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The Role of CARMA1 in T Cells

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Abstract

Caspase recruitment domain-containing membrane-associated guanylate kinase protein-1 (CARMA1), a member of the membrane associated guanylate kinase (MAGUK) family of kinases, is essential for T lymphocyte activation and proliferation via T cell receptor (TCR) mediated NF-κB activation. Recent studies suggest a broader role for CARMA1 regulating other T cell functions as well as a role in non-TCR mediated signaling pathways important for lymphocyte development and functions. In addition, CARMA1 has been shown to be an important component in the pathogenesis of several human diseases. Thus, comprehensively defining its mechanisms of action and regulation could reveal novel therapeutic targets for T cell-mediated diseases and lymphoproliferative disorders.

Keywords

CARMA1; T cell; T cell receptor; NF-κB

I. INTRODUCTION

T lymphocytes are critical components of the immune response and play a major role in the defense against infections and tumors. The capacity of these cells to detect specific pathogen- or disease-specific antigens and provide immunologic memory, defines the adaptive immune response. However, inappropriate or excessive activation of T cells can cause non-infectious inflammatory diseases or lead to hematologic malignancies. Thus, an understanding of the molecular mechanisms that regulate T lymphocyte activation and functions is necessary to understand immune defense and disease, and to develop effective therapies for T cell-mediated disorders and malignancies.

T cell development and the activation of naïve T cells is dependent on T cell receptor (TCR) binding to cognate peptide-major histocompatibility complex (pMHC) complexes on antigen presenting cells (APCs). In addition, subsequent T cell proliferation and survival is dependent on signaling through the TCR, and TCR signaling strength can influence the development and function of effector T cells, memory T cells and the formation of regulatory T cells (Treg).[1–6] Therefore, the intensity and nature of T cell-mediated inflammatory reactions depends on the strength of the TCR stimulus both initially and during the immune response.[1–3] In addition, dysfunction in the TCR signaling pathway can contribute to lymphoproliferative disorders.[4]

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The scaffold protein caspase recruitment domain-containing membrane-associated guanylate kinase protein-1 (CARMA1), a member of the MAGUK family of kinases, has been shown to be an essential component in TCR signaling in T lymphocytes and B cell receptor (BCR) signaling in B lymphocytes. Scaffold proteins help physically assemble multiple signaling components in response to a specific stimulus, thus helping cells organize protein complexes to provide specificity in signaling pathways.[5] This could involve a simple association to increase the efficiency of interactions between molecules (Figure 1A) or more complex control over multiple partners allowing several outputs from a single stimulus (Figure 1B). In addition, scaffold proteins like CARMA1 are often targets of regulation and may lead to a biphasic response with enhancement of signaling at low to moderate levels and inhibition at high levels (Figure 1C). Thus, scaffold proteins allow cells to regulate signaling for specific pathways and selectively shape output behaviors.[5]

CARMA1 is expressed selectively in T and B lymphocytes where in resting T cells it resides in a closed and inactive form. Multiple studies have now demonstrated that following engagement of the TCR, CARMA1 is activated, localizes to the TCR signalosome at the plasma membrane, and then forms multi-protein complexes that initiate NF-κB and c-jun N-terminal kinase (JNK) signaling cascades.[6–8] Consistent with this, CARMA1-deficient T lymphocytes have impaired antigen-induced proliferation and activation, and have enhanced cell death with TCR stimulation.[9] Recent data has suggested that CARMA1 is activated and regulated via complex interactions with numerous proteins and that CARMA1 may help mediate signaling in other non-TCR pathways in T cells.[10, 11] Overall, it appears that CARMA1 is a signaling protein in T cells that has a complex role in modulating T cell activation and functions. The fact that mutations in CARMA1 can be associated with immunodeficiency, allergy, autoimmunity, and lymphoproliferative disorders demonstrates its importance for lymphocyte function,[12–18] and suggests that CARMA1 is critical for T cell-mediated inflammation. Furthermore, since CARMA1 has multiple protein interactions and phosphorylation sites, it could be a prime target for therapeutic intervention. Herein, we will review the current state of knowledge regarding the role of CARMA1 in mediating T cell functions as well as its role in T cell mediated immunity and inflammation.

II. STRUCTURE AND FUNCTION OF CARMA1

A. Structural Features of CARMA1

CARMA1 is a scaffolding protein comprised of five domains connected by intervening stretches of linker regions. The domains are: 1) an N-terminal caspase activation and recruitment (CARD) domain; 2) a coiled-coil (CC) domain; 3) a PDZ homology domain (Postsynaptic Density Protein [PSD95], Drosophila Disc Large Tumor Suppressor [DLG1] and Zona Occludans-1 protein [ZO1]); 4) a SRC homology 3 (SH3) domain; and 5) a guanylate kinase (GUK) domain. Among these, the PDZ, SH3 and GUK domains together constitute the membrane associated guanylate kinase (MAGUK) domain, a catalytically inactive domain that is evolutionarily conserved in proteins. This domain plays a major role in cellular adhesion, formation of multi-protein complexes, and signal transduction.[9, 19, 20] It appears that this region may be critical for localization of CARMA1 to the plasma membrane and for CARMA1 multimerization, two processes thought to be important for CARMA1 function.[8, 9, 21–24] Among the CARMA1 domains, only the PDZ domain has been shown to be dispensable for CARMA1 driven NF-κB activation.[21, 24–27]

CARD domains are protein motifs that facilitate multi-protein binding via CARD-CARD interactions. These motifs have long been associated with a multitude of outcomes such as caspase activation, apoptosis and inflammasome assembly in innate immunity. Accumulating evidence over the last decade strongly suggests that CARD domain containing proteins such as CARMA1 also play a crucial role in the adaptive immune

system by the regulation of NF-κB and JNK transcription factor pathways.[12, 14, 22, 28–31] Importantly, the CARD domain in CARMA1 interacts with a CARD motif in B-cell CLL-lymphoma 10 (Bcl10), which helps mediate NF-κB activation following TCR engagement.[32, 33]

The coiled-coil motif is a common domain found in many types of proteins. Its primary role is to facilitate oligomerization of proteins,[34, 35] and it has been postulated that this region is needed for CARMA1 oligomerization following activation and may help mediate binding to mucosa-associated lymphoid tissue lymphoma translocation gene 1 (MALT1), another essential protein for NF-κB activation following TCR engagement.[22, 23, 36] The domain appears to be critical for CARMA1 function based on studies in a mouse strain with a mutation in this domain of CARMA1.[25]

