direct. While mTOR is found in two different protein

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complexes (mTORC1and mTORC2), we hypothesize that MALT1 specifically associates with and regulates mTORC1, since Akt phosphorylation at S473, a direct target of mTORC2, is normal in Carma1-deficient T cells (37, 174, 176). Further study will be necessary to fully understand the underlying molecular mechanism(s) of this previously unappreciated pathway.

Given the known role of the CBM complex in the NF-κB pathway, we considered the possibility that the impairment of mTOR activation in Carma1 or MALT1 deficient T cells (or treatment with z-VRPR-fmk) might be a secondary effect of decreased NF-κB activity. However, a small molecule IKK inhibitor did not impair S6 phosphorylation in primary CD4+ T cells, suggesting that regulation of S6K and S6 activity by Carma1 and MALT1 is independent of their roles in NF-κB activation. Regulation of NF-κB by the CBM complex also occurs in other cell types, for example monocytes, where the Carma1 homolog CARD9 regulates activation of NF-κB (68). Knowing that these proteins have similar functions in other cell types, we are interested in determining if activation of mTOR is another pathway that they might regulate.

We often noted increased basal S6 phosphorylation in Carma1-deficient Jurkat T cells, which may be due to a persistent inhibition of mTORC1, as this has been shown to activate insulin receptor and, and thus S6 phosphorylation (196). Nonetheless, use of extended nutrient starvation and investigation into additional S6 phosphorylation sites (S240/244, which are indicative of activation, Fig. 3-1C) confirmed our findings that Carma1 is indeed a positive regulator of TCR/CD28-dependent mTOR activation (data not shown). Furthermore, T cells from Carma1 KO mice did not have increased basal S6 phosphorylation (Fig. 3-1G). Finally, treatment of primary T cells with the MALT1 inhibitor z-VRPR-fmk did not result in increased basal S6 phosphorylation, although it did significantly impair CD3/CD28 -induced S6 phosphorylation.

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The MALT1 inhibitor z-VRPR-fmk partially decreases NF-κB activation in lymphocytes (97). This compound specifically targets the paracaspase domain of MALT1 (as opposed to classical caspases), and has provided evidence for catalytic activity of MALT1, which was previously thought to act solely as an adaptor protein. Our data provide evidence for another function for MALT1 catalytic activity - i.e. activation of mTORC1. The mechanism by which MALT1 catalytic activity induces activation of this novel pathway is still unclear, but we hypothesize that there might be additional intermediary molecules leading to mTOR pathway activation after TCR/CD28 engagement. For example, Kawadler et al., demonstrated that MALT1 mediated cleavage of caspase 8 leads to lymphocyte proliferation (197). Since caspase 8 may also be important for S6K activity (198), caspase 8 is another potential link between MALT1 and S6K activation. In addition, MALT1-mediated cleavage of A20 and CYLD have been implicated in NF-κB induction (98, 199), although neither has previously been implicated in mTOR regulation. A recent study showed that MALT1 can cleave the protein Regnase-1 (aka MCPIP1), which normally functions to restrain T cell activation, through destabilization of specific mRNA's, including those that encoding several cytokines (200). Finally, while MALT1 can cleave Bcl10 close to its C-terminus, this event appears to regulate lymphocyte adhesion but not NF-κB (97). Thus, MALT1 is not exclusively a regulator of NF-κB induction, but participates in other pathways as well.

The major functional connections that we have been able to make thus far between the MALT1/mTOR pathway involve regulation of T cell proliferation and metabolism, but not early activation. The former is consistent with the fact that primary mouse T cells with reduced (heterozygous) S6 expression were shown to have decreased cellular proliferation, but normal blasting (177). We have also noted that MALT1 activity is dispensable for activation-induced

blasting of T cells, as well as upregulation of early activation markers like CD69. The ribosomal protein S6 is a critical regulator of protein translation. It is also one of the main downstream effectors of mTORC1, and as such is thought to play a critical role in oncogenesis involving defects along the mTOR signaling cascade (115, 118, 123, 140).

Our data have also revealed a role for MALT1 in regulating increased metabolism upon T cell activation, a process important for T cells to meet the changing needs of activated and proliferating T cells (131). The mTOR pathway is an important regulator of this shift (131, 201). Based on the totality of our data, we propose that MALT1 regulation of metabolism in T cells occurs at least, in part, through mTOR. We also assessed expression of CD71 and CD98, nutrient receptors which facilitate proliferation of T cells upon activation (193, 194). We noted increased expression of both after stimulation; however treatment with the MALT1 inhibitor did not impair this upregulation (Fig. 3-7D). These data, along with our ECAR results (Fig. 4-7B), suggest that treatment with the MALT1 inhibitor, z-VRPR-fmk, does not completely inhibit mTOR activation. Thus, we would not expect to see as potent an effect as that seen with the more direct mTOR inhibitor rapamycin (Fig. 3-7). Numerous reports have now described additional important functions for mTOR signaling pathways in the regulation of peripheral T cell differentiation to different effector or regulatory lineages, as well as the regulation of memory T cell development (130, 131). For example, mTORC1 promotes the development of pro-inflammatory Th17 cells, while suppressing the development of anti-inflammatory regulatory T cells. Such effects of mTOR in T cells appear to be mediated, at least in part, through regulation of the metabolic re-programming that accompanies T cell activation and expansion (120, 131). Further studies of these aspects of mTOR function are therefore warranted, as possible additional downstream consequences of signaling from Carma1 and MALT1.
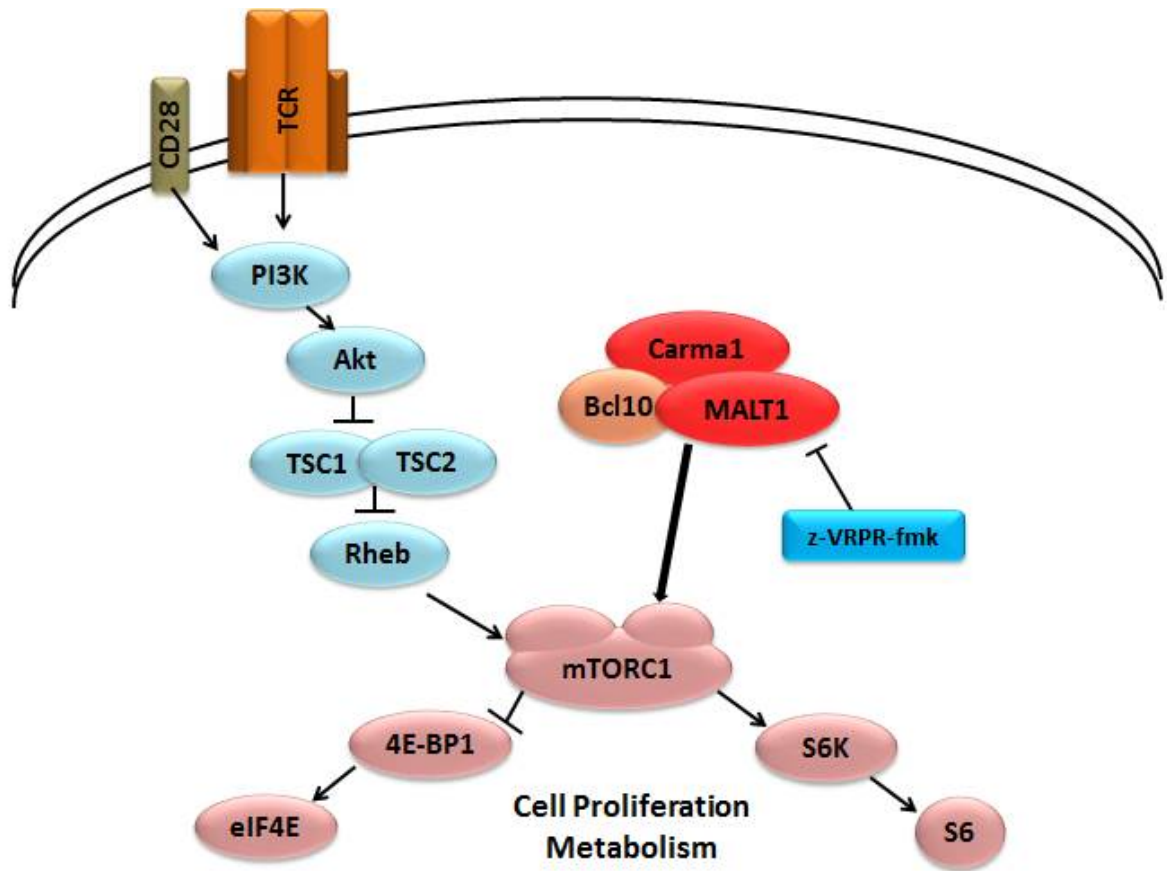
Figure 3-8 Model for mTORC1 activation in T cells. Carma1 and MALT1 regulate activation of mTORC1 with TCR and CD28 stimulation in T cells.

