Phosphorylation and ubiquitination of the I κ B kinase complex by two distinct signaling pathways

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The I κ B kinase (IKK) complex serves as the master regulator for the activation of NF- κ B by various stimuli. It contains two catalytic subunits, IKK α and IKK β , and a regulatory subunit, IKK γ /NEMO. The activation of IKK complex is dependent on the phosphorylation of IKK α / β at its activation loop and the K63-linked ubiquitination of NEMO. However, the molecular mechanism by which these inducible modifications occur remains undefined. Here, we demonstrate that CARMA1, a key scaffold molecule, is essential to regulate NEMO ubiquitination upon T-cell receptor (TCR) stimulation. However, the phosphorylation of IKK α / β activation loop is independent of CARMA1 or NEMO ubiquitination. Further, we provide evidence that TAK1 is activated and recruited to the synapses in a CARMA1-independent manner and mediate IKK α / β phosphorylation. Thus, our study provides the biochemical and genetic evidence that phosphorylation of IKK α / β and ubiquitination of NEMO are regulated by two distinct pathways upon TCR stimulation.

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Introduction

NF- κ B is a family of transcription factors that play pivotal roles in immune, inflammatory, and antiapoptotic responses. NF- κ B is sequestered in the cytosol of unstimulated cells through the interactions with a class of inhibitor proteins,

called I κ Bs, which mask the nuclear localization signal of NF- κ B and prevent its nuclear translocation (Baldwin, 1996). Various stimuli induce the activation of the I κ B kinase (IKK) complex, which then phosphorylates I κ Bs. The phosphorylated I κ Bs are ubiquitinated and then degraded through the proteasome-mediated pathway (Karin and Ben-Neriah, 2000). The degradation of I κ Bs releases NF- κ B to translocate into nucleus and induces the expression of various genes.

The IKK complex is composed of at least three subunits: two catalytic subunits, IKK α and IKK β , and an essential regulatory subunit, IKK γ /NEMO (Karin and Ben-Neriah, 2000). Various stimuli such as viral and bacterial infections, mitogen phorbol esters (PMA), antigens, and proinflammatory cytokines like tumor necrosis factor- α (TNF α) and interleukin 1 (IL-1) activate NF- κ B by initiating distinct signaling pathways that eventually converge on IKK (Hayden and Ghosh, 2004). Activation of IKK is associated with phosphorylation of serines 177 and 181 in the activation loop of IKK β , whereas phosphorylation of equivalent sites (Serines 176 and 180) in IKK α was shown to be dispensable for NF- κ B activation (Karin and Ben-Neriah, 2000). Genetic studies show that NEMO is required for the activation of the NF- κ B by facilitating the formation of the high-molecular-weight IKK complex (Yamaoka *et al*, 1998). Additionally, recent studies suggest that Lys63 (K63)-linked ubiquitination of NEMO is essential for activating IKK (Tang *et al*, 2003; Zhou *et al*, 2004). However, how phosphorylation of IKK α / β and K63-linked ubiquitination of NEMO is interconnected and induce IKK activation remain poorly understood.

NF- κ B is one of the key transcriptional factors activated by T-cell receptor (TCR) engagement. TCR-induced NF- κ B is important for the proliferation, differentiation, and survival of T cells (Schulze-Luehrmann and Ghosh, 2006). TCR signaling is initiated by interaction of T cells with antigen-major histocompatibility complex (MHC) on the surface of antigen-presenting cells (APC). The contact region of T cell engaging APC is known as immunological synapse (IS) or supramolecular activation clusters (SMACs) (Bromley *et al*, 2001), which are characterized by the assembly of the TCR complex, signaling molecules, and lipid rafts in T cells (Xavier and Seed, 1999). SMAC is segregated into the lipid-rich central core, the central SMAC (c-SMAC), and the peripheral zone, the peripheral SMAC (p-SMAC) (Monks *et al*, 1998). TCR-induced recruitment of the IKK complex to the IS is essential for NF- κ B activation (Wang *et al*, 2004). A recent study suggests that the recruitment of IKK to the c-SMAC is dependent on CARMA1 (Hara *et al*, 2004).

CARMA1 is a scaffold molecule that is constitutively associated with the membrane and is recruited to the IS following TCR stimulation (Gaide *et al*, 2002; Wang *et al*, 2002). Various studies have shown that CARMA1 is essential for TCR-induced IKK activation and functions downstream of protein kinase C θ (PKC θ) (Gaide *et al*, 2002; Wang *et al*, 2002; Egawa *et al*, 2003; Newton and Dixit, 2003). Earlier genetic

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studies indicate that PKC θ is required for TCR-induced NF- κ B activation (Sun *et al*, 2000). Recent studies suggest that PKC θ phosphorylates CARMA1 (Matsumoto *et al*, 2005; Sommer *et al*, 2005), leading to the recruitment of BCL10 (B-cell lymphoma-10) and MALT1 (mucosa-associated lymphoid tissue protein-1, also known as paracaspase) to the IS (Gaide *et al*, 2002; Egawa *et al*, 2003; Che *et al*, 2004; Wang *et al*, 2004). Genetic studies demonstrate that BCL10 and MALT1 are required for antigen receptor-induced NF- κ B activation (Ruland *et al*, 2001; Ruefli-Brasse *et al*, 2003; Ruland *et al*, 2003). However, how BCL10 and MALT1 mediate antigen receptor-induced NF- κ B activation remains controversial. One study suggests that MALT1 functions as a ubiquitin E3 ligase to induce NEMO ubiquitination (Zhou *et al*, 2004), whereas another study proposes that BCL10-associated TRAF6 functions as the E3 ligase to ubiquitinate NEMO following TCR stimulation (Sun *et al*, 2004).