The region linking the coiled-coil domain to the MAGUK domain (linker region) appears to stabilize CARMA1 in an inactive or "closed" form (Figure 2).[30] This is suggested by studies which demonstrated that deletion of this region leads to constitutive activation of NF-κB and high-level CARMA1 activation.[27] Phosphorylation of serine residues in this region by PKCβ in B cells or PKCθ in T cells leads to activation of the protein presumably by "opening" up its conformation and allowing it to interact with other proteins as well as form multimers with itself.[27, 37]

T cell activation typically involves the interaction of the TCR with pMHC complexes and also the interaction of T cell and APC co-stimulatory molecules at the immunological synapse. Formation of the immunological synapse during the interaction of T cells and APCs is the first step in shaping of the T cell driven adaptive immune response. The CARD-CC-MAGUK domains in CARMA1 ideally suits its involvement in plasma membrane tethering, oligomerization and formation of multi-protein complexes at the immune synapse.[38]

B. Binding Partners of CARMA1

In resting T cells, CARMA1 is constitutively expressed and primarily localized under the cytoplasmic membrane in an inactive conformation.[8, 30] TCR stimulation leads to the activation of PKCθ, which phosphorylates and activates both CARMA1 and TGFβ associated kinase 1 (TAK1).[39, 40] After activation CARMA1 acquires an active conformation (Figure 2) and is recruited to the immunological synapse where it self-associates in multimers and interacts with multiple other proteins to initiate signaling cascades.[8, 27, 37] Overexpression and mutation studies of CARMA1 and its binding partners suggest that oligomerization of the proteins is both necessary and sufficient for activation of downstream signaling pathways such as NF-κB.[22, 40] Many of the proteins that interact with the CARMA1 signalosome are kinases and ubiquitylating enzymes which probably help regulate CARMA1 activity (Table 1).

Once activated, CARMA1 binds to Bcl10 via CARD-CARD interactions, and then this complex binds the immunoglobulin-like domains in MALT1 via the distal end of the Bcl10 CARD domain to complete the assembly of the CARMA1-Bcl10-MALT1 (CBM) complex at the TCR signalosome. There may also be direct association between CARMA1 and MALT1 through the C–C domain.[36] The CBM complex is essential for activation of transcription factors such as NF-κB and JNK (see signaling sections), and is critical for the recruitment and activation of the IκB kinase (IKK) complex to the TCR signalosome (Figure 3). Other proteins also interact with CARMA1 and may have a role in mediating its activity. The adhesion and degranulation-promoting adapter protein (ADAP) has been shown to interact with CARMA1 and may help translocate CARMA1 to lipid rafts at the TCR signalosome.[41] Of note, ADAP-deficient T cells have impaired TCR-induced NF-κB

activation. Another study has demonstrated that phosphoinositide-dependent kinase 1 (PDK1) binds to CARMA1 and may help recruit it to lipid rafts.[42] However, it is not entirely clear whether PDK1 is necessary for TCR-induced NF-κB activation as experiments have given contradictory results.[42, 43] It also seems likely that CARMA1 binds to a different membrane-associated protein to localize to the cytoplasmic membrane.[30] Other kinases in addition to PKCθ and PDK1 have been shown to associate with CARMA1 and/or phosphorylate it including AKT, TAK1, RIP, PKCδ, hematopoetic progenitor kinase-1 (HPK1), IKKβ, casein kinase 1α (CK1α) and calmodulin-dependent protein kinase II (CaMKII). However, it is unclear how critical all of these are for optimal CARMA1 function.[11, 37, 42–50] The CBM also associates with the ubiquitin ligase TNF receptor-associated factor 6 (TRAF6) which ubiquitinates Bcl10 and MALT1 which may facilitate IKK complex recruitment to the CBM via binding to the IKKγ subunit.[40] TRAF6 may also directly activate the IKK complex by ubiquination of IKKγ.[51] Other ubiquitin ligases such as TRAF2, Cbl-b, and STUB1 have also been shown to bind to CARMA1 and may regulate its function.[11, 47, 52] A complete list of proteins found to interact with CARMA1 is shown in Table 1.

C. CARMA1-Mediated Signaling Pathways

1. NF-κB Signaling—Activation of NF-κB is critical for multiple facets of T cell driven adaptive immune responses, including T cell development, activation, polarization, proliferation and survival.[53, 54] Engagement of the TCR in T cells is one of the major triggers for activation of NF-κB, and this signaling pathway is dependent on CARMA1.[12, 14] Exactly how CARMA1 helps mediate NF-κB activation is not fully understood, however, many of the key steps and mediators are now known.

Five individual transcription factors (RelA or p65, RelB, c-Rel, p50 and p52) constitute the NF-κB family of proteins. Among these, RelA and c-Rel form heterodimers with p50, and RelB heterodimerizes with p52 to form functional units of NF-κB. These complexes, when allowed to translocate to the nucleus, will then induce transcriptional activation of NF-κB target genes.[55] NF- κB can be activated via the classical (or canonical) pathway which depends on p50, RelA, or c-Rel, or by the alternative pathway that involves RelB and p52. TCR-induced NF-κB activation is through p50, RelA, and c-Rel, so for this review we will focus on the classical pathway.[7, 14]

In resting T cells NF-κB is sequestered in the cytoplasm by association with the inhibitor of κB (IκB). This protein prevents nuclear translocation of the transcription factor by masking its nuclear localization motif. NF-κB is activated when the IκB complex is degraded, which allows the transcription factor to travel to the nucleus and initiate gene transcription. Stimulation of cells with a wide range of stimuli ultimately results in activation of the IKK complex, which is made up of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ or NEMO). Once activated, IKK phosphorylates IκB which then triggers ubiquitination and degradation of the protein, allowing NF-κB to translocate into the nucleus. All stimuli that activate the classical NF-κB pathway utilize IKK, however, specificity is obtained upstream of IKK by signaling intermediates such as CARMA1, which tend to be both cell- and pathway-specific mediators of NF-κB activation. In TCR-induced NF-κB activation, it is the formation of the CBM complex that ultimately activates IKK and initiates NF-κB activation.