4.0   SERINE 231 OF BCL10 REGULATES NF-KB ACTIVATION IN T CELLS

4.1   INTRODUCTION

Bcl10 is a ubiquitously expressed protein that plays roles in both apoptosis and NF-κB activation (33, 58, 202). Bcl10 is essential for T cell activation and Bcl10 KO T cells are unable to proliferate. This phenotype is also shared with Bcl10 associated proteins- Carma1 and MALT1. Upon TCR engagement and co-stimulation Bcl10 and its associated protein MALT1, are recruited to Carma1, where other proteins, IKK, PDK and TRAF6 bind and activate NF-κB through degradation of the inhibitory subunit, IκB (36, 57, 59, 203). In the absence of Bcl10, this "signalosome" of proteins is unable to form and NF-κB is unable to be activated through this pathway (58, 204). However, NF-κB can be activated through other receptors and pathways, specifically through the TNF and TLR receptors (205, 206).

An area of great interest is the mechanism(s) by which Bcl10 is regulated. Bcl10 is a primary target for post translational modifications in the CBM complex. Bcl10 contains a CARD domain and a serine/threonine (S/T) rich domain at its C-terminus. The CARD domain binds to Carma1, whilst the S/T domain is ubiquitinated and phosphorylated by multiple proteins (69). For example, Bcl10 is phosphorylated by IKK, CamKII, RIP2 and Akt at multiple sites (69) and is also ubiquitinated by cIAP-2 and Itch (69, 190). Phosphorylation and ubiquitination lead to

contraction of Bcl10 signaling and reduced NF-κB activation (69, 190). On the other hand, phosphorylation by RIP2 and Akt both positively regulate Bcl10 and downstream signaling (69).

Based on our current knowledge of regulation of Bcl10 we were interested in identification of other positive regulators of Bcl10 and subsequent NF-κB activation. We have identified a previously unappreciated role for serine 231 in T cells. We show that this site is necessary for optimal NF-κB activation and IL-2 production. We also show that this site is most probably not phosphorylated by Akt but potentially PKC.

4.2 METHODS AND MATERIALS

Antibodies and Reagents

Phospho Akt substrate was obtained from Cell Signaling Technology, Inc. (Danvers, MA). Bcl10 and MALT1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin antibody was from Sigma (St. Louis, MO). Antibody specific to the Jurkat TCR (C305), purified from the C305.2 hybridoma, was from ATCC (Manassas, VA). Biotin conjugated anti-mouse CD3, CD4 and CD28 were obtained from BD Biosciences (San Jose, CA). Anti-mouse-CD3 and -CD28 antibodies were obtained from Life Technologies (Carlsbad, CA). Donkey anti-rabbit-HRP, was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Recombinant human TNFα was from R&D Systems (Minneapolis, MN). Phorbol-12-myristate-13-acetate

(PMA), ionomycin and Akt Inhibitor VII (Akti1/2) were from Calbiochem/EMD (Billerica, MA). Bisindolylmaleimide VIII acetate was from Enzo Life Sciences (Farmingdale, NY). Phospho-sopecific antibody to serine 231 in Bcl10 was generated as previously described (15).

Cells

Jurkat T cells, Jurkat T cell mutants, Bcl10sh Clone 3.20, Clone 3.21, Bcl10shRNA, a fast-growing variant of the mouse T cell clone D10 cell and the human B cell line Raji were maintained as previously described (167).

Luciferase Assays

Jurkat T cells were transfected with 15 ug of NF-κB luciferase reporter vector by electroporation, cultured in complete medium (RPMI, 5% BGS and penicillin/ streptomycin) for 16-20 hrs. Cells were pre-treated with inhibitor for 30 mins, before stimulation with anti-TCR and anti-CD28, PMA and ionomycin or rhTNFα for 6 hours. Luciferase assays were performed as previously described (15).

Western Blotting and Immunoprecipitation

Cells were lysed with NP-40 lysis buffer (1% NP-40, 1 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl) or with RIPA buffer (1% NP-40, 150 mM NaCl, 25 mM Tris-HCl, sodium deoxycholate, SDS) for immune-precipitation (IP) experiments), protease inhibitors (aprotinin, pepstatin, 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF) and leupeptin) and phosphatase inhibitors (sodium fluoride, beta-glycerophosphate and sodium orthovandate). Proteins were resolved on 8-

10% SDS-PAGE gels, transferred to PVDF membrane and blocked in 4% BSA. Membranes were probed with the specified primary antibodies, followed by HRP-conjugated secondary antibodies. Proteins were detected using the SuperSignal West Pico ECL substrate (Thermo Scientific) and imaged on a Kodak Image Station. β-actin was used as a loading control for western blot experiments.

Stable Cell Line

Jurkat T cells were transiently transfected with Bcl10shRNA (Qiagen, Valencia CA) and two days later, seeded in media containing G418 antibiotic. Six weeks later cells were collected and analyzed for Bcl10 knockdown by western blotting.(186)

4.3   RESULTS

4.3.1 Generation of Bcl10-deficient Jurkat T cells

Expression of Bcl10 is required for activation of NF-κB after TCR and CD28 stimulation (33, 36, 58). Moreover, post-translational regulation of Bcl10 itself has been shown to be a critical regulator of NF-κB activation (69, 82, 190, 207). We were interested in further elucidating Bcl10's role in T cell activation. First, we reduced Bcl10 expression in Jurkat T cells, by

transfection with Bcl10shRNA. This provided a system where we could re-express wild type and mutant forms of Bcl10 and assess the potential effects on NF-κB activation. We generated several Jurkat T cell lines stably expressing Bcl10shRNA (data not shown). We found that clone 3.20 had more than a 50% reduction in Bcl10 expression compared with another clone (3.21) or parental Jurkat T cells (Fig. 4-1A). We then assessed the ability of this cell line to induce NF-κB activation. We noted that while stimulation with TCR and CD28 antibodies or PMA and ionomycin activated an NF-κB luciferase reporter in parental Jurkat T cells, there was significantly less NF-κB activation in Bcl10shRNA clone 3.20, as predicted (Fig 4-1B).

We then re-expressed wild type Bcl10 in clone 3.20 by transient transfection, in a knock-down/add-back experiment. We could detect Bcl10 protein in clone 3.20 cells transfected with Bcl10 (Fig 4-1C). However, the NF-κB activity did not reflect re-expression of Bcl10 as expected, and we saw no increase in NF-κB in clone 3.20 cells transfected with Bcl10, compared with empty vector transfected cells (Fig 4-1D). Of note, clone 3.20 was still capable of NF-κB activation, as stimulation with TNF (which does not require Bcl10) induced NF-κB activation (Fig 4-1D) (33, 58).