It has been suggested that TGF- β -activated kinase-1 (TAK1) serves as an upstream kinase that phosphorylates IKK α/β at its activation loop (Wang *et al*, 2001). Several recent studies demonstrate that TAK1 indeed plays an essential role in the activation of the IKK complex in signaling pathways induced by IL-1, TNF α , Toll-like receptor (TLR) family members, and receptor activator of NF- κ B (RANK) ligand (Sato *et al*, 2005; Shim *et al*, 2005). Several lines of evidence also suggest that TAK1 is essential for antigen receptor-induced IKK activation. Targeted disruption of TAK1 in chicken DT40 B-cells inhibited B-cell receptor (BCR)-induced IKK activation (Shinohara *et al*, 2005). The role of TAK1 in TCR signaling was first addressed by suppression of TAK1 expression using a small interference RNA (siRNA) approach that blocked TCR-induced IKK activation in Jurkat T cells (Sun *et al*, 2004). Owing to embryonic lethality of TAK-null mice, conditional deletion of TAK1 in B and T cells was carried out (Sato *et al*, 2005; Liu *et al*, 2006; Wan *et al*, 2006). These studies reported an essential role of TAK1 in maturation and activation of lymphocytes, but suggested that TAK1 may not be involved in NF- κ B activation in mature lymphocytes. The observed discrepancies of TAK1's role in antigen receptor-mediated NF- κ B activation in the conditional deletion of TAK1 in primary mouse T and B cells as opposed to targeted deletion of TAK1 in chicken B cells or siRNA-mediated knockdown of TAK1 in human Jurkat T cells can be explained by inefficient deletion of floxed TAK1 by Cre recombinase (Sato *et al*, 2005; Wan *et al*, 2006). The residual TAK1 in these cells could account for the observed NF- κ B activation (Chen *et al*, 2006; Liu *et al*, 2006). Thus, the role of TAK1 in BCR and TCR signaling pathways remains controversial.

It is clear that various stimuli induce the activation of IKK through the phosphorylation of IKK α/β and the K63-linked ubiquitination of NEMO. However, the specific requirements for the inducible phosphorylation and ubiquitination of IKK are poorly understood. In this study, we have investigated the mechanism of activation of the IKK complex in the context of TCR stimulation. We demonstrate that CARMA1 mediates the ubiquitination of NEMO in the TCR signaling pathway and this ubiquitination is dependent on the proper localization of CARMA1 in lipid rafts of the IS. In contrast, phosphorylation of IKK α/β is independent of CARMA1 and the K63-linked ubiquitination of NEMO. The phosphorylation of IKK α/β is likely to be mediated by TAK1 in a PKC-dependent manner. Together, our results provide the biochemical and genetic

evidence that the phosphorylation of IKK α/β and NEMO ubiquitination are mediated through two distinctly regulated signaling pathways.

Results

Phosphorylation of IKK α/β is independent of CARMA1 and BCL10

It has been shown that signal-dependent phosphorylation of IKK α/β at its activation loop is associated with the activation of the IKK complex. Previous studies have demonstrated that CARMA1 and BCL10 are essential for TCR-induced IKK activation (Ruland *et al*, 2001; Gaide *et al*, 2002; Wang *et al*, 2002; Egawa *et al*, 2003; Hara *et al*, 2003; Newton and Dixit, 2003). However, it remains to be determined how CARMA1 and BCL10 are involved in the regulation of IKK activation. To determine if CARMA1 is required for TCR-induced IKK phosphorylation, we analyzed TCR-induced phosphorylation of IKK α/β in wild-type (WT) or CARMA1-deficient Jurkat T (JPM50.6) cells. Surprisingly, the signal-induced phosphorylation of IKK α/β was comparable in Jurkat and JPM50.6 cells upon CD3-CD28 costimulation (Figure 1A), indicating that the inducible phosphorylation of IKK α/β is independent on CARMA1. The inducible phosphorylation of IKK α/β was further confirmed by immunoprecipitating IKK α/β from CD3-CD28- or PMA/ionomycin-stimulated Jurkat and JPM50.6 cells (Supplementary Figure 1). However, consistent with previous observations (Wang *et al*, 2002), the phosphorylation and degradation of I κ B α and phosphorylation of I κ B β were observed only in WT, but not in JPM50.6 cells following CD3-CD28 costimulation (Figure 1A and Supplementary Figure 2), indicating that the kinase activity of the IKK complex was defective in CARMA1-deficient cells following TCR stimulation. To further confirm this observation, we performed an *in vitro* kinase assay by immunoprecipitating the IKK complex from variously stimulated Jurkat and JPM50.6 cells. Consistent with the above results, the IKK immunoprecipitated from CD3-CD28 costimulated or PMA/ionomycin-treated JPM50.6 cells failed to phosphorylate GST-I κ B α , whereas the IKK complex immunoprecipitated from WT Jurkat T cells could effectively phosphorylate GST-I κ B α (Figure 1B). In contrast, TNF α stimulation effectively induced IKK activity in both cell lines (Figure 1B). Similarly, we observed that the autophosphorylation of IKK complex in response to CD3/28 or PMA/ionomycin stimulation is dependent on CARMA1 (Supplementary Figure 3). Thus, CARMA1 regulates the catalytic activity of IKK complex upon TCR stimulation.

JPM50.6 cells were generated by a somatic mutagenesis approach (Wang *et al*, 2002). To rule out the possibility that other mutation(s) in the cell line might contribute to the observed IKK defect, we used JPM50.6 cells reconstituted with an expression vector encoding WT CARMA1 (JPM50.6WT) to confirm our findings. Again, although the inducible phosphorylation of IKK α/β in JPM50.6 and JPM50.6WT was comparable, only the IKK complex in JPM50.6WT cells could phosphorylate I κ B α (Figure 1C). Together, these results indicate that CARMA1 is essential for activation of IKK upon CD3-CD28 or PMA/ionomycin stimulation, and the phosphorylation of IKK α/β alone is insufficient to activate IKK.

Figure 1 TCR-induced phosphorylation of IKK α/β is independent on CARMA1 or BCL10. (A) Jurkat or JPM50.6 cells (8×10^6 /sample) were stimulated with or without anti-CD3 and anti-CD28 (6 and 3 μ g/ml, respectively) for various time points. Cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting with indicated antibodies. (B) Jurkat or JPM50.6 cells (8×10^6 /sample) were stimulated with anti-CD3-CD28, PMA/ionomycin, or TNF α for various time points. The IKK complex was immunoprecipitated using anti-IKK α/β , and the immunoprecipitate was incubated with GST-I κ B α (1–62) in the kinase reaction buffer for 30 min at 30°C. The reaction mixtures were subjected to SDS-PAGE and analyzed by autoradiography. (C) JPM50.6 cells or JPM50.6 cells reconstituted with wt CARMA1 (JPM50.6WT) were stimulated as indicated. Cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting as described in (A). (D, E) Peripheral lymph nodes T cells from wt, CARMA1 $^{-/-}$, or BCL10 $^{-/-}$ mice were stimulated with PMA and ionomycin (D) or plate-bound anti-CD3-CD28 (E). Cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting using indicated antibodies.