The exact mechanism(s) by which the CBM complex leads to NF-κB activation has not been fully delineated. Some studies have suggested that the CBM complex facilitates the phosphorylation of IKKβ via the TAK1 kinase.[40, 44] Whereas a more recent study suggests that the phosphorylation of IKKα and IKKβ occurs via TAK1 independently of CARMA1, suggesting that the major mechanisms by which the CBM complex initiates IKK activation

is via recruitment of IKKγ to the TCR signalosome leading to its polyubiquitination.[39] Consistent with this, studies have shown that MALT1 functions as an ubiquitin ligase that induces polyubiquitination of IKKγ leading to activation of IKK,[56] while other studies have suggested that TRAF6 associates with the CBM and is the primary ligase involved in the polyubiquitination of IKKγ.[40] A different study has suggested that TRAF6 directly ubiquinates MALT1 which then leads to association of MALT1 and IKK.[51] It is also possible that other E3 ligases are involved in the process.

Another potential mechanism by which the CBM mediates NF-κB activation involves the paracaspase activity of MALT1.[36] Among the members of the CBM complex, MALT1 is the only enzymatically active component with a caspase like functional domain whose catalytic activity is enabled upon its assembly in the CBM complex. Following TCR stimulation, MALT1 in the CBM signalosome can cleave Bcl10, the NF-κB inhibitory protein A20 (a deubiquitinating enzyme), and the NF-κB subunit protein RelB at the same consensus cleavage site.[57–59] The protease activity of CBM-associated MALT1 has been linked to several functional outcomes in T-cells following TCR stimulation. For example, cleavage of Bcl10 by CBM-associated MALT1 in activated T-cells was not essential for NF-κB activation or IL-2 production, but enhanced T cell adhesion to integrin ligands such as fibronectin.[58] In this context, it has been proposed that MALT1 in the CBM complex may have a dual function – the first being driven by MALT1 in the CBM complex that degrades IkB proteins to allow nuclear translocation of NF-κB, and the second being driven by MALT1 protease activity that degrades RelB to prolong the DNA binding capacity of RelA- and c-Rel- containing NF-κB complexes through a yet unclear mechanism.[59] Caspase-8 has also been shown to be associated with the CBM complex following TCR stimulation,[60] and it was later shown that MALT1 directly induces Caspase-8 activation to induce NF-κB stimulation and IL-2 production.[61]

2. JNK Signaling—The JNK family is comprised of the ubiquitously expressed kinases JNK1 and JNK2 and the tissue restricted kinase JNK3. Activation of the TCR triggers the activation of JNK, which helps mediate T-cell proliferation and differentiation.[62–65] Unlike constitutively expressed transcription factors such as NF-κB, TCR stimulation induces the expression of JNK, while CD28 co-stimulation is necessary for phosphorylation and activation of JNK.[66] Following TCR activation, CARMA1 and Bcl10 form a molecular scaffold on which the kinases TAK1, MKK7 and JNK2 are assembled, leading to JNK2 phosphorylation.[12, 30, 67] Phosphorylated JNK2, together with the independently phosphorylated JNK1, induce the activation of the transcription factor AP-1, which regulates T cell proliferation and survival.[68] AP-1 is a heterodimeric protein formed by the members of the Jun family (c-Jun, JunB and JunD) with Fos or ATF/CREB families of transcription proteins.[69] Engagement of the TCR leads to transcriptional upregulation of c-Jun, which is then capable of upregulating its own expression through a feedback amplification loop.[70, 71] T cells from CARMA1-deficient mice fail to activate JNK following TCR stimulation suggesting that CARMA1 mediates JNK activation.[67, 72] Consistent with this, TCR-induced accumulation of c-Jun is severely impaired in a CARMA1-deficient T cell line,[67] and it appears that deficiency of CARMA1 leads to enhanced ubiquitination and the subsequent degradation of c-Jun.[73] Despite these functional relationships between CARMA1 and JNK, deficiency of CARMA1 does not lead to defective AP-1 activity in T cell lines or primary T cells following TCR stimulation.[21, 26, 67, 72] The uncoupling of CARMA1 dependent JNK phosphorylation from AP-1 activity therefore suggest that signaling through JNK1 and other Jun family members can still drive AP-1 activity in the absence of CARMA1.[12, 30] Since JNK phosphorylation leads to the activation of a plethora of transcription factors, it remains to be determined if CBM-complex driven JNK signaling in T cells participates in the activation of transcription factors other than AP-1, and how important CBM-mediated JNK activation is for overall T cell functions independent of NF-κB activation. It is also not clear

whether CBM-mediated JNK activation depends on unique binding partners and regulatory pathways different from those needed for NF-κB activation.

D. Regulation of CARMA1

1. Phosphorylation of CARMA1—While the conserved domains in CARMA1 drive its function, the activation of CARMA1 in T cells is largely determined by the phosphorylation status of key residues throughout the protein. These phosphorylation events presumably change the conformation of CARMA1 allowing it to interact with downstream binding partners. The dominant activation signal seems to be phosphorylation of serine residues S552 and S645 (S564 and S657 for murine CARMA1) in the linker region that connects the coiled-coil and PDZ domains by PKCθ in T-cells and PKCβ in B-cells. This phosphorylation event is essential for the assembly of the CBM complex and the subsequent activation of NF-κB and JNK.[27, 37] Multiple other kinases have been shown to phosphorylate and regulate CARMA1 function (Table 2). These include, CaMKII, which localizes to the immune synapse following TCR activation and phosphorylates CARMA1 at residue S109 (S116 in mice) to enhance NF-κB activation.[45] HPK1 also phosphorylates CARMA1 at residues S549, S551, and S552, of which the residues S549 and S551 are obligatory for TCR-induced NF-κB activation and IL-2 production.[48] In addition, IKKβ has been shown to phosphorylate CARMA1 at S555 and is important for NF-κB and JNK activation as well as CBM complex formation.[43] CARMA1 T110 has been shown to be phosphorylated with antigen receptor activation, and mutation of the residue impairs CBM formation.[43] However, the kinase that acts on T110 has not been identified. On the other hand, mutation of two other residues in murine CARMA1, S620 (targeted by CK1a) and S649 (targeted by PKCθ), resulted in enhanced activation of NF-κB upon TCR stimulation, suggesting that phosphorylation of these residues may serve to downregulate CARMA1 driven NF-κB activation.[49, 74] In a recent report, PKCδ was co-purified with the CARMA1 signalosome upon TCR activation, and overexpression of PKCδ inhibited TCR driven NF-κB activation in T cells by interfering with the physical interactions between MALT1 and TRAF6.[50] However, the kinase activity of PKCδ was dispensable for this activity and does not seem to play a role in limiting CBM complex driven T cell responses.