The reconstitution results for clone 3.20 compelled us to utilize a different system. We obtained a Bcl10shRNA T cell line that had previously been validated (190). We transfected these Bcl10shRNA cells with a Bcl10-expressing vector, or the empty vector, and assessed NF-κB activity. Thus, re-expression of Bcl10 increased NF-κB activity in these cells, as compared with empty vector-transfected cells (Fig 4-1E). This increase was seen in both unstimulated and TCR and CD28-stimulated Bcl10shRNA cells (Fig 4-1E).
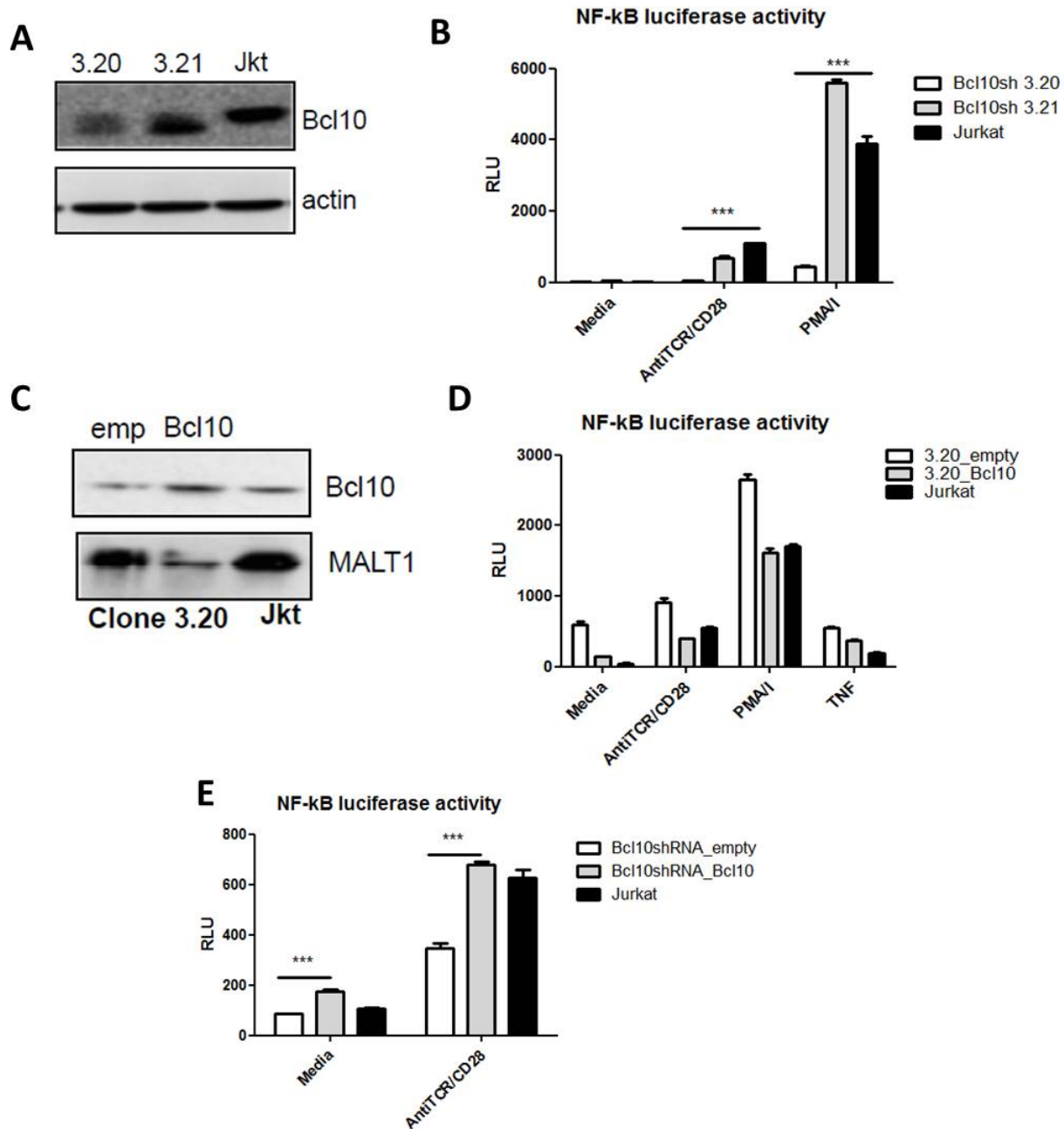
Figure 4-1 Generation of Bcl10 knockdown Jurkat T cells. (A and C) Bcl10 expression in Bcl10 shRNA stably transfected Jurkats (A) or transiently transfected with wt Bcl10. (B, D) NF-κB luciferase activity in Bcl10shRNA clones 3.20, 3.21 and Jurkats (B) or clone 3.20 reconstituted with Bcl10 (D) and stimulated for 6 hours. (E) NF-κB luciferase activity in Bcl10shRNA cells reconstituted with Bcl10 and stimulated for 6 hours. All values represent mean + S.E.M of triplicates of a single experiment. Data are representative of three independent experiments.

4.3.2 Serine 231 of Bcl10 regulates NF-κB activation in T cells

Previous work identified serine 231 (s321) as a potentially important site for Bcl10 regulation after TNF-a stimulation of the breast cancer cell line MCF-7 (208). S231 lies near the C-terminus of the Bcl10 and the preceding amino acids, RSRTV, identify S231 as a potential Akt substrate (Fig 4-2A) (15). A vector expressing Bcl10 with S231 mutated to alanine had been generated previously in the lab. Mutation of serine to alanine prevents phosphorylation of serine 231. To understand if phosphorylation of Bcl10 at this site is important for NF-κB activation in T cells, we transiently transfected Bcl10shRNA cells with mutant (Bcl10 S231A) or wild type Bcl10 (Bcl10 wt) and assessed NF-κB activation by luciferase assay. We noted that stimulation through the TCR and CD28 induced NF-κB activation in Bcl10shRNA cells expressing either mutant or wild type Bcl10. However, wild type Bcl10 was able to induce significantly more NF-κB activation, compared with mutant Bcl10 (Fig 4-2B). We also assessed the functional significance of serine 231 by IL-2 ELISA. NF-κB drives expression of the cytokine IL-2 (12) and, based on our luciferase data, we predicted that mutation of serine 231 would also affect the ability of T cells to produce IL-2. We noted that transfection with either wild type or mutant Bcl10 could rescue some IL-2 expression in Bcl10 knockdown cells. However, this rescue was significantly more efficient in cells expressing wild type vs. Bcl10 (S231A) (Fig 4-2C). Luciferase and IL-2 ELISA experiments were supplemented with 50ng/ml TNF-α to induce a robust enough readout for measurement (Fig 4-2B-C). While the TNF-dependent signal does not require Bcl10, the PMA-dependent signal does require Bcl10 and we noted significant differences in the ability of wild type Bcl10 to rescue NF-κB activation, compared to mutant Bcl10.
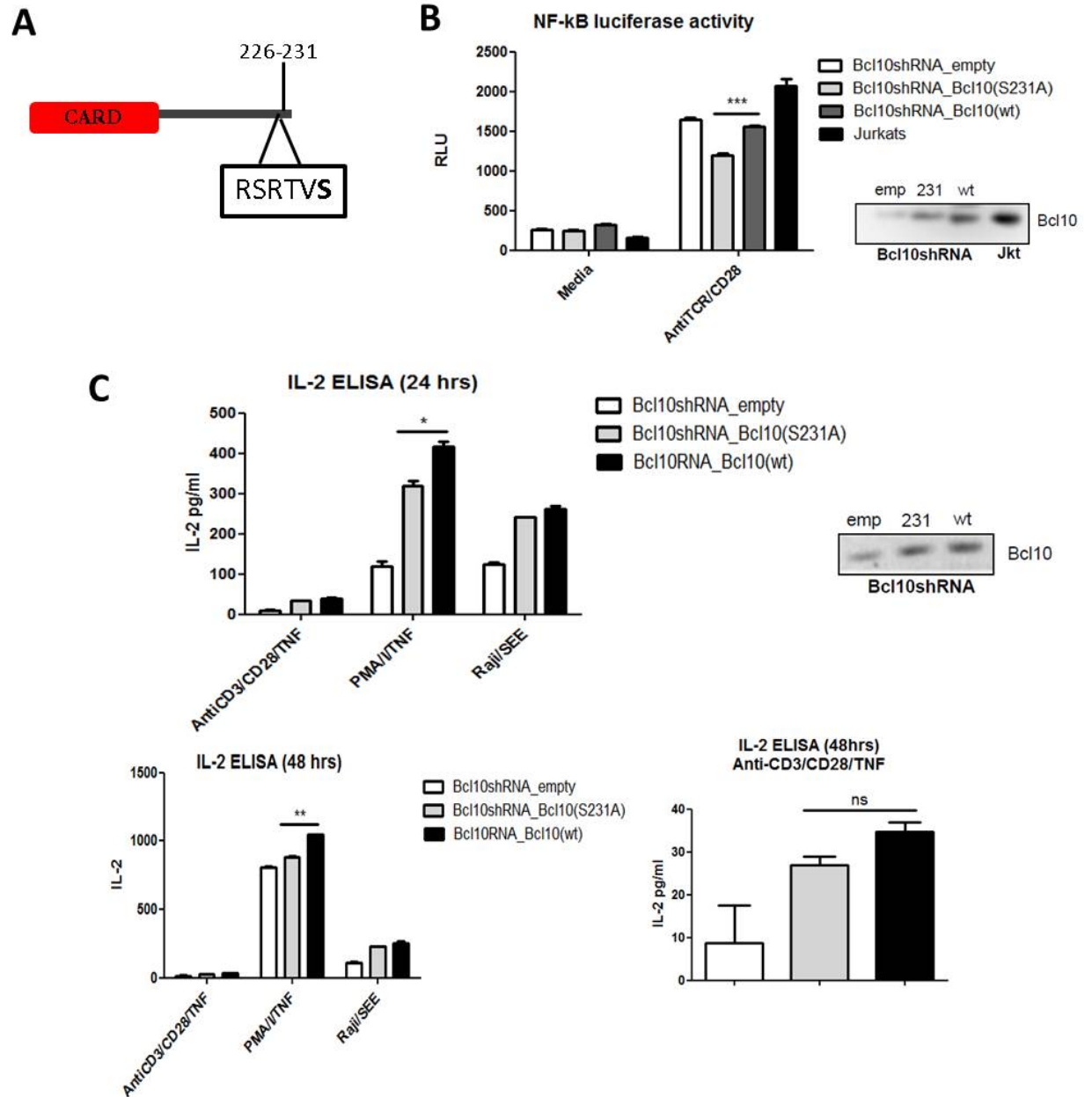
Figure 4-2 Serine 231 of Bcl10 is required for NF-κB activation in T cells. (A) Schematic of Bcl10 showing phosphorylation site at S231. (B) NF-κB luciferase activity in Bcl10shRNA cells transfected with 10ug of empty vector, Bcl10 (S231A) and Bcl10 (wt) expressing vectors and Jurkat T cells (*left*) and stimulated for 6 hours; western blot of Bcl10 expression in transfected Bcl10shRNA cells (*right*). (C) IL-2 expression in Bcl10shRNA cells transfected with mutant or wt Bcl10; IL-2 expression at 24 hours and Bcl10 expression (*top*) and expression at 48 hr (*bottom*). All values represent mean + S.E.M of triplicates (luciferase assay) or duplicates (ELISA) of a single experiment. Data are representative of three independent experiments.