To further confirm our findings, primary T cells from WT, CARMA1 $^{-/-}$, and BCL10 $^{-/-}$ mice were stimulated with PMA/ionomycin. We observed that the levels of IKK α/β phosphorylation in all of these cells were comparable. However, IKK-mediated I κ B α phosphorylation was observed only in WT T cells but not in T cells lacking CARMA1 or BCL10 (Figure 1D). Similarly, CD3-CD28 costimulation also effectively induced IKK α/β phosphorylation in WT, CARMA1 $^{-/-}$, and BCL10 $^{-/-}$ T cells (Figure 1E). To determine whether the observed phenomenon is T-cell-specific, we purified primary splenic B cells from WT, CARMA1 $^{-/-}$, and BCL10 $^{-/-}$ mice and stimulated these B cells with PMA/ionomycin or anti-IgM/CD40. Although IKK α/β was inducibly phosphorylated to a similar extent in these cells, the I κ B α phosphorylation in response to PMA stimulation was observed only in WT cells (Supplementary Figures 4 and 5). Together, these results demonstrate that although the signal-induced IKK α/β phosphorylation is independent of CARMA1 and BCL10 upon stimulation of antigen receptors, the phosphorylated IKK α/β in CARMA1- and BCL10-deficient cells is insufficient to phosphorylate I κ B α , indicating that CARMA1

and BCL10 regulate IKK through additional signaling events other than phosphorylation.

CARMA1 is essential for the ubiquitination of NEMO

Earlier studies suggest that the ubiquitination of NEMO is an essential step for IKK activation (Tang *et al*, 2003), and it has also been shown that overexpressed BCL10 induces NEMO ubiquitination via MALT1 (Zhou *et al*, 2004). As CARMA1 functions upstream of BCL10 and recruits BCL10 to the IS, we next examined whether the ubiquitination of NEMO is dependent on CARMA1 in the TCR signaling pathway. JPM50.6 and JPM50.6WT cells were stimulated with or without PMA/ionomycin. We found that NEMO was ubiquitinated following the stimulation in JPM50.6WT cells but not in JPM50.6 cells (Figure 2A). Consistent with the above observations (Figure 1), although phosphorylation and degradation of I κ B α were dependent on CARMA1 and correlated with the ubiquitination of NEMO, the inducible phosphorylation of IKK α/β was neither dependent on CARMA1 nor on the ubiquitination of NEMO (Figure 2B). In addition, no degradation of ubiquitinated NEMO was observed in these cells,

Figure 2 CARMA1 is required for the signal-induced ubiquitination of NEMO. (A, B) JPM50.6WT and JPM50.6 cells (2×10^7 /sample) were stimulated with or without PMA/ionomycin for various time points. The cell lysates were subjected to immunoprecipitation using anti-NEMO (A) or directly SDS-PAGE (B) and then analyzed by immunoblotting using indicated antibodies. (C) JPM50.6WT and JPM50.6 cells (3×10^7 /sample) were stimulated with or without PMA/ionomycin for various time points. NEMO was immunoprecipitated using anti-NEMO, and the resulting immunoprecipitates were subjected to SDS-PAGE and immunoblotting with indicated antibodies.

suggesting that NEMO undergoes a K63-linked ubiquitination. Although CD3-CD28 costimulation also induced NEMO ubiquitination, this inducible ubiquitination was much weaker than that induced by PMA/ionomycin (Supplementary Figure 6). Thus, we were unable to directly compare the ubiquitination induced by CD3-CD28 costimulation in WT Jurkat cells with JPM50.6 cells. Nevertheless, it is well documented that PMA/ionomycin stimulation bypasses the proximal signaling components and directly activates PKC. Therefore, our results suggest that CARMA1 is required for mediating NEMO ubiquitination in the TCR signaling pathway.

As BCL10 was shown to induce K63-linked ubiquitination of NEMO (Zhou *et al*, 2004), and NEMO directly interacts with CARMA family proteins (Stilo *et al*, 2004), we next examined if CARMA1 facilitates the interaction between NEMO and BCL10. Endogenous NEMO was immunoprecipitated from JPM50.6 and JPM50.6WT cells with or without PMA/ionomycin stimulation. The immunocomplexes were analyzed for BCL10 and CARMA1. We found that NEMO could associate with BCL10 and CARMA1 only in JPM50.6WT cells but not in JPM50.6 cells (Figure 2C). Thus, these results suggest that CARMA1 facilitates the interaction between NEMO and BCL10.

NEMO, but not its ubiquitination, is required for IKK α / β phosphorylation

Previous studies suggest that the C-terminal Zn-finger (ZF) domain of NEMO is required for its ubiquitination (Sun *et al*, 2004; Zhou *et al*, 2004), Lys399 in the ZF domain of NEMO was identified as the ubiquitination site (Zhou *et al*, 2004). Therefore, we next determined whether NEMO or its ubiquitination is required for IKK α / β phosphorylation. To address this question, we used a NEMO-deficient Jurkat T-cell line (Harhaj *et al*, 2000) and reconstituted these cells with full-length NEMO (NEMO-FL), NEMO deleted off the ZF domain

(NEMO- Δ C), or NEMO with Lys399 residue mutated to arginine (NEMO-K399R) (Figure 3A). We found that these NEMO proteins were effectively assembled into the IKK complex (Figure 3B). The reconstituted cells were then stimulated with or without PMA/ionomycin or TNF α . As expected, the reconstitution of NEMO-deficient cells with NEMO-FL restored the signal-induced IKK and NF- κ B activation, whereas NEMO- Δ C failed to do so (Figure 3C). These results confirm previous findings that the C-terminal ZF domain of NEMO plays an important role in IKK activation (Makris *et al*, 2002; Tang *et al*, 2003).