While phosphorylation by kinases generally leads to the assembly of the CBM complex and T cell activation, counter-regulatory mechanisms such as dephosphorylation by phosphatases may also be present to downregulate T cell activation. There is now accumulating evidence that there are specific phosphatases that target members of the CBM complex to limit NF-κB activation in T cells. A subunit of the serine/threonine protein phosphatase PP2A (PPP2R1A) was constitutively associated with CARMA1 in resting and activated cells.[75] Upon T cell activation, PP2A removed PKCθ dependent phosphorylation of CARMA1 at residue S645, leading to the attenuation of NF-κB responses. Similary, the calcium dependent phosphatase calcineurin dephosphorylated Bcl10 to disrupt the formation of CBM complexes and NF-κB activation.[76, 77] Most recently, another protein phosphatase (PP4R1) subunit was found to be associated with the CBM complex following TCR stimulation.[78] PP4R1 is a component of the phosphatase holoenzyme PP4, which dephosphorylated IKKα and IKKβ to suppress NF-κB activation in T cells, a phenomenon that reduced CARMA1-mediated NF-κB activity.[78]

In summary, CARMA1 is phosphorylated at multiple sites by a diverse set of kinases including PKCθ/β, IKKβ, CaMKII, HPK1, and CK1α. Whether other kinases, such as PDK1, TAK1, and Akt, which are known to associate with CARMA1, also phosphorylate CARMA1 remains to be determined. Phosphorylation of S608 and S637, or dephosphorylation of other sites mediates negative regulation of CARMA1 function. The fact that CARMA1 has such a complex array of phosphorylation sites suggests that the

activity of this protein must require tight regulation for effective T cell function. Exactly how all of these events modulate CARMA1 binding activity as well as NF-κB and JNK activation remains to be fully determined.

2. Ubiquitination of CARMA1—Not surprisingly, post-translational modifications that target CARMA1 for degradation also downregulate TCR-mediated NF-κB activation. For example, ubiquitination of lysine residues in the SH3 and GUK domains of CARMA1, are induced following TCR activation and lead to proteasomal degradation of CARMA1.[79] In addition, elimination of these ubiquitination sites in CARMA1 resulted in elevated NF-κB and JNK activation and increased persistence of CARMA1 protein in activated lymphocytes. In natural killer T cells (NKT cells), repeated doses of the agonistic ligand α-galactosylceramide leads to the ubiquitination and degradation of CARMA1 by the E3 ligase Cbl-b, inducing NKT cell anergy.[80] Similar processes also regulate Bcl10 as demonstrated by a recent study which showed that TCR activation in primary T cells was associated with ubiquitination of Bcl10 and degradation by the autophagy dependent proteolysis machinery.[81] Inhibition of Bcl10 autophagy resulted in enhanced activation of NF-κB upon TCR stimulation. Multiple ubiquitinating and deubiquitinating enzymes have been shown to target the CARMA1 signalosome (Table 1), however, more work is needed to fully understand which enzymes regulate CARMA1 activity and how this effects T cell functions.

III.Role of CARMA1 in TCR signaling

A. TCR signaling pathway

TCR induced T-cell activation is a critical event in adaptive immune responses. The ability of a T cell to recognize an antigen is dictated by the interaction of the TCR and peptides presented in the groove of the MHC molecule on APCs. This TCR-pMHC interaction initiates a complex process that transforms a naïve T cell into a cell capable of executing effector functions.[82] The TCR is composed of two ligand binding chains (TCRα and TCRβ) which form a complex with a cluster of four different CD3 signaling invariant chains organized into three dimers (CD3γε, CD3δε, and CD3ζζ). These chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails.[83, 84] The TCR-CD3 complex is accompanied by a CD4 molecule in T helper cells or a CD8 molecule in cytotoxic cells.

It is thought that engagement of the TCR complex with the appropriate pMHC triggers the TCR signaling pathway via TCR aggregation and conformational changes in the complex that induces changes in the CD3 subunits.[85] These conformational changes allow phosphorylation of the ITAMs by the protein tyrosine kinases Lck and Fyn and the subsequent recruitment of the Syk kinase ζ-associated protein of 70 kDa (ZAP-70) to the complex. This is followed by the recruitment and phosphorylation of linker for activation of T cells (LAT),[86–88] which serves as a platform for several signaling proteins. The recruitment of multiple proteins to the TCR as well as lipid rafts creates the TCR signaling complex at the point of contact with the APC forming the immune synapse. Sequential amplification of downstream signals leads to the recruitment of an array of molecules to the immunological synapse and activation of a network of signaling pathways (reviewed in Smith-Garvin *et al*[85]). These dynamic and highly regulated signaling complexes induce PLCγ1-dependent calcium- and diacylglycerol (DAG)-mediated responses. TCR-mediated calcium and DAG release is instrumental in activation of PKCθ which then activates CARMA1 and starts the cascade that stimulates NF-κB and AP-1 activity as outlined in previous sections.[85] Co-stimulatory signals from proteins such as CD28 augment these signaling pathways.

The signaling pathways activated in response to TCR engagement include NF-κB, AP-1 (via JNK activation), and NFAT.[85] Once activated, these transcription factors mediate the expression of numerous genes involved in T cell activation. Furthermore, the profile and degree of activation of these pathways will ultimately determine aspects of the T cell response including effector functions. The magnitude of activity of these pathways is mediated in part by the strength of the TCR signal. Consistent with this, TCR signal strength has been shown to determine short-term T cell activity as well as long-term T cell functions and inflammatory events.[89] These include effects on T cell proliferation, cytokine production, and cytotoxicity.[89–93] These data suggest that alterations in the activity of downstream signaling proteins in the TCR cascade could be utilized to influence T cell-mediated inflammatory disorders.[3] We propose that in T cells CARMA1 has as its primary function the amplification of TCR signaling, providing a mechanism to enhance signals from pMHC complexes. It is unclear if the output generated by CARMA1 is digital (switch-like) or analog (the greater the CARMA1 activity the stronger the TCR signal), but prior work has suggested that TCR-mediated NF-κB activation is digital in nature and that the digitization occurs upstream of activation the IKK complex.[94] This suggests that CARMA1 may amplify the TCR signal above a threshold that leads to NF-κB activation. However, CARMA1 could alter the duration of NF-κB signaling, modulate JNK signaling in an analog fashion, or modulate NF-κB signaling via interactions with co-stimulatory pathways. Overall, we feel that CARMA1 provides a mechanism to fine-tune TCR-mediated signaling in response to an antigen.