4.3.3   Bcl10 is not phosphorylated by Akt in T cells

Our data showing a requirement for serine 231 in Bcl10-dependent NF-κB activation indicate a role for phosphorylation of this site. As mentioned previously, the most probable kinase was Akt, based on the sequence around S231 (188). We tested this hypothesis by pretreating D10 mouse T cells with an allosteric Akt inhibitor (see chapter 2) at various concentrations, and assessed phosphorylation with an Akt phospho-substrate antibody. We immunoprecipitated Bcl10 from CD3/CD28-stimulated D10 cells and observed phosphorylation; however, treatment with the Akt inhibitor did not alter this phosphorylation (Fig 4-3A). We generated a polyclonal antiserum that specifically recognizes the phosphorylated from of Bcl10 at serine 231(15). Using this antibody, we assessed Bcl10 phosphorylation at S231 and observed basal phosphorylation even under unstimulated conditions (Fig 4-3B). Akt also shares some substrate specificity with PKC, so we assessed Bcl10 phosphorylation at serine 231 in the presence of the PKC inhibitor BIM (188). Bcl10 phosphorylation was impaired after BIM treatment (Fig 4-3B). Thus, our results suggest that Akt does not regulate Bcl10 phosphorylation at serine 231, which instead may be a target of PKC.
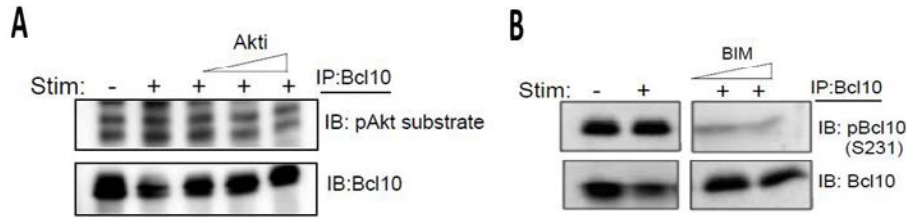
Figure 4-3 Bcl10 is not phosphorylated by Akt in T cells. (A) Bcl10 IP of D10s pretreated with varying concentrations of Akti, stimulated with anti-CD3/CD28 for 30 minutes and probed with a phospho Akt substrate antibody. (B) Bcl10 IP of Jurkats pretreated with BIM for 1 hour, stimulated with anti-CD3/CD28 for 30 minutes and probed with a phospho-specific Bcl10 antibody (panels are from the same blot). Data are representative of three independent experiments.

4.4   DISCUSSION

Engagement of the TCR and CD28 activates the NF-κB pathway through the Carma1, MALT1 and Bcl10 complex (32-34, 66). MALT1 is the only member of this complex known to have catalytic activity, while Carma1 and Bcl10 primarily serve as adaptor proteins, allowing for docking of other proteins necessary for NF-κB activation (69, 98). Importantly Bcl10 has been shown to be a critical regulator of TCR induced NF-κB, and is regulated in a complex fashion by phosphorylation, ubiquitination and degradation (69, 190). Many of these modifications lead to attenuation of NF-κB activity (69, 190). Here, we have characterized another mechanism by which Bcl10 is regulated in T cells, which translates to effects on NF-κB and IL-2. Thus, we

have shown that phosphorylation of serine 231 of Bcl10 is necessary for optimal NF-κB activation with TCR and CD28 stimulation.

The Cheng group had previously identified serines 231 and 218 of Bcl10 as targets of Akt phosphorylation after TNF-α stimulation (208). They also showed that Bcl10 forms a complex with Bcl13 and translocates to the nucleus (208). Our results using an Akt specific inhibitor (Akti 1/2) suggest that Bcl10 is not regulated by Akt, at least in T cells. We assessed inhibition of Bcl10 phosphorylation after Akt inhibition in D10 cells and saw no difference in phosphorylation profiles between untreated and inhibitor-treated cells. We also assessed Bcl10 S231 phosphorylation with a phospho-specific antibody and found that Akt inhibitor treatment did not impair S231 phosphorylation (data not shown). The study by the Cheng group assessed the ability of Akt ability to phosphorylate Bcl10 with TNF-α stimulation in the breast cancer cell line MCF-7 (208). It is possible that there is differential signaling to Bcl10 in different types of cells and, more importantly, with different modes of stimulation (e.g. TNF receptor versus TCR). Little is known about the mechanism by which TNF induces Akt activation leading to NF-κB activation, and as such we are unable to conclude whether Bcl10 signaling in T cells parallels that of TNF stimulation. More studies assessing direct Bcl10 phosphorylation by Akt (e.g. by in vitro kinase assay) after TCR and CD28 stimulation, as well as knockdown of Akt in T cells, will be needed to confirm our findings.

Interestingly, the Akt phospho-substrate antibody showed that Bcl10 is indeed phosphorylated, although this phosphorylation did not seem to be mediated by Akt. This antibody recognizes serines/threonines within the consensus sequence RxRxxS/T which also shares substrate sequence specificity with other AGC kinases (eg. RSK, S6K and RSK) (188). We noted that another AGC kinase, PKC, may regulate S231 phosphorylation. However the

PKC consensus substrate specificity, R/KxSxK/R, does not correlate as well with the amino acids surrounding 231 (188). BIM serves as a pan-PKC inhibitor, so we are unable to determine which PKC isoform may be regulating Bcl10 phosphorylation and downstream function (170).

Our data suggest another mechanism by which the NF-κB pathway is positively regulated, i.e. through phosphorylation of Bcl10 at serine 231. There are many questions that remain to be answered about this, including the kinetics of Bcl10 phosphorylation, involvement of other Bcl10 phosphorylation sites (i.e. whether there is hierarchical phosphorylation) and whether phosphorylation facilitates binding to Bcl10 of other proteins necessary for NF-κB activation.

5.0   ROLE FOR MALT1 DEPENDENT S6 PHOSPHORYLATION IN ABC-DLBCL

5.1   INTRODUCTION

We have shown that MALT1 catalytic activity is necessary for activation of the mTORC1 complex in T cells. Recently, inhibition of MALT1 is shown to be effective in impairing growth and viability of Diffuse Large Cell Lymphoma (DLBCL) cell lines (111, 114). DLBCL is the most common form of non-Hodgkin's lymphoma, accounting for 20-40% of all cases reported (77, 209). DLBCL is divided into three major sub-types: germinal center B-cell like (GCB), activated B-cell like (ABC) and primary mediastinal B-cell lymphoma. These characterizations are primarily based on gene expression profiling of individual DLBCL cases (77, 109, 209). Of interest to us is the ABC-DLBCL sub type, which relies on hyper-activation of the NF-κB pathway for growth (112, 210). In addition, the proteins Carma1, Bcl10, MALT1 and A20 are all implicated in continued activation of NF-κB (77, 209, 211-213). Clinically, ABC-DLBCL shows the least favorable prognosis, in comparison with other DLBCL sub types. Thus, understanding the mechanism(s) by which ABC-DLBCL maintain their proliferation and survival may lead to the development of new and more efficacious therapies.

Several groups have assessed the effects of genetic and pharmacological manipulation in ABC-DLBCL (mostly using cell lines), focusing on Carma1 and/or MALT1. The Staudt group has identified mutations in Carma1, specifically in its coiled coil domain, in 10% of ABC-

DLBCL cases studied (77). These mutations were shown to potentiate NF-κB activation in both unstimulated and stimulated B cells (77). Additionally, mutations in the deubiquitinase A20, leading to increased NF-κB activation, have been identified in ABC-DLBCL (78).