Significantly, we found that NEMO-K399R was only partially defective and could still significantly rescue PMA/ionomycin-induced NF- κ B activation in NEMO-deficient cells (Figure 3C), suggesting that ubiquitination of additional sites in NEMO may also contribute to IKK activation. However, NEMO- Δ C, as well as the NEMO-FL or NEMO-K399R, effectively restored the inducible IKK α / β phosphorylation in NEMO-deficient cells (Figure 3D). Together, these results indicate that NEMO but not its ubiquitination is essential for the inducible IKK α / β phosphorylation, whereas IKK activation correlates with NEMO ubiquitination.

CARMA1 induces K63-linked ubiquitination of NEMO mainly at Lys399

To determine whether CARMA1 can induce NEMO ubiquitination, we coexpressed CARMA1 and NEMO with plasmids encoding WT, K63-only, or K48-only variant of ubiquitin into HEK293 cells. We found that overexpressed CARMA1 induces K63-linked but not K48-linked ubiquitination of NEMO (Supplementary Figure 7). To determine whether Lys399 of NEMO is the ubiquitination site induced by CARMA1, we coexpressed CARMA1 with different mutants of NEMO in HEK293 cells (Figure 3E). We found that CARMA1 induced ubiquitination of NEMO-FL but not NEMO- Δ C. In contrast, CARMA1 induced much weaker ubiquitination of NEMO-

Figure 3 NEMO, but not its ubiquitination, is essential for IKKα/β phosphorylation. (A) Schematic representation of NEMO constructs. Domains: CC1, coiled coil 1; CC2, coiled coil 2; LZ, leucine zipper; ZF, zinc-finger. (B) NEMO-deficient Jurkat T cells (NEMO^{-/-}) were reconstituted by transducing with lentiviral vectors encoding NEMO-FL, NEMO-ΔC, or NEMO-K399R. Mock-infected NEMO^{-/-} cells were used as negative controls. NEMO was immunoprecipitated from the NEMO-reconstituted cells (mock, FL, ΔC, and K399R) (8 × 10⁶/sample) and the immunoprecipitates were probed for the constitutive association with IKKα/β. (C) The NEMO-reconstituted cells (mock, FL, ΔC, and K399R) (1 × 10⁷/sample) were stimulated with or without PMA/ionomycin or TNFα for 30 min. Nuclear extracts (10 μg) from these cells were incubated with ³²P-labeled NF-κB or OCT-1 probes, followed by electrophoresis and autoradiography (upper panels). IKKα/β were immunoprecipitated from the cell lysates and subjected to *in vitro* kinase assay as described in Figure 1B (lower panels). (D) The NEMO-reconstituted cells (8 × 10⁶/sample) were stimulated with PMA/ionomycin for various time points. Cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting using indicated antibodies. (E) NEMO constructs were coexpressed with or without CARMA1 in the presence of HA-K63-ubiquitin. NEMO constructs were immunoprecipitated and analyzed by immunoblotting.

K399R (Figure 3E). Together, these results suggest that CARMA1 induces K63-linked ubiquitination of NEMO, and Lys399 is the main ubiquitination site.

Subcellular localization of CARMA1 is critical for NEMO ubiquitination

The IS has recently been implicated as the area where the ubiquitination of signaling components in the TCR pathway may occur (Lee *et al*, 2003; Wiedemann *et al*, 2005). As CARMA1 is essential for the recruitment of NEMO to lipid rafts of IS (Che *et al*, 2004; Hara *et al*, 2004), and is required for the signal-induced ubiquitination of NEMO (Figure 2), we next determined whether CARMA1-mediated recruitment of NEMO to the lipid rafts is required for its ubiquitination. To address this question, we examined whether CARMA1(L808P), a CARMA1 mutant that is defective in IS localization (Supplementary Figure 8) (Wang *et al*, 2004), could mediate TCR-induced ubiquitination of NEMO. Although WT CARMA1 could mediate the signal-induced ubiquitination of NEMO, CARMA1(L808P) failed to do so

(Figure 4A). As CARMA1 facilitates the association of BCL10 with NEMO (Figure 2C), we next examined whether CARMA1(L808P) could facilitate the association of BCL10 with NEMO. We found that the signal-induced complex formation of BCL10 and NEMO was defective in JPM50.6L808P cells (Figure 4B). Together, our data indicate that CARMA1 localization in the lipid rafts is essential for facilitating the association of BCL10 with NEMO, and NEMO ubiquitination likely occurs in the lipid rafts. Moreover, consistent with the above observations, the inducible IKKα/β phosphorylation was observed in JPM50.6L808P cells to a similar extent as in JPM50.6WT cells (Figure 4C). To further confirm that the defect of CARMA1(L808P) results in a defect in TCR-induced NF-κB activation, we stimulated JPM50.6WT and JPM50.6L808P cells with PMA/ionomycin or TNFα and found that NF-κB activation induced by PMA/ionomycin but not TNFα was defective in JPM50.6L808P cells (Figure 4D). Together, these results indicate that the localization of CARMA1 in lipid raft is required for NF-κB activation and plays an important role in NEMO ubiquitination, whereas

Figure 4 Localization of CARMA1 in the lipid rafts is critical for NEMO ubiquitination. (A) JPM50.6 cells (2×10^7) reconstituted with CARMA1 (50.6WT) or CARMA1(L808P) (50.6L808P) were stimulated with or without PMA/ionomycin for various time points. The immunoprecipitates were subjected to SDS-PAGE and analyzed by immunoblotting. (B) For detecting CARMA1-mediated association of BCL10 and NEMO in 50.6WT and 50.6L808P cells, co-immunoprecipitation was performed as described in Figure 2C. (C) The above lysates were also directly subjected to SDS-PAGE and analyzed by immunoblotting using indicated antibodies. (D) 50.6WT and 50.6L808P cells (1×10^7 /sample) were stimulated with or without PMA/ionomycin or TNF α for 30 min. Nuclear extracts (10 μ g) from these cells were incubated with 32 P-labeled NF- κ B or OCT1 probe, followed by electrophoresis and autoradiography.

IKK α / β phosphorylation is independent of the subcellular localization of CARMA1 and NEMO.