B. Role of Carma1 in naïve T cells

1. T cell development—Naïve T cells emerge from the thymus after undergoing a complex selection process that produces cells that react to a highly diverse set of foreign-pMHC complexes without reacting with self-antigens for the most part.[95, 96]. Although the development of T cells in the thymus requires TCR signaling, studies in CARMA1-deficient mice have revealed normal cell numbers in the thymus and normal T cell numbers and ratios in lymph nodes.[97] However, there is some variation in the numbers of thymocyte subsets within the double-negative compartment ($CD4^{-}CD8^{-}$). This may be due to premature maturation of these cells as well as an accelerated rate of apoptosis. Not surprisingly, this thymic phenotype is very similar to that described in Bcl10-deficient mice.[98] The impact of these thymocyte subset abnormalities does not seem to be significant. Overall it appears the development of naïve T cells is largely unaffected by CARMA1-deficiency despite the dependence of the selection process on TCR signaling. However, whether alterations in CARMA1 activity will affect the TCR repertoire of naive T cells has not been determined.

2. T cell activation—Naïve T cells congregate in secondary lymphoid tissues and migrate continuously from one lymphoid organ to another via the blood and lymph. In the lymph node, T cells enter from the blood thru the HEV into the T cell zone in a process mediated by homing receptors CD62L and CCR7. Naïve T cells in the periphery constitute a stable, diverse and functional repertoire of cells, maintained by homeostatic mechanisms.[99] The activation of a naïve T cell depends on strong TCR binding to cognate pMHC complexes on APCs.[85, 100] As discussed in the previous sections, CARMA1 is required for optimal activation of T cells, and deletion of CARMA1 abolishes TCR-induced activation of JNK and NF-κB. Interestingly, it appears that activation of naïve $CD8^{+}$ T cells is not as impaired as it is in $CD4^{+}$ T cells, suggesting that these cells are less dependent on CARMA1 and that an alternate pathway may be active in $CD8^{+}$ T cell activation (unpublished observations).

3. T cell proliferation—An effective adaptive immune response relies on the capability of activated cells to undergo rapid expansion. A productive engagement of the TCR signal guides the fate of the cells towards a proliferative state. The early activation responses that

induce T cell proliferation and signal amplification, is characterized by the secretion of pro-inflammatory cytokines (i.e. IL-2) and upregulation of the alpha chain of the IL-2 receptor (CD25). T cell proliferation and the expression of these cytokines are highly dependent on NF-κB as well as AP-1 activation.[85] As discussed in the previous section, TCR-mediated NF-κB and AP-1 activation is dependent on CARMA1, thus induction of gene expression of these cytokines and their effects on proliferation is also dependent on CARMA1. This has been demonstrated in studies where deficiency in CARMA1 correlates with impaired proliferation signals.[72, 97] In addition, CARMA1-deficient $CD4^+$ and $CD8^+$ T cells show defective proliferation and display markedly abrogated cytokine production. These data demonstrate that T cell proliferation is intrinsically dependent on the formation of the CBM complex.[31, 101]

4. T cell survival—An effective T cell-mediated immune response against infection requires expansion of antigen-specific T cells against the invading pathogen, and then contraction of T cell numbers after the pathogen is cleared. The downregulation of the immune response is important for limiting organ damage from the inflammatory response, but memory T cells specific for the pathogen must be maintained to preserve cellular immunity. Thus, signals that control T cell survival after activation are important components of adaptive immunity. TCR-mediated NF-κB activation provides an important survival signal to activated T cells. In addition, protection of cells against activation induced cell death during the immune response is mediated by an increase of co-stimulatory signals,[102] and cytokines such as IL-2, IL-4, IL-7 and IL-15.[103, 104] The survival of activated T cells is not fully dependent on CARMA1,[72, 97] however, recruitment of PKCθ to the immunological synapse and its association with CARMA1 upregulates the anti-apoptotic molecule Bcl-x, suggesting an indirect requirement of CARMA1 in T cell survival.[105] This is also demonstrated by studies in which CARMA1-deficient T cells have a high rate of apoptosis following activation, and in human studies of lymphomas which have demonstrated that mutations in CARMA1 provide high-level proliferative and survival signals in malignant cells.[15, 16, 97, 106, 107]

C. Role of CARMA1 in effector and memory T cells

TCR induced activation of naïve $CD4^+$ T cells lead to proliferation and differentiation into effector T cells that fight infection by producing cytokines that promote a number of activities that help eliminate invading pathogens.[108] As stated above the success of an effector T cells is attained upon initial T cell activation, a process largely dependent on CARMA1. In addition, effector T cells likely continue to encounter antigen and receive TCR signals which provide survival signals and enhance their effector function.[109] Following the clearance of infection and the reduction in TCR signaling, many of the effector cells are eliminated via apoptosis. However, some of the effector cells appear to differentiate into memory T cells that provide long-term cellular immunity to the specific pathogen. These cells can then be reactivated in recall responses to recurrent infections where they lead to a rapid and robust immune reaction.[110–113]

While the role of CARMA1 and CBM complex has been extensively investigated in T cell activation, proliferation and survival responses, there is a paucity of studies that describe the role of CARMA1 and the CBM complex in effector and memory T cells. The signals requirements for the re-activation of memory T cells are not the same as those in naïve and effector T cells,[114–116] and Bcl10 seems to be dispensable for activation of memory T cells,[117] suggesting that CARMA1 may not be critical for TCR signaling in effector and memory T cell responses. However, we have recently demonstrated that CARMA1 is necessary for optimal effector T cell and memory T cell function.[109] Deletion of CARMA1 from T cells after activation lead to impaired re-activation of memory T cells and reduced

allergic inflammation in a model of memory T cell recall responses. In addition, CARMA1-deficient effector T cells produced less allergic inflammation in an adoptive transfer model of allergic airway inflammation compared to wild-type effectors. Interestingly, there was no survival defect in the CARMA1-deficient memory cells. These data suggest that TCR signaling in effector and memory T cells is also dependent on CARMA1 activity. Whether the signaling outputs from CARMA1 differ in these cell types compared to naïve T cells is unclear at this time.