More recently, inhibitors of MALT1 paracaspase activity (Chapter 1) have shown promise in controlling growth of ABC-DLBCL cell lines, in comparison to other sub-types of DLBCL (101, 102, 110, 114). Along with decreased cellular proliferation in the presence of MALT1 inhibitors, activation of NF-κB and its downstream targets were also decreased (101, 102, 110). Based on our findings, showing a requirement for Carma1 and MALT1 in activation of the mTORC1 pathway in T cells, we were interested in understanding if this pathway may also play a role in ABC-DLBCL.

5.2   METHODS AND MATERIALS

Antibodies and Reagents

Alexa Flour 647-conjugated antibody to pS6 (S235/236) was obtained from Cell Signaling Technology, Inc. (Danvers, MA). Phorbol-12-myristate-13-acetate (PMA), ionomycin was from Calbiochem/EMD (Billerica, MA). MALT1 inhibitor z-VRPR-fmk was obtained from A.G. Scientific (San Diego, CA).

Cells

The DLBCL cell lines, OCI-Ly1, OCI-Ly3 and OCI-Ly7, were from Dr. Mark Minden (Ontario Cancer Institute, Ontario, Canada) were maintained in IMDM media (Life Technologies, Grand Island, NY), with 10% Bovine Growth Serum and 1% penicillin/ streptomycin (Mediatech, Herndon, VA).

Flow Cytometry

Flow cytometry staining was performed as previously described (167). In short, cells were starved in 1% bovine serum albumin, in PBS for one hr at 37°C; for indicated experiments cells were also pre-treated with inhibitor during starvation period. After stimulation cells were fixed with 1.5% paraformaldehyde at RT for 15 minutes and permeablized with cold methanol on ice. Cells were then washed three times with buffer (PBS, 1% BSA) and stained with phospho-specific antibodies at room temperature. Samples were analyzed on a BD LSRII.

5.3   RESULTS

5.3.1   z-VRPR-fmk does not inhibit basal S6 phosphorylation in DLBCL cells

To address a potential role for MALT1 in regulating the mTORC1 pathway, we first assessed the ability of a MALT1 inhibitor (z-VRPR-fmk) to inhibit S6 phosphorylation in DLBCL cell lines.

We utilized the GCB-DLBCL cell line OCI-Ly1 and OCI-Ly7, and the ABC-DLBCL cell line OCI-Ly3. We pretreated these cells for one hour with z-VRPR-fmk and assessed S6 phosphorylation. We did not observe any effect on S6 phosphorylation in these particular cell lines (Fig. 5-1A) after treatment with the MALT1 inhibitor. In previously published work by the Thome group, treatment for extended periods of time with z-VRPR-fmk was shown to inhibit cellular proliferation in ABC-DLBCL cell lines (100, 110). As such, we also assessed S6 phosphorylation in DLBCL cell lines pretreated with z-VRPR-fmk for 24 and 48 hours (Fig. 5-1B). Similar to the results above with short-term pretreatment, there was no impairment in S6 phosphorylation after extended inhibitor treatment. However, we did note significantly higher S6 phosphorylation in the ABC-DLBCL cell line OCI-Ly3 with MALT1 inhibitor treatment, which was not seen in the GCB-DLBCL cell line OCI-Ly1 and OCI-Ly7 (Fig. 5-1B). These results show that inhibition of MALT1 catalytic activity with z-VRPR-fmk does not impair basal S6 phosphorylation in DLBCL cells.
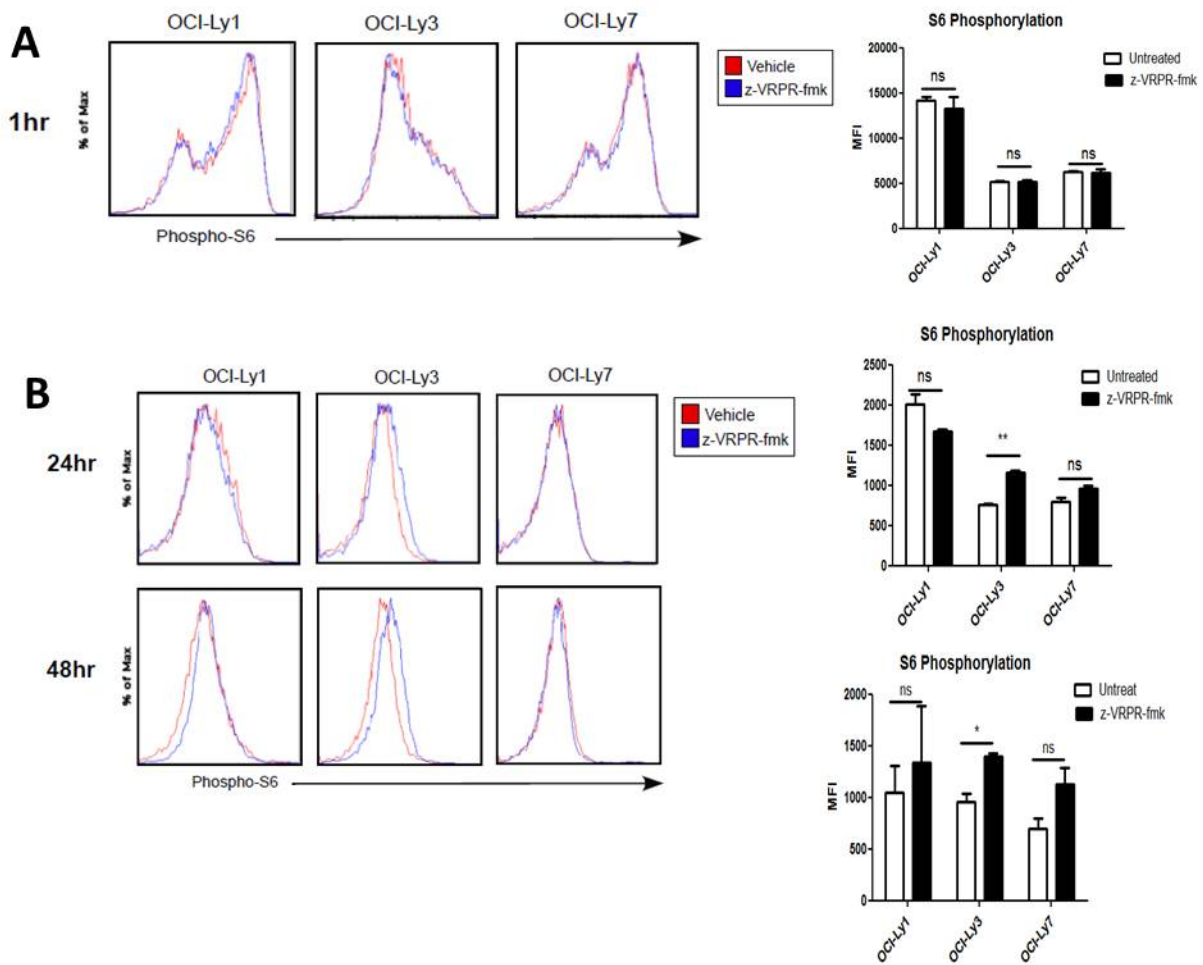
Figure 5-1 z-VRPR-fmk does not inhibit basal S6 phosphorylation in DLBCL cells. (A) Histograms (*left*) or mean fluorescence intensity (MFI) (*right*) of S6 phosphorylation in GCB-DLBCL cell lines (OCI-Ly1, OCI-Ly7) and ABC-DLBCL cell line (OCI-Ly3) pretreated for 1 hr with z-VRPR-fmk. (B) Same as A, but pretreated for 24 hours (*top*) or 48 hours (*bottom*) with z-VRPR-fmk. All values represent mean + S.E.M of duplicates of a single experiment.