TAK1 regulates IKK phosphorylation in a CARMA1-independent manner in the TCR pathway

Based on the above results, we hypothesize that there is a kinase phosphorylating IKK α / β in TCR signaling pathways in a CARMA1-independent manner. We speculate that TAK1 may be such a kinase, as it has been shown that TAK1 phosphorylates IKK (Wang *et al*, 2001) and participates in several innate immune signaling pathways to mediate IKK activation (Sato *et al*, 2005; Shim *et al*, 2005). To determine whether TAK1 regulates IKK, we immunoprecipitated TAK1 from Jurkat or JPM50.6 cells and found that TAK1 inducibly associated with IKK in a CARMA1-independent manner following CD3-CD28 costimulation (Figure 5A). To determine if CARMA1 is essential for TAK1 activation in the TCR pathway, we examined the kinase activity of TAK1 in Jurkat and JPM50.6 cells and found that TAK1 from both types of cells could effectively phosphorylate the recombinant MKK6 to a similar extent in response to CD3-CD28 costimulation (Figure 5B, top panel). To determine whether TAK1 could phosphorylate IKK β , the immunoprecipitated TAK1 was subjected to an *in vitro* kinase assay using a kinase-deficient IKK β , IKK β (K44A), as the substrate. We found that TAK1 from both Jurkat and JPM50.6 cells could inducibly phosphorylate IKK β at its activation loop to a similar extent (Figure 5C, top panel). Together, our results

demonstrate that TAK1 can be activated and inducibly associates IKK in a CARMA1-independent manner in the TCR signaling pathway.

To determine whether TAK1 is required for TCR-induced NF- κ B activation, we used a TAK1-specific inhibitor, 5Z-7-oxozeaenol (Ninomiya-Tsuji *et al*, 2003), to inhibit TCR-induced TAK1 activation. We found that this inhibitor blocked IKK α / β phosphorylation in both Jurkat and JPM50.6 cells, and I κ B α phosphorylation in Jurkat cells in response to TCR or PMA/ionomycin stimulation (Figure 6A, upper panels), indicating that TAK1-mediated IKK α / β phosphorylation is essential for TCR- and PMA/ionomycin-induced I κ B α phosphorylation and degradation. Consistent with the role of PKC in the TCR pathway, a PKC inhibitor, GF109203X, also blocked IKK α / β and I κ B α phosphorylation, suggesting that PKC isoforms might be involved in TAK1 activation (Figure 6A, upper panels). As the internal control, similar levels of ERK1/2 phosphorylation were detected with or without these inhibitors (Figure 6A, middle panels). To address whether PKC functions upstream of TAK1, we investigated if PKC could regulate TAK1 activation upon TCR or PMA/ionomycin stimulation. We found that TAK1 activation was significantly inhibited with the pretreatment of the PKC inhibitor in both Jurkat and JPM50.6 cells (Figure 6A, lower panels). Together, these results suggest that PKC functions upstream of TAK1 and TAK1 regulates IKK α / β phosphorylation in the TCR signaling pathway.

Figure 5 TAK1 is activated in a CARMA1-independent manner and associates with IKK upon TCR stimulation. Jurkat and JPM50.6 cells were stimulated with anti-CD3-CD28 for indicated time points. TAK1 was immunoprecipitated by using anti-TAK1 from these cells. The immunoprecipitates were subjected to immunoblotting using indicated antibodies (A) or to an *in vitro* kinase assay using either His-MKK6 (B) or IKKβ(K44A) (C) as substrates. In (C), lane 9 is a negative control using non-immune antibodies for stimulated Jurkat T cell lysates, whereas lane 10 is the untreated substrate incubated in kinase reaction buffer only. Kinase reaction mixtures were then subjected to SDS-PAGE and analyzed by autoradiography or immunoblotting using indicated antibodies.

To further test this model, we pretreated Jurkat cells with PKC or TAK1 inhibitor and then immunoprecipitated IKK and examined its kinase activity using GST-IκBα as the substrate. We found that TCR, PMA, or TNFα stimulation robustly activated IKK and induced IκBα degradation (Figure 6B), leading to NF-κB activation in DMSO-treated cells (Figure 6C). However, TAK1 inhibitor blocked the activity of IKK and IκBα degradation (Figure 6B), and the subsequent NF-κB activation (Figure 6C), in response to TCR, PMA, or TNFα stimulation. Consistent with the selective role of PKC in TCR, but not in TNFα, signaling pathway, the PKC inhibitor selectively blocked TCR- or PMA-induced, but not TNFα-induced, IKK activation and IκBα degradation (Figure 6B) and NF-κB activation (Figure 6C). Together, our results suggest that TAK1 functions downstream of PKC in a CARMA1-independent pathway and mediates TCR-induced NF-κB activation.

Finally, to provide the genetic evidence that TAK1 is required for IKKα/β phosphorylation, we analyzed chicken DT40 B cells deficient in either CARMA1 or TAK1 (Shinohara *et al*, 2005). We found that IKKα/β was phosphorylated in WT and CARMA1-deficient (CARMA1^{-/-}) B cells following

PMA/ionomycin stimulation, but this inducible phosphorylation was significantly reduced in TAK1-deficient (TAK1^{-/-}) B cells (Figure 6D). Consistent with the above results, phosphorylation of IκBα was significantly impaired in both CARMA1^{-/-} and TAK1^{-/-} DT40 B cells (Figure 6D). These results further support our findings that IKKα/β phosphorylation is mediated by TAK1 in a CARMA1-independent manner.

TAK1 is inducibly redistributed in T cells in a CARMA1-independent manner

CARMA1 is a scaffold protein that controls the recruitment of downstream signaling components to IS (Egawa *et al*, 2003; Che *et al*, 2004; Hara *et al*, 2004; Wang *et al*, 2004). To determine whether CARMA1 regulates the subcellular localization of TAK1 in a physiological condition, superantigen (*Staphylococcus enterotoxin E*, SEE)-primed Raji B cells were used as APCs to stimulate T cells. To visualize IS, Jurkat and JPM50.6 cells were labeled with FITC-cholera toxin B (FITC-CTxB) that binds to GM1 in lipid rafts. The formation of IS was defined by the aggregation of lipid rafts, whereas endogenous TAK1 was detected using anti-TAK1 antibodies. Without SEE stimulation, we did not observe the aggregation of lipid rafts in Jurkat and JPM50.6 cells in all Raji-Jurkat conjugates, and TAK1 was distributed throughout cells (Figure 7A and B, upper panels). However, lipid rafts were aggregated in the contact site of T cells with SEE-primed Raji cells (Figure 7A and B, lower panels), and majority of TAK1 was recruited to the contact site of Jurkat and JPM50.6 cells with Raji cells (Figure 7A and B, lower panels).