D. Role of CARMA1 in regulatory T cell development

The maintenance of peripheral tolerance and establishment of controlled T-cell responses is dependent of the generation of regulatory $CD4^+$ T cells (Tregs). Tregs can be generated from a bone marrow derived progenitor cell in the thymus (thymic Tregs) or from a naïve T cells during an inflammatory response in the periphery (peripheral Tregs).[118] Tregs have been characterized by their ability to suppress immune responses and regulate peripheral tolerance.[119] CARMA1-deficiency has been shown to alter the frequency of Tregs demonstrating its requirement for Treg cell lineage commitment.[120–122] The role of CARMA1 in each type of Tregs is discussed below.

1. Thymic Tregs—Thymic Tregs are characterized by their involvement in the regulation of autoimmune interactions via active suppression of self-reactive T cells.[123] These cells evolve from bone marrow progenitor cells that acquire linage commitment and mature in the thymus. Tregs develop from moderately self-reactive T cells that have escaped negative selection in a process that requires TCR triggering, CD28 costimulation, and stimulation by IL-2.[124–127] After selection in the thymus, these cells move to the periphery where they constitute a small percentage of the total peripheral $CD4^+$ T cell population.[128] However, the majority of Treg cells present in the periphery are of thymic origin as peripheral Treg cell generation has specific prerequisites.[129]

Although TCR signaling is known to be an essential requirement for thymic Treg development, the signaling molecules that regulate Treg development are still a work in progress.[129, 130] Prior work has shown that CARMA1 is necessary for Treg development,[121, 122, 131, 132] as is PKCθ, Bcl10, and IKK.[122, 133–135] Interestingly, the defect in Treg development with PKCθ-deficiency is not as severe as the defect with CARMA1-deficiency. This may be due to a role for CARMA1 in IL-2 signaling via CD25, which is necessary for optimal Treg development.[132] Overall these data suggest that TCR induced NF-κB signaling via CARMA1 is necessary for thymic Treg development. Recent data suggests that CARMA1-mediated activation of NF-κB provides a survival signal that opposes the clonal deletion of self-reactive T cells allowing the development of organ-specific Tregs.[121, 136]

2. Peripheral Tregs—In contrast to thymic Tregs, peripheral Tregs develop from naïve T cells that acquire their suppressive activity as a consequence of activation in the setting of a unique set of stimulatory conditions.[137–139] Peripheral Tregs seem tailored to respond to foreign antigens and neoantigens (such as tumor antigens), but it is likely that they are also generated in response to self-antigens and synergize with thymic Tregs in the control of autoimmunity.[140] Studies suggest that the mechanisms for differentiation of peripheral Tregs differ from thymic Tregs.[141, 142] However, CARMA1 is also important for optimal peripheral Treg development,[122] although it is possible to induce peripheral Tregs in CARMA1-deficient T cells *in vitro* and *in vivo* with very strong TCR stimulation.[122, 143, 144] It has been suggested that the different requirements for CARMA1 for generation of thymic derived Tregs versus peripheral Tregs might be a consequence TCR signaling strength, but further supportive evidence remains to be generated.[129]

E. Differential role of CARMA1 in different T cell subtypes

Following activation, $CD4^+$ and $CD8^+$ T cells differentiate into effector cells. Depending on the conditions during activation the effector cells can have different properties as defined by their functions and cytokine production. $CD4^+$ T cells can develop into multiple different effector subtypes such as Th1, Th2, and Th17 cells. $CD8^+$ T cell subtypes are not as well defined but may also develop different functional properties based on the conditions during activation. The specific role of CARMA1 in effector differentiation has not been fully delineated, but it does appear that CARMA1 has a differential role in the development of various T cell subsets.

1. T helper subtypes—$CD4^+$ effector T cells are polarized into specific subsets which are defined by the cytokine profile secreted by the cells. The secreted cytokines then help mediate the immune response and help dictate the nature of the inflammatory response. The most common helper T cell subtypes are Th1, Th2, and Th17.

The factors that mediate the polarization of a T cell into a particular subtype are complex, but may be partly determined by the strength of the TCR signal, and thus could be influenced by CARMA1 activity.[89] There are few reports investigating the function of CARMA1 in $CD4^+$ T cell subtype commitment. Reports from our group and others have confirmed the relevance of CARMA1 in allergic airway inflammation, a process highly dependent on the generation of Th2 effector cells.[109, 145, 146] Furthermore, a mouse line with a hypoactive mutant form of CARMA1 develops spontaneous allergic disease, probably due to an increased propensity for T cells to form Th2 cells and decreased Treg development.[120] Most recently, it has been shown that CARMA1 directs polarization towards a Th2 phenotype via its regulation of JunB and GATA3 transcription factors.[73] A role of CARMA1 in Th1 mediated inflammatory processes has not been well established. Studies suggest that Th1 polarization is impaired in CARMA1-deficient $CD4^+$ T cells, but the defect may not be as profound as with Th2 cells.[106, 147] However, these studies have indicated that CARMA1 induced NF-κB activation is essential for the induction of Th17 differentiation. These authors indicate a relatively selective role for CARMA1 in Th17 differentiation, that is independent of CARMA1-mediated survival and proliferative responses.[106] Interestingly, in contrast to other studies, they suggest that reported defects in Th1 and Th2 differentiation in CARMA1-deficient T cells are largely due to a defect in cell cycle progression. Altogether, these observations confirm the requirement of CARMA1 for T cell differentiation, although the importance of CARMA1 may differ under different polarizing conditions.