5.3.2 Induction of S6 phosphorylation in DLBCL after PMA and Ionomycin stimulation

Although we did not observe an impairment of S6 phosphorylation after MALT1 inhibition in unstimulated cells, we hypothesized that treatment with the inhibitor may only be effective when the DLBCL cell lines are stimulated. This was based on our findings in T cells (Chapter 3) where t z-VRPR-fmk inhibited S6 phosphorylation in a human T cell line cells and in primary mouse T cells, after TCR/CD28 stimulation (Chapter 3). PMA activates PKCs (Chapter 1) through activation of the Ras/ Raf pathway and ERK (214-216), but the ability of PMA and ionomycin to induce S6 phosphorylation in DLBCL cell lines has not yet been reported. We stimulated the three DBLCL cell lines described above with PMA and ionomycin for up to one hour, and noted increased S6 phosphorylation at all time points assessed - 15, 30 and 60 minutes (Fig. 5-2). We found different kinetics for S6 phosphorylation with the different cells lines, but consistently found increased S6 phosphorylation at 30 minutes (Fig. 5-2). Our results show that stimulation with PMA is indeed able to induce S6 phosphorylation in the DLBCL cells tested.
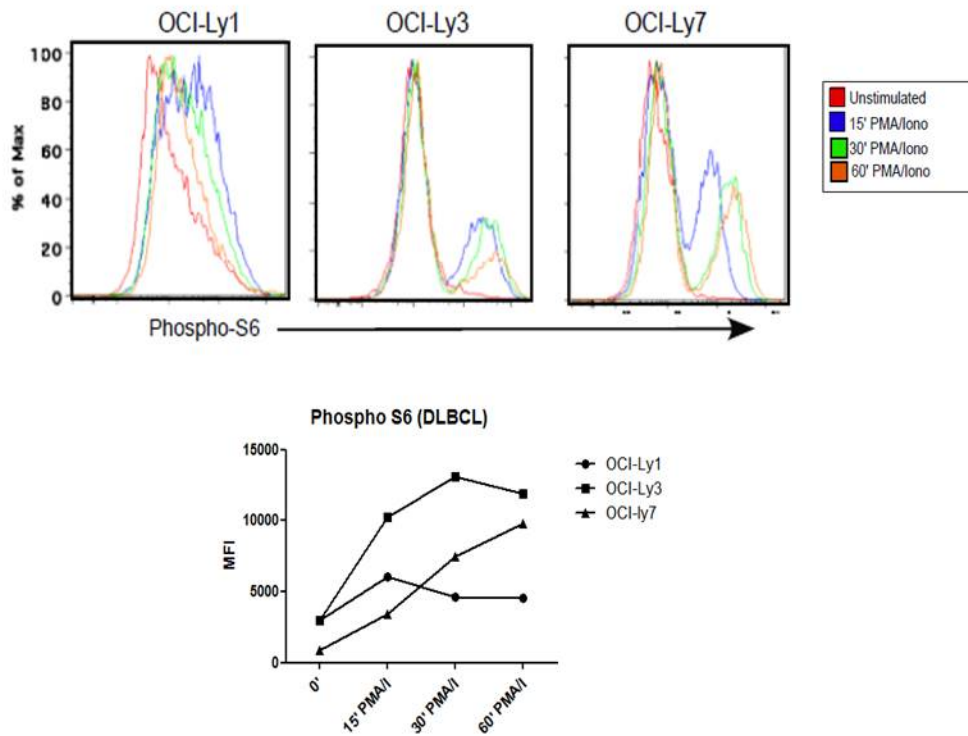
Figure 5-2 Induction of S6 phosphorylation in DLBCL after PMA and ionomycin stimulation. DLBCL cell lines were stimulated for 15, 30 and 60 minutes with PMA (50ng/ml) or ionomycin (1uM) and S6 phosphorylation assessed by flow cytometry- histograms (*top*), MFI (*bottom*).

5.3.3 MALT1 regulates inducible S6 phosphorylation in ABC-DLBCL cells

After determining a suitable time point (30 minutes) for induction of S6 phosphorylation with PMA/ionomycin stimulation (Fig. 5-2), we next assessed the effect of MALT1 inhibition on inducible S6 phosphorylation. OCI-Ly1 and OCI-Ly3 cell lines were pre-treated with the MALT1 inhibitor z-VRPR-fmk, stimulated with PMA/ionomycin, and S6 phosphorylation was assessed by flow cytometry. We did not observe a consistent difference between these cell lines in the levels of basal S6 phosphorylation, nor was basal S6 phosphorylation in either cell line significantly affected by z-VRPR pre-treatment (Fig. 5-3A). Interestingly, however, PMA/ionomycin-induced S6 phosphorylation was reduced by z-VRPR-fmk in ABC-type OCI-Ly3 cells, but not in OCI-Ly1 (GCB) cells (Fig. 5-3A). We also assessed another GCB-DLBCL cell line (OCI-Ly7) and, consistent with the above results, did not see any impairment in PMA/ionomycin-inducible S6 phosphorylation by MALT1 inhibitor treatment (Fig. 5-3B). These findings suggest that S6 is a previously unknown downstream target of dysregulated MATL1 activity in ABC-DLBCL, which may further our understanding of molecular events that contribute to this sub-type of lymphoma.
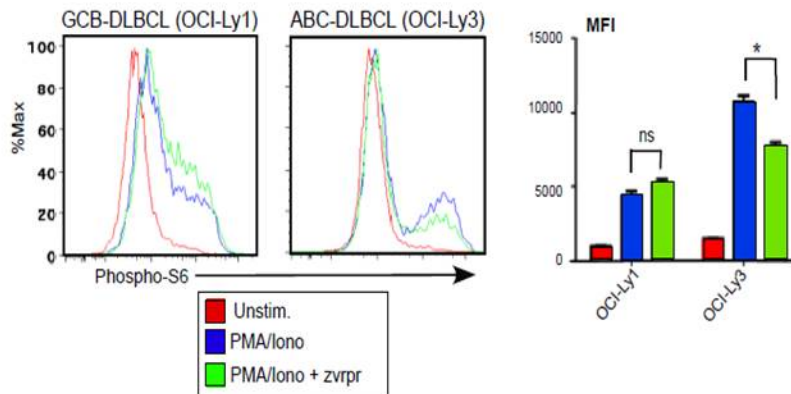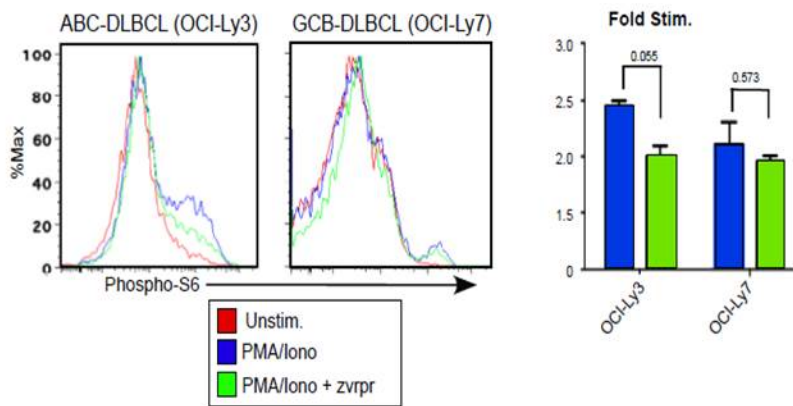
Figure 5-3 MALT1 regulates inducible S6 phosphorylation in ABC-DLBCL cells. (A) OCI-Ly1 (GCB) and OCI-Ly3 (ABC) DLBCL cells were pre-treated with z-VRPR-fmk (75 mM) for four hours and stimulated with PMA (50 ng/mL) and ionomycin (1 mM) for 30 minutes. S6 phosphorylation was assessed by flow cytometry. (B) Same as (A) using OCI-Ly7 (GCB) with OCI-Ly3 (ABC) DLBCL. Data are duplicate points from a single experiment, representative of three that were performed. Values represent mean +/- S.E.M; n=2. Unpaired Student's t-test was used for statistical analysis, * p< 0.05; **p <0.01.

5.4 DISCUSSION

Carma1 and MALT1 play key roles in the progression of DLBCL, specifically the ABC type. We found that a MALT1 inhibitor reduced S6 phosphorylation in an ABC-DLBCL cell line, but not two different GCB-DLBCL lines, consistent with published data (110, 114). We were not surprised to find that the effect of z-VRPR-fmk on S6 phosphorylation in the ABC-DLBCL cells was somewhat modest, as other published data have only shown partial inhibition of NF-κB in these cells, even with higher concentrations of z-VRPR-fmk (110, 210). Additionally, ribosomal protein S6 is regulated by a multitude of other pathways, so the effects of MALT1 on this pathway may be at least partially redundant with other regulators.

We were initially surprised by our preliminary results showing no inhibition of S6 phosphorylation by z-VRPR-fmk in the ABC-DLBCL cell line OC-Ly3 under unstimulated conditions (Fig. 5-1). Based on published work assessing the effects of z-VRPR-fmk on DLBCL growth, we expected to observe some inhibition of S6 phosphorylation (110, 114). However, our results showing z-VRPR-fmk inhibition of inducible S6 phosphorylation are similar to our findings in T cells (Chapter 3). These results indicate that MALT1 needs to be "activated" through PKC and Carma1 for eventual phosphorylation of S6, and that this maybe specific to ABC-DLBCL, which already displays CBM dysregulation.