To rule out the possibility that the observed redistribution of TAK1 was due to the nonspecific staining of anti-TAK1 antibodies, we transfected the expression vector encoding a fusion protein of TAK1 and green fluorescence protein (GFP). Similarly, we found that TAK1-GFP was distributed throughout T cells that engaged with SEE-untreated Raji cells (Figure 7C and D, top panels). However, TAK1-GFP was recruited to the contact site of JPM50.6WT and JPM50.6 cells that engaged with SEE-primed Raji cells (Figure 7C and D, middle panels). Of note, although TAK1-GFP in T cells was effectively redistributed in a CARMA1-independent manner (Figure 7C and D, middle panels), the XZ confocal axis analysis showed that about 60% TAK1-GFP was recruited to the central region of lipid raft area (c-SMAC) in JPM50.6WT cells (Figure 7C, bottom panels, and Supplementary Figure 9), whereas TAK-GFP was localized in both peripheral region (p-SMAC) and central region (c-SMAC) of the synapse in JPM50.6 cells (Figure 7D, bottom panels, and Supplementary Figure 9).

To further determine whether TAK1 is redistributed to the lipid rafts in a CARMA1-dependent manner, we examined the TAK1 redistribution in primary T cells from WT or CARMA1^{-/-} mice. We found that TAK1 was also redistributed following TCR capping stimulation. However, similar to that in JPM50.6 cells, although TAK1 was recruited to the cytoplasm membrane following the stimulation, it was not concentrated into the center of lipid raft region (Supplementary Figure 10). Together, these results indicate that TAK1 receives a TCR signal and is recruited to the cytoplasm membrane in a CARMA1-independent manner. However, CARMA1 appears to further recruit TAK1 into the lipid raft.

As our results suggest that TAK1 functions upstream of IKK, we next determined whether IKK responds to TCR

Figure 6 PKC regulates TAK1, leading to IKK α/β phosphorylation and NF- κ B activation in antigen receptor signaling pathway. (A) Jurkat and JPM50.6 (8×10^6 /sample) were preincubated with DMSO, PKC inhibitor, GF109203X (100 nM for 15 min) (GF), or TAK1 inhibitor, 5Z-7-oxozeaenol (2 μ M for 30 min) (OXO), followed by stimulation with anti-CD3/CD28 antibodies or PMA/ionomycin. Cell lysates were prepared and subjected to SDS-PAGE followed by immunoblotting with indicated antibodies (upper panels). Part of these lysates was immunoprecipitated using anti-TAK1 and then subjected to *in vitro* kinase assay using MKK6 as substrates (lower panels). (B) The IKK complex was immunoprecipitated from Jurkat T cells following pretreatment with the above TAK1 or PKC inhibitors and stimulated with or without anti-CD3-CD28, PMA/ionomycin, or TNF α . The resulted immunoprecipitates were subjected to an *in vitro* kinase assay using GST-I κ B α as substrates and detected by autoradiography. The resulted immunoprecipitates and cell lysates were also analyzed by immunoblotting using indicated antibodies. (C) By inhibiting TAK1 and PKC using the same conditions as in (A), nuclear extracts were prepared from Jurkat cells stimulated with or without anti-CD3-CD28, PMA/ionomycin, or TNF α , and subjected to EMSA using 32 P-labeled NF- κ B or Oct-1 probes. (D) wt, CARMA1^{-/-}, and TAK1^{-/-} chicken DT40 B cells were stimulated with PMA plus ionomycin for various time points. Total cell lysates were subjected to SDS-PAGE, followed by immunoblotting with indicated antibodies.

stimulation as TAK1 does. As IKK α/β forms a tight complex with NEMO (Karin and Ben-Neriah, 2000) and this complex survives 2 M urea treatment (Rothwarf *et al*, 1998), we monitored the subcellular localization of the IKK complex by monitoring the localization of NEMO. Indeed, we found that NEMO was redistributed to the contact site of T cells with SEE-primed Raji cells (Figure 7E and F, middle panels). This redistribution of NEMO was independent of CARMA1 as it occurred in both JPM50.6 and JPM50.6 cells reconstituted with CARMA1 (JPM50.6WT) (Figure 5E and F). Interestingly, the XZ confocal axis analysis also showed that NEMO colocalized with lipid rafts in the presence of CARMA1 (Figure 5E, bottom panels), whereas it was localized on the peripheral region of the synapse and excluded from the lipid rafts in JPM50.6 cells (Figure 5F, bottom panels). Together, these results suggest that, similar to TAK1, the IKK complex could also receive a TCR signal independent of CARMA1 and is recruited to the contact site of T cells with APCs. However,

CARMA1 is required for the recruitment of IKK and TAK1 into the lipid raft upon TCR stimulation.

To determine CARMA1-dependent signaling complexes, we immunoprecipitated PKC θ from Jurkat and JPM50.6 cells. Consistent with the above results, TAK1 and IKK complex formed a complex with PKC θ in a CARMA1-dependent manner following CD3-CD28 costimulation (Figure 8A). In contrast, BCL10 and MALT1 were only assembled into the PKC θ -containing complex in the presence of CARMA1 (Figure 8A). Thus, PKC θ , TAK1, and IKK assemble into a complex in a signal-dependent but CARMA1-independent manner, whereas BCL10 and MALT1 require CARMA1 to associate with the PKC θ -TAK1-IKK complex. Together, our results suggest a model in which TAK1 functions downstream of PKC and phosphorylates IKK α/β in a CARMA1-independent manner, and, together with CARMA1-dependent ubiquitination of NEMO, leads to activation of the IKK complex and NF- κ B in antigen receptor signaling pathways (Figure 8B).