2. NKT/CD8αα/NK Cells—As mentioned, CARMA1-deficient mice have impaired development of Tregs presumably due to effects on the strength of TCR signaling. NKT and CD8αα T cells are T cell subtypes that are also selected by the nature and strength of the TCR signals.[122, 148] However, NKT cell development is not dependent on CARMA1 expression and there are actually increased numbers of CD8αα T cells in the gut of CARMA1-deficient mice.[122] NK cells also develop in CARMA1-deficient animals (unpublished observations), however, CARMA1 seems to be necessary for NK cell-mediated effector functions through TAK1.[149]

IV. CARMA1 role in non-TCR Signaling Pathways

TCR signaling has thus far remained the most extensively investigated mechanism that leads to the formation of the CBM complex in T cells. Recently, it has been shown that co-stimulatory molecules such as OX40 can also induce assembly and recruitment of CBM complexes to lipid rafts in T cells in an antigen-independent manner.[11] TCR independent engagement of OX40 with OX40L from antigen presenting cells led to the assembly of a

complex of proteins consisting of CARMA1, Bcl10, MALT1, TRAF2, RIP and IKK.[11] Furthermore, formation of the OX40 signalosome that contained the CBM complex has been shown to be essential for the prolonged survival of antigen-experienced T cells in the absence of the antigen. It has been well known that OX40 signaling contributes to the formation of memory T cells.[150, 151] Therefore, the association of CBM signalosome to OX40 in the absence of antigen signals suggests that this could be an important mechanism that guides effector T cells into the memory T cell phase.

There are additional examples for the association of CBM complexes with T cell co-stimulatory molecules. For example, engagement of the T cell co-stimulatory molecule CD26 with its APC-expressed ligand caveolin-1 resulted in the association of CARMA1 to the cytoplasmic tail of CD26, and induced T-cell proliferation, IL-2 production, and NF-κB activation in a TCR-dependent manner.[152] Furthermore, ligation of CD26 by caveolin-1 recruits a complex consisting of CD26, CARMA1, Bcl10, and IKKβ to lipid rafts.[152] The serine/threonine kinase Akt has been shown to play a role in CD28 mediated NF-κB activation.[153–156] It appears that Akt binds to CARMA1 and cooperates with other upstream signaling elements to help induce NF-κB activity.[10, 46] CARMA1 has also been shown to be necessary for optimal IL-2 signaling via CD25.[132] Although the mechanisms for this interaction are not known, it appears that CARMA1-deficient $CD4^+$ T cells have defective JAK1 and STAT5 phosphorylation. Overall these data suggest that co-stimulatory pathways may help fine-tune TCR signaling responses by modulating CARMA1 providing further sensitivity and specificity in the pathway.

V. Role of CARMA1 in disease

Given the essential role of CARMA1 for lymphocyte survival, proliferation, and activation, it is not surprising that studies have found that changes in CARMA1 activity are associated with lymphoproliferative disorders. The most well-established association is with gain of function mutations and B cell lymphomas, most notably diffuse large B cell lymphoma.[14] Other studies have linked persistent CARMA1 activity to congenital B cell lymphocytosis and to T cell lymphomas/leukemias.[16, 157] Most of these mutations and variants are associated with enhanced CARMA1-mediated NF-κB signaling or CARMA1 overexpression.[158, 159] Analysis suggests that these mutations often disrupt inhibitory domains on CARMA1 that normally prevent binding to other proteins.[158] These data suggest that CARMA1 could be a potential therapeutic target in certain T and B cell lymphoma/leukemia and may have a role in other lymphoproliferative disorders.

CARMA1 has also been linked to other diseases. Recently a case report has demonstrated that CARMA1-deficiency in humans leads to profound immunodeficiency,[17] and a recent genome-wide association study has identified a susceptibility locus for atopic dermatitis within the CARMA1 gene.[18, 160] These data are especially intriguing since the “Unmodulated” mutant mouse strain that carries a CARMA1 single nucleotide variant that decreases TCR-induced NF-κB signaling spontaneously develops allergic dermatitis.[25, 120] Furthermore, Th2 polarization of T cells and the development allergic airway inflammation is critically dependent on CARMA1 expression in mouse models.[109, 145, 146] These data suggest that CARMA1 activity may be an important factor for allergy and could be targeted to modulate allergic sensitization. Another genome-wide association study has also linked allelic variation in CARMA1 to inflammatory bowel disease,[18] and given new data on the role of CARMA1 in Th1- and Th17-type inflammation,[106, 147, 161] there may also be a role for CARMA1 in other inflammatory diseases such as organ transplant rejection and autoimmunity.

VI. Conclusion

The primary function of scaffold proteins is to facilitate the formation of protein complexes that then work to amplify signaling pathways. The unique expression patterns and localization of these proteins also provide cell- and pathway-specific activity. We propose that in T cells CARMA1 has as its primary function the amplification of TCR signaling providing a mechanism to enhance signals from pMHC complexes and to fine-tune TCR-mediated signaling in response to an antigen. Prior research has demonstrated that CARMA1 plays an important role in T cell activation, survival, differentiation, effector/memory cell function, and in the pathogenesis of lymphoproliferative and inflammatory diseases. Thus, comprehensively defining its mechanisms of action and regulation could reveal novel therapeutic targets for T cell-mediated diseases.

Abbreviations

TCR	T cell receptor
pMHC	peptide-major histocompatibility complex
APC	antigen presenting cells
BCR	B cell receptor

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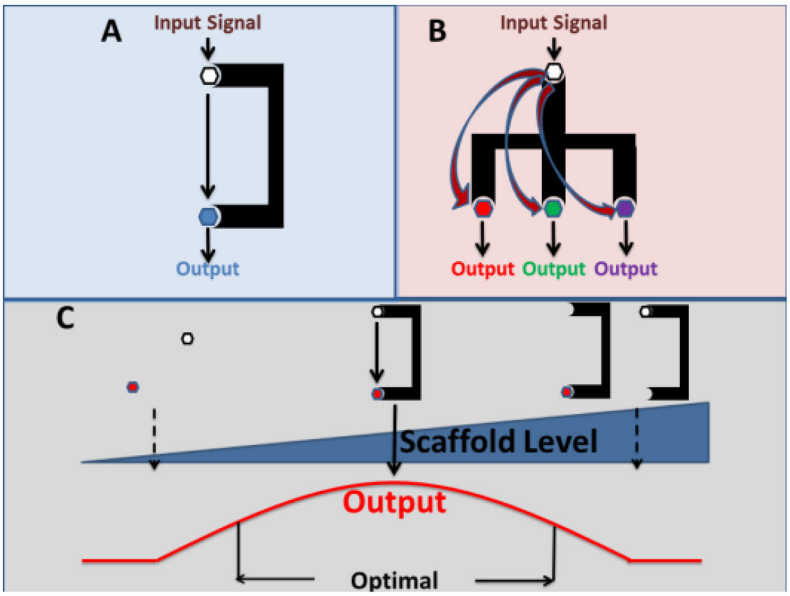

Figure 1.
Scaffold proteins can mediate pathway input to a single output (**A**) or multiple outputs (**B**). **C**) Biphasic relationship of scaffold protein levels to output. Reduced output at high levels of scaffold mediated by sequestration of proteins away from each other.