We did note increased basal S6 phosphorylation in OCI-Ly3 cells treated for longer time points (24 and 48 hours, Fig. 5-1B). This may be due in part to feedback inhibition through mTOR, which has been shown to potentiate Akt activation through expression of insulin receptor substrate (IRS) and continued mTOR signaling (217, 218). Under basal (or steady state) conditions, continued inhibition of the mTORC1 pathway by z-VRPR-fmk may activate this

feedback pathway, leading to increased S6 phosphorylation. Thus, further studies assessing IRS expression and Akt activation with z-VRPR-fmk treatment will need to be performed.

More recently, small molecule inhibitors of MALT1 have also shown substantial efficacy in inhibiting *in vitro* growth of ABC-DLBCL, but not GCB-DLBCL, which was attributed to differential activation of the NF-κB pathway (101, 102, 219). Thus, it will be of interest to further investigate the role(s) MALT1 may play in ABC-DLBCL progression, specifically in the mTORC1 pathway.

6.0 SUMMARY AND DISCUSSION

Induction of T cell responses upon TCR and co-stimulatory engagement involves a myriad of signaling events leading to transcriptional, translational and post-translational changes (4, 10). Understanding these events is critical for deciphering how T cells work in normal and pathological settings. Here, we have identified a previously unappreciated role for the proteins PKC, Carma1 and MALT1 in the induction of the mTORC1 pathway, downstream of TCR and CD28 engagement. Our findings provide new insight into the additional mechanisms through which the mTORC1 pathway is regulated in T cells and uncover alternative functions for Carma1 and MALT1, beyond their known roles in the NF-κB and JNK2 pathways.

6.1 TCR-DEPENDENT S6 PHOSPHORYLATION REQUIRES PKC

Phosphorylation of the ribosomal protein S6 after TCR and CD28 stimulation requires PKC, Carma1 and the MEK/ERK pathways. Based on previously published work on receptor tyrosine kinases (RTKs), PKC has been shown to activate the mTORC1 pathway and also to activate S6 independently of mTORC1, through p90S6K (134, 161, 220-223). However, the mechanism by which PKC regulates S6 phosphorylation after TCR stimulation has not been investigated. The

majority of published findings on activation of S6 after TCR and CD28 stimulation are consistent with PI3K/Akt leading to mTORC1 and subsequent S6 and 4E-BP1 activation. However, studies by the Cantrell group have suggested the existence of an alternate pathway that includes PDK (147, 173, 224). They showed that Akt is dispensable for T cell metabolism through mTOR and HIF1α, moreover, inhibition of cytotoxic T lymphocytes (CTL) with either an Akt or PI3K inhibitor did not inhibit phosphorylation of S6, S6K or 4E-BP1 (147, 149, 173). Based on our results, we hypothesize that PDK lies upstream of PKC to induce downstream signaling events. PDK phosphorylates several isoforms of PKC, contributing to its activation in T cells.

A fundamental question which still remains is the precise mechanism by which PKC regulates S6 phosphorylation through Carma1 and the MEK/ERK pathways. Our data show a requirement for both; however we are unable to determine whether these pathways are linked or if they occur independently. It is more likely that these pathways occur independently to induce S6 phosphorylation, as Carma1 is not required ERK activation in T cells. We propose a model where Carma1 and MALT1 regulate mTORC1 activation potentially through direct modification of the mTOR complex itself (Fig. 6-1).

Another critical factor to be determined is the isoform(s) of PKC that may be involved TCR-induced S6 phosphorylation. Stimulation through the TCR and CD28 induces both the generation of DAG and an increase in intracellular Ca^{2+}, through cleavage of PIP_2 by PLCγ, so both novel and classical PKCs can be activated (4, 10). Our experiments attempting to narrow down the isoform responsible, using rottlerin (an inhibitor of novel PKCs), did not prove fruitful as it also inhibited Akt phosphorylation. It would be interesting to assess S6 phosphorylation in the presence of PMA (synthetic DAG analogue) but the absence of Ca^{2+}, to determine if this is

sufficient to induce S6 phosphorylation comparable to PMA and ionomycin (induces release of intracellular Ca^{2+}), thus differentiating between the necessity for a novel PKC or both novel and classical isoforms (42, 43, 162). PKC θ is one of the primary isoforms that is activated after TCR and CD28 stimulation, leading to activation of the NF-κB and MEK/ERK pathways, and we predict it also serves a similar role in activation of S6 (29). However, based on the different mechanisms through which PKC regulates S6 phosphorylation, we also predict that PKC θ may be more relevant for Carma1-mediated activation of mTORC1 and that other isoforms may mediate MEK/ERK-dependent S6 phosphorylation in T cells. Genetic approaches (siRNA/shRNA/ knockout systems) may be optimal to understand the potential differential requirements of PKC isoforms in S6 phosphorylation in T cells.

6.2   CARMA1 AND MALT1 ARE REQUIRED FOR MTORC1 ACTIVATION IN T CELLS

We show that Carma1 and MALT1 are required for activation of the mTORC1 pathway downstream of PKC. We are one of the first groups linking Carma1 to the mTORC1 pathway. Our data primarily focus on T cells, and thus we are unable to conclude if Carma1 also plays a significant role in mTORC1 activation in B cells. We predict that similar to our findings in T cells, Carma1 and MALT1 are critical for mTORC1 activation in B cells. However, due to the necessity of PKCβ in phosphorylation of Carma1 there may be differential signaling upstream of

Carma1 after B cell receptor engagement, as well as different requirements for co-stimulatory molecules (CD80/86, ICOSL and OX40L) (225).

Another member of the CARD family, Card9, also associates with MALT1 and Bcl10 in cells of the myeloid lineage to induce activation of the NF-κB pathway. This pathway does not integrate signals from antigen receptors, but rather innate receptors like Dectin, TREM, Mincle and NOD2 (225). Due to the nature of the activation, we hypothesize alternate signaling mechanisms for mTORC1 activation in these cells, through Card9 and MALT1. Additionally, we expect differential activation of mTORC1, depending on which receptor is activated.

To further understand how Carma1 is regulating mTORC1 activation in T cells, we would like to assess the necessity of specific domains in Carma1 that are required for mTORC1. Our results showing the nonobligatory role for Bcl10 in this pathway, which binds to the CARD domain, indicate that this may not be required, however the CARD domain may be a docking site for a currently unknown protein (31, 66). The coiled coil (CC) and PKC responsive (PRD) domains in Carma1 are both essential for downstream function as MALT1 is predicted to bind to the former, and the latter is necessary for activation by PKC (31, 66). The MAGUK domain is necessary for localization of Carma1 at the membrane, allowing for activation by PKC (31, 66). Carma1 is also regulated by CKI, Akt and IKKβ (31, 66), whether phosphorylation by these kinases is necessary for Carma1 mediated regulation of the mTORC1 has yet to be determined.

We show that the widely expressed protein, MALT1 is also essential for mTORC1 activation in T cells, providing another pathway that MALT1 regulates. Of note, MALT1 inhibition (at least with z-VRPR-fmk) does not inhibit JNK activation in T cells. Currently, this paracaspase has been shown to regulate activation of NF-κB, c-FLIP$_L$ and the β$_1$ integrin ligand fibronectin, and now the mTORC1 pathway (96, 100, 197). Functionally, this inhibition

translates to impaired T cell proliferation (NF-κB, mTORC1 and c-FLIP$_L$ pathways), T cell adhesion (Bcl10 and β$_1$ ligand fibronectin) and T cell metabolism (mTORC1 pathway) (96, 100, 197). Continued research in this area will be needed to define the mechanism through which MALT1 regulates the mTORC1 pathway, specifically identifying MALT1 substrates. None of the previously mentioned MALT1 targets (Table 1) have been shown to directly affect mTORC1 activation in any cell type studied; as such, it more likely that a yet unidentified target for MALT1 is acting in this novel pathway.

There may be additional roles for MALT1 beyond its paracaspase activity. It is possible that MALT1 serves as an adaptor protein to facilitate mTORC1 activation, leading to activation of S6/S6K and 4E-BP1. TRAF6, a well-characterized MALT1 associated protein, modulates mTORC1 activation through association and ubiquitination leading to impaired autophagy and cell proliferation after amino acid stimulation (226). TCR and co-stimulation may also induce association of TRAF6, mTORC1 and MALT1, leading to downstream function. To further understand possible differential requirements of the MALT1 protein or it paracaspase activity, assessment of transcriptional differences between MALT1 KO T cells and WT T cells treated with z-VRPR-fmk will need to be performed. Based on the growing recognition of the importance of mTOR in T cell differentiation and metabolism, focus should be placed on the role of Carma1 and MALT1 in controlling T cell lineage-specific transcription factors, and associated co-activators, as well as on genes necessary for T cell metabolism (for both oxidative phosphorylation and glycolysis).