Figure 7 TAK1 is recruited to the immunological synapse in a CARMA1-independent manner. (A) Jurkat T cells or (B) JPM50.6 cells were labeled with FITC-conjugated cholera toxin B (FITC-CtxB) to localize the lipid rafts (green). The cells were incubated with SEE-untreated (upper panels) or SEE-treated (lower panels) Raji cells. The cells were stained with mouse anti-TAK1 and followed with ALEXA-conjugated goat anti-mouse antibodies for the localization of TAK1 (red). The formation of immunological synapse and the recruitment of TAK1 were visualized by confocal microscopy. (C) JPM50.6WT cells or (D) JPM50.6 cells were transfected with vectors encoding TAK1-GFP. Twenty-four hours after transfection, the transfected cells were first labeled with ALEXA-conjugated CTxB (red) and then incubated with SEE-untreated (top panels) or SEE-primed (middle and bottom panels) Raji cells. Images of Raji-T-cell conjugates and localization of the lipid rafts (red) are shown in DIC plus red and red-only channels. TAK1-GFP is shown in green. Yellow in the merge indicates the colocalization of TAK1-GFP with lipid rafts. The colocalization of TAK1-GFP and GM1-associated lipid rafts at the immunological synapse in SEE-primed cells is also shown in XZ focal axis images. (E) JPM50.6WT or (F) JPM50.6 cells were labeled with FITC-CtxB (green). The cells were incubated with SEE-untreated (upper panels) or SEE-primed (lower panels) Raji cells to allow the formation of Raji cell-T-cell conjugates. The cells were stained with Rabbit anti-NEMO antibodies and followed with ALEXA-conjugated goat anti-rabbit antibodies for the localization of NEMO (red). The formation of immunological synapse and the recruitment of NEMO were visualized using confocal microscopy. Images of Raji cell-T-cell conjugates and localization of the lipid rafts (green) are shown in DIC plus green, green-only, and red-only channels. Yellow in merge indicates the colocalization of NEMO with lipid rafts. The colocalization of NEMO and GM1-associated lipid rafts at the immunological synapse in SEE-treated cells is also shown in XZ focal axis images. All results are representative of three independent experiments. Asterisks indicate Raji cells.

Discussion

Various stimuli activate the IKK complex, leading to NF- κ B activation. It has been shown that IKK α/β is inducibly phosphorylated (Karin and Ben-Neriah, 2000), whereas NEMO in the IKK complex undergoes a K63-linked ubiquitination upon TCR stimulation (Tang *et al*, 2003; Zhou *et al*, 2004). However, the molecular mechanism leading to phosphorylation and ubiquitination of the IKK complex is not fully understood. Previous studies indicate that CARMA1 is required for the activation of IKK following TCR stimulation (Lin and Wang, 2004). In this study, we demonstrate that the defect of the IKK complex in CARMA1-deficient cells is due to lack of NEMO

ubiquitination following TCR stimulation. Significantly, we find that CARMA1 deficiency does not affect the inducible IKK α/β phosphorylation. This phosphorylation is mediated by TAK1 in a CARMA1-independent pathway (Figure 6). Therefore, these results demonstrate that two distinct pathways control the phosphorylation and ubiquitination of the IKK complex upon TCR stimulation, in which NEMO ubiquitination is dependent on CARMA1 and is likely to be mediated through the downstream BCL10-MALT1-TRAF6 complex, whereas PKC regulates TAK1, leading to IKK α/β phosphorylation in a CARMA1-independent pathway (Figure 8).

Our data show that NEMO inducibly associates with BCL10 in a CARMA1-dependent manner, suggesting that CARMA1

Figure 8 The role of CARMA1 in antigen receptor signaling pathway. (A) Jurkat or JPM50.6 cells (4×10^7 /sample) were stimulated with or without anti-CD3/CD28 antibodies for various time points. PKCθ was immunoprecipitated using anti-PKCθ. The immunoprecipitates were subjected to SDS-PAGE and analyzed by immunoblotting using indicated antibodies. Lane 'L' is the cell lysate from unstimulated Jurkat T cells and lane '7' is the immunoprecipitated sample of stimulated Jurkat T cells using non-immune antibodies. Lane 'M' is a molecular weight marker. (B) The working model of antigen receptor-induced signaling events. In the wt cells, stimulation of antigen receptors leads to activation and initiation of receptor proximal signaling events that lead to activation of PKC. The activated PKC further activates TAK1. TAK1 associates and phosphorylates IKKα/β in a CARMA1-independent manner, whereas PKC phosphorylates CARMA1 which leads to the recruitment of the BCL10-MALT1-TRAF6 complex. The complex induces the ubiquitination of NEMO. The combined IKKα/β phosphorylation and NEMO ubiquitination activate the IKK complex, leading to the phosphorylation and degradation of IκBα, and subsequent NF-κB activation. In CARMA1-deficient cells, the BCL10-MALT1-TRAF6 complex is not activated and thus fails to induce the ubiquitination of NEMO. Without NEMO ubiquitination, the IKKα/β phosphorylation alone is insufficient to activate the IKK complex.

facilitates the formation of a complex containing BCL10, MALT1, and IKK/NEMO, leading to NEMO ubiquitination. This result is consistent with the earlier proposed model that the activated BCL10 and MALT1 induce NEMO ubiquitination (Sun *et al*, 2004; Zhou *et al*, 2004). Of note, the signal-induced NEMO ubiquitination is difficult to be detected (Supplementary Figure 6). In WT Jurkat cells, we can only detect a very low level of NEMO ubiquitination even with PMA/ionomycin stimulation (data not shown). The most evident NEMO ubiquitination was observed in CARMA1-reconstituted JPM50.6 cells (Figure 2), in which CARMA1 level is relatively higher than that in Jurkat cells (data not shown), suggesting that CARMA1 is a limiting factor for regulating NEMO ubiquitination. Additionally, CD3-CD28 costimulation, in comparison with PMA/ionomycin stimulation, only slightly induced NEMO ubiquitination (Supplementary Figure 6), suggesting that either NEMO ubiquitination is very transient or only a very small amount

of NEMO is ubiquitinated following CD3-CD28 costimulation. However, we demonstrate that the NEMO ubiquitination induced by PMA/ionomycin stimulation, which mimics TCR stimulation and potentially activates IKK, is dependent on CARMA1 (Figure 2), suggesting that CARMA1 is indeed required for TCR-induced NEMO ubiquitination. Therefore, our data indicate that activation of the IKK complex is not only dependent on IKKα/β phosphorylation, but also on CARMA1-mediated NEMO ubiquitination upon TCR stimulation, although our data cannot rule out a possibility that an unknown CARMA1-dependent signaling event in addition to NEMO ubiquitination may also regulate IKK activation.