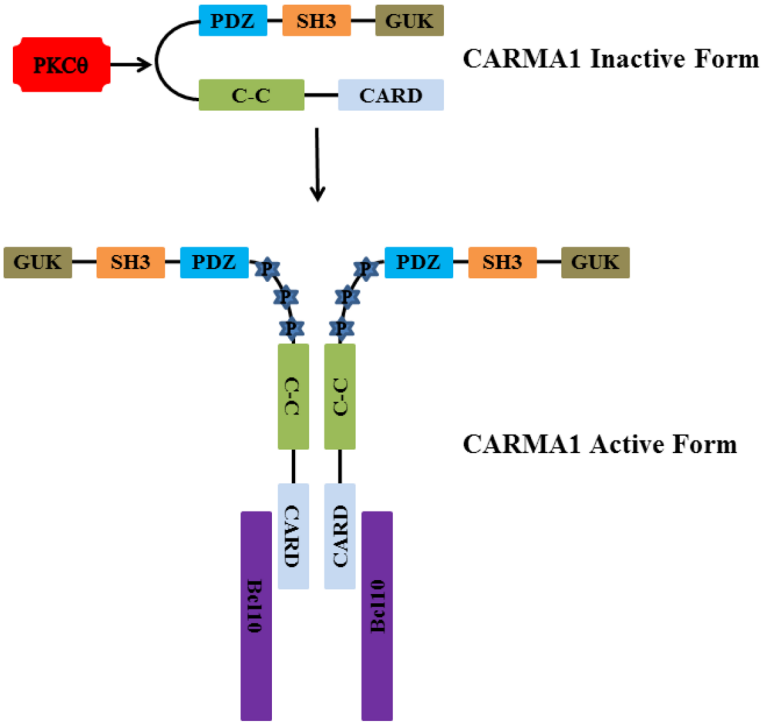

Figure 2.

Proposed model of TCR-mediated activation of CARMA1 via PKCθ in T cells. Phosphorylation (P) of the linker region between the C–C and PDZ domains results in opening up of the CARMA1 protein allowing Bcl10 to bind to the CARD domain. This then initiates further downstream signaling.

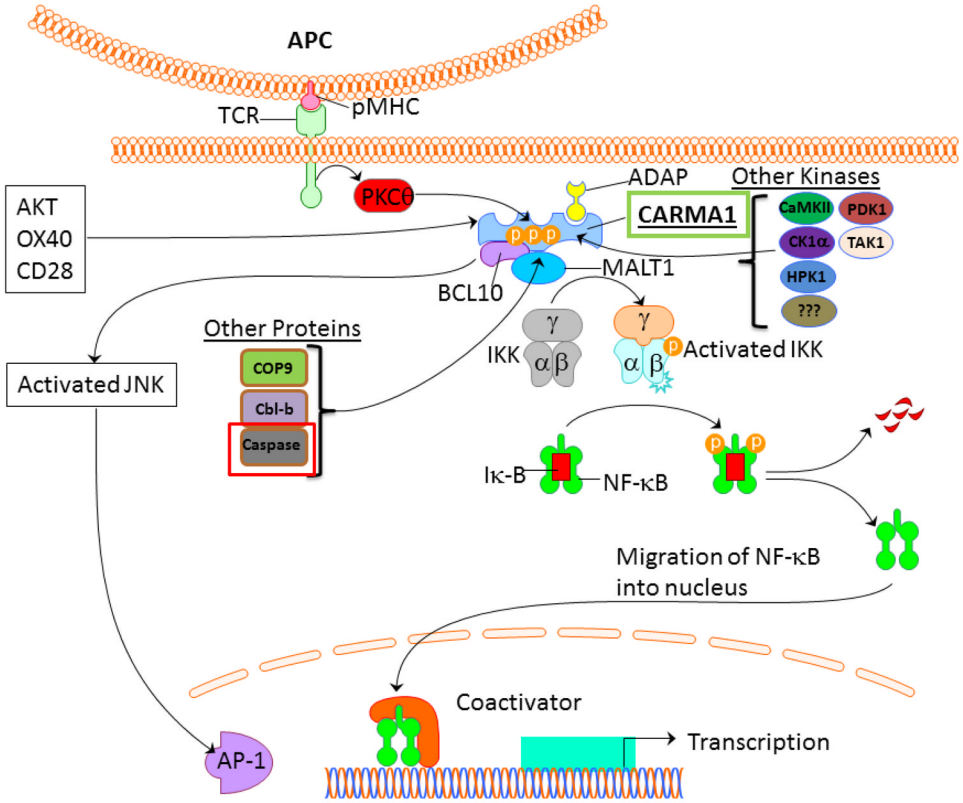

Figure 3.
Schematic of TCR signaling pathway leading to NF-κB and JNK activation.

Table 1

Proteins that Interact with the CARMA1 Signalsome

Kinaseses	**Ubiquitin Ligases**	**Other**
PKC θ/β	UBC13-UEV1A	Bcl10
IKK Complex	TRAF6	MALT1
PDK1	TRAF2	ADAP
CaMKII	cIAP2	Caspase 8
HPK1	NEDD4	Net1[162]
CK1α	ITCH	
Akt	CBL-b	
TAK1	COP9	
RIP2	STUB1	
MKK7 (JNK activation)	CYLD (de-ubiquitylating enzyme)	
Calcineurin (phosphatase) PP2A (phosphatase)	A20 (de-ubiquitylating enzyme)	

Table 2

CARMA1 Phosphorylation Sites

Human CARMA1	S109	T110	S551	S552	S555	S565	S608	S637	S645
Mouse CARMA1	S116	T117	S563	S564	S567	S577	S620	S649	S657
Kinase	CaMKII	PKCθ	HPK1	PKCθ	IKKβ	?	CK1α	PKCθ	PKCθ
Effect of Mutation									
NF-κB	↓	↓	↓	↓	↓	↓	↑	Normal	↓
JNK	?	↓	?	↓	↓	?	Normal	?	↓

Adapted from[14].