Surprisingly, we found that Bcl10, a MALT1 binding partner, is not required for activation of the mTORC1 pathway, using both Jurkat T cells knocked down in Bcl10 and primary murine Bcl10 KO T cells. While these findings were initially confounding, as Carma1,

MALT1 and Bcl10 generally work as a complex to activate NF-κB and JNK pathways, work by Pomerantz and colleagues showed that the kinesin GAKIN competes with Bcl10 to bind the CARD domain of Carma1, to negatively regulate NF-κB activity (195). Of note, binding of GAKIN did not perturb association of Carma1 with other proteins (TRAF 6 and IKKγ) and translocated it from the immunological synapse (IS) (195). Bcl10 constitutively associates with MALT1 and is thought to stabilize MALT1 protein expression (31). In the absence of Bcl10 or MALT1 there is reduced expression of MALT1 or Bcl10, respectively (31). We did note reduced Bcl10 in MALT1 shRNA cells Fig. 4-3A), however reconstitution of these cells with WT MALT1 restored NF-κB activity (data not shown) (190).

We also show that activation of NF-κB is not necessary for S6 phosphorylation (Fig. 4-4E) a finding consistent with a very recent study using IKKβ KO T cells (227). A major goal which remains is understanding differential functional outcomes downstream of Carma1 and MALT1 leading to activation of the NF-κB versus mTORC1 pathways. Both pathways contribute to T cell proliferation and metabolism (4, 183, 228), e.g. IKKα regulates oxidative metabolism in muscle cells (229). To address whether IKK also regulates metabolism in activated T cells, one could perform microarray experiments in stimulated CD4+ T cells with z-VRPR-fmk treatment, or z-VRPR-fmk in CD4+ T cells expressing constitutively active IKKβ (230). Utilizing these mice might allow the identification of MALT1 paracaspase-dependent genes that are impaired independently of NF-κB. In conjunction with these experiments, it may also be used to also perform microarray experiments comparing stimulated CD4+ T cells from Bcl10 KO mice and CD4+ T cells from MALT1 KO or Carma1 KO mice. Bcl10 is dispensable for TCR and CD28-dependent mTORC1 activation in T cells, so in this way differences in

genetic profiles between Bcl10 KO and MALT1 KO or Carma1 KO could be attributed to the mTORC1 pathway.

6.3   MALT1 CATALYTIC ACTIVITY REGULATES S6 PHOSPHORYLATION IN
ABC-DLBCL

Understanding distinct signaling pathways downstream of antigen receptors is essential in unraveling the molecular mechanisms that drive disease. We show that inhibition of MALT1 is able to prevent stimulation-dependent S6 phosphorylation in the ABC-DLBCL sub-type. Our data provide evidence for additional mechanisms through which MALT1, and potentially also Carma1, may be driving cellular growth in this DLBCL sub-type, apart from effects on NF-κB (109). It would be interesting to review the genetic signature of ABC-DLBCL, previously ascertained by the Staudt group, for potential mTORC1 target genes (109). Several groups have shown that inhibition of MALT1 induces cell death specifically in the ABC-DLBCL sub-type, attributing most effects to inhibition of the NF-κB pathway (101, 102, 111, 114). Going forward, emphasis should be placed on understanding other cellular pathways that Carma1 and MALT1 may regulate.

6.4 SERINE 231 OF BCL10 IS REQUIRED FOR NF-KB ACTIVATION IN T CELLS

The previous chapter addresses regulation of Bcl10 in its C-terminus. We show that serine 231 is required for TCR and CD28 mediated NF-κB activation and IL-2 production in T cells. Previously published work shows Akt as the kinase phosphorylating this site, however our data do not confirm this finding and that inhibition of Akt does not affect Bcl10 phosphorylation (208). We also specifically assessed S231 phosphorylation in the presence of an Akt inhibitor, and no change was noted (data not shown), however inhibition with a pan-PKC inhibitor, inhibited phosphorylation at S231. We are unsure if PKC directly phosphorylates this site, or if PKC activates another kinase which directly phosphorylates S231. This site has substrate specificity for the AGC kinases: S6K, RSK and serum and glucocorticoid-inducible kinase (GSK) (188). PKC may regulate one of these kinases leading to Bcl10 phosphorylation.

6.5 CONCLUSION

We have identified a novel signaling pathway in T cells, whereby TCR and CD28 co-stimulation activate PKC, Carma1 and MALT1 which induces mTORC1 activation leading to T cell proliferation and increased metabolic output (Fig. 6-1). mTORC1 is a critical player in T cell activation and differentiation, through increased expression of metabolism-associated genes (HIF1α, c-myc amino acid transporters and glycolytic proteins) and inhibition of Foxp3 expression (Treg signature transcription factor). Thus, understanding how this pathway is

activated specifically through the T cell antigen receptor is essential for our understanding of T cell responses to different pathogens. In the cases of aberrant T cell activation- autoimmune disease-identification of novel targets will provide new courses for therapies which do not fully cease T cell activity, and yet still provide relief from disease.
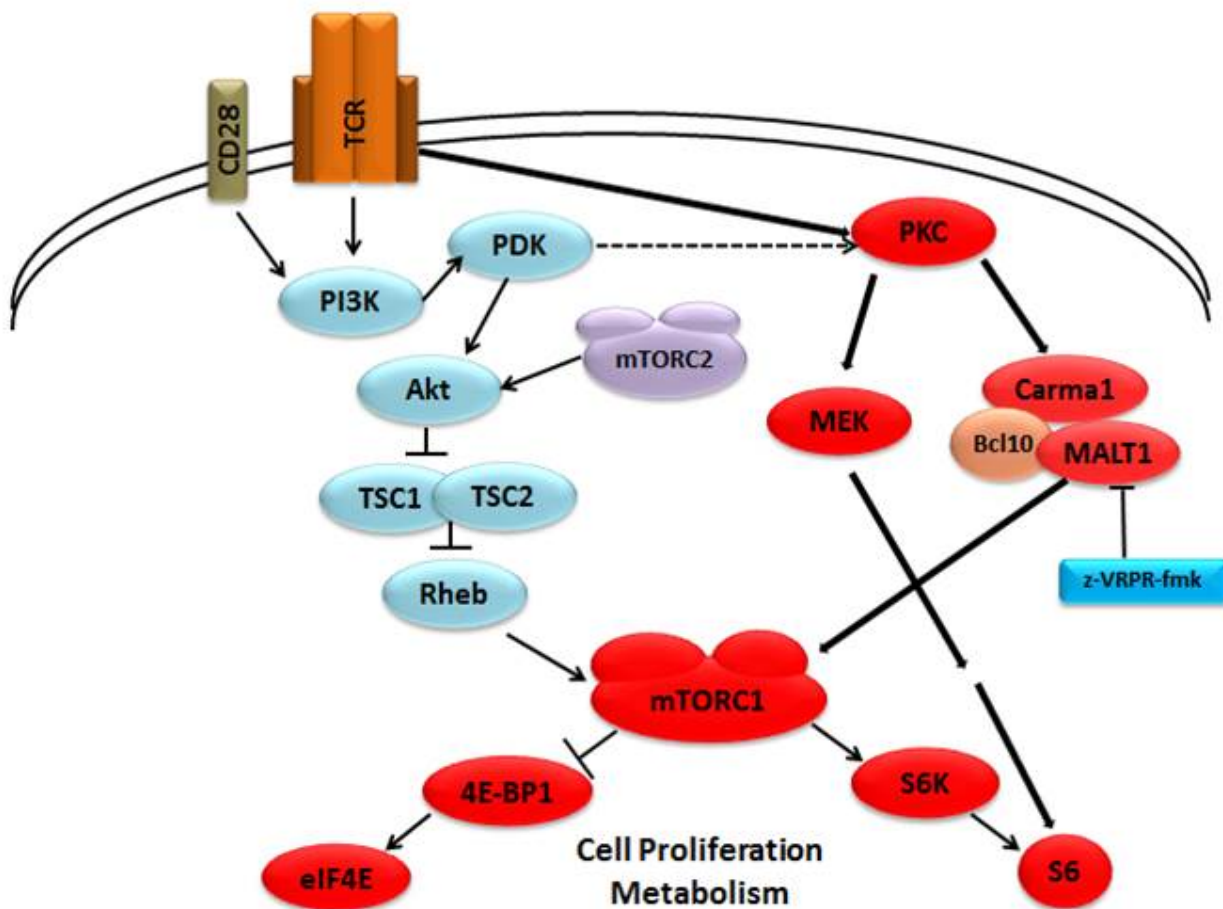
Figure 6-1 Model for PKC, Carma1 and MALT1 regulation of mTORC1 activation in T cells.

Acknowledgements

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