Although the definitive role of TAK1 in antigen receptor-induced NF-κB activation remains controversial (Sato *et al*, 2005; Liu *et al*, 2006; Wan *et al*, 2006), our results suggest that TAK1 mediates IKKα/β phosphorylation through a CARMA1-independent signaling pathway upon TCR stimulation. We provide several lines of evidence to support the role

of TAK1 in the regulation of IKK α / β phosphorylation. First, we show that TAK1 is inducibly associated with IKK, and TCR-activated TAK1 can phosphorylate IKK β . Second, we find that a TAK1-specific inhibitor can effectively block TCR-induced IKK α / β phosphorylation and NF- κ B activation. Finally, we find that the signal-induced IKK α / β phosphorylation is significantly impaired in chicken TAK1-deficient DT40 B cells. Therefore, our results suggest that TAK1 is a kinase mediating CARMA1-independent IKK α / β phosphorylation in antigen receptor signaling pathways. However, it is possible that other kinases may also play a compensatory or complementary role to TAK1 in mediating IKK α / β phosphorylation.

Consistent with our working hypothesis (Figure 8B), TAK1 induces IKK phosphorylation through a CARMA1-independent pathway upon TCR activation, we find that TAK1 is redistributed to the cytoplasm membrane, and is recruited to the IS following TCR stimulation in WT and CARMA1-deficient T cells. However, we also find that TAK1 is predominantly localized to the central region of lipid rafts (c-SMAC) in the presence of CARMA1 upon TCR stimulation, whereas TAK1 spans the peripheral region (p-SMAC) and central region (c-SMAC) of lipid rafts in the absence of CARMA1 (Figure 7D and Supplementary Figure 10). These results suggest that although TAK1 can be activated and is recruited to the IS through a CARMA1-independent pathway upon TCR stimulation, CARMA1 further recruits TAK1 into c-SMAC.

Recently, Hara *et al* (2004) demonstrated that NEMO, along with IKK α / β , is recruited to the IS, but is excluded from the lipid raft-enriched c-SMAC region in CARMA1 null T cells. Consistent with the study, we observed that NEMO was redistributed and recruited to the IS in a CARMA1-independent manner, but was mainly localized in the peripheral region of the lipid raft (Figure 7F). On the other hand, NEMO is colocalized with the lipid rafts in the presence of CARMA1 (Figure 7E). Thus, CARMA1 is essential for NEMO recruitment to the lipid rafts. Although our results show that CARMA1-dependent recruitment of NEMO to the lipid raft is correlated with NEMO ubiquitination (Figure 4A), we cannot distinguish whether the inducible NEMO ubiquitination is only dependent on CARMA1-mediated signal or on the microenvironment of lipid rafts, or both of these events. To address this question, it is necessary to disrupt lipid rafts in cells. However, we have not been able to achieve this condition, as depleting lipid rafts, using chemicals such as M β CD, induces stress responses and activates NF- κ B and JNK (data not shown). Nevertheless, our results suggest that CARMA1-mediated NEMO recruitment to the lipid rafts plays an important role in NEMO ubiquitination and is required for NF- κ B activation, whereas IKK α / β phosphorylation is independent of CARMA1 or NEMO ubiquitination.

Consistent with the findings of Lee *et al* (2005), we observed an association of PKC θ with TAK1 and the IKK complex in a CARMA1-independent manner (Figure 8A),

indicating that TAK1 and IKK complex are recruited through an undefined, but PKC θ -dependent and CARMA1-independent, pathway to p-SMAC region following TCR stimulation. This undefined pathway is sufficient to activate TAK1 and subsequently induce phosphorylation of IKK α / β . The TCR-induced TAK1 activation is likely through a PKC-dependent pathway, leading to the phosphorylation of IKK α / β (Figure 6). Thus, PKC θ not only phosphorylates CARMA1, but also regulates TAK1 activation to induce IKK phosphorylation in the TCR signaling pathway. However, further studies are needed to investigate how PKC regulates TAK1 activation.

Materials and methods

Plasmids and antibodies

The plasmid encoding TAK1-GFP was constructed by inserting TAK1 cDNA into *NheI/EcoRI* sites of pEGFP-N2 vector (BD Bioscience, San Jose, CA). The NEMO constructs were obtained by PCR amplification and subsequently cloning the PCR fragment into a lentivirus vector. Antibodies specific for Myc(A14), TAK1(C-9 and M-579), IKK α / β (H-744), ubiquitin(P4D1), NEMO(FL-419 and B-3), Bcl10(H-197), ERK2(C-14), phospho-IKK α / β (Ser-176), phospho-IKK α / β (S-181), and β -actin (C-2) were obtained from Santa Cruz Biotechnology. Antibodies for phospho-IKK α / β (Ser180/181) and phospho-ERK1/2 were purchased from Cell Signaling Technologies. Anti-IKK β antibodies were from BD Bioscience and anti-FLAG antibodies were from Sigma. Recombinant human TNF α was obtained from Endogen Inc. Anti-human CD3, anti-human CD28, and goat anti-rabbit IgG were obtained from BD Bioscience (San Diego, CA). Anti-mouse CD3 and anti-mouse CD28 were from BD Pharmingen. Anti-CARMA1 N-terminal antibodies were provided by Dr Margot Thome.

Cell lines and mice

The generation and maintenance of JPM50.6, JPM50.6WT, and JPM50.6L808P were as described previously (Wang *et al*, 2002, 2004). CARMA1 and Bcl10-null mice were described previously (Hara *et al*, 2003; Xue *et al*, 2003) and provided by Drs Josef Penninger and Demin Wang, respectively. All of the animal experiments were performed in compliance with the institutional guidelines and according to the protocol approved by Institutional Animal Use and Care Committee of the University of Texas, MD Anderson Cancer Center.

Please see additional methods in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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