The CARMA1-Bcl10 Signaling Complex Selectively Regulates JNK2 Kinase in the T Cell Receptor-Signaling Pathway

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SUMMARY

Members of the c-Jun NH_2-terminal kinase (JNK) family play crucial roles in cell activation, differentiation, and apoptosis. Although many studies have indicated that JNK1 and JNK2 have functional differences and redundancy, the upstream signaling pathway that selectively activates JNK1 or JNK2 remains unknown. In this study, we have revealed a selective mechanism of JNK activation, in which JNK2, but not JNK1, was regulated by CARMA1, a scaffold molecule, after stimulation of the T cell receptor (TCR). This CARMA1-dependent regulation of JNK2 worked through the scaffold molecule Bcl10, which was inducibly associated with JNK2 and served as a JNK-interacting protein (JIP)-like scaffold to assemble the kinases JNK2, MKK7, and TAK1. Finally, we showed that CARMA1- and Bcl10-mediated JNK2 activation had a critical role in regulating the amount of c-Jun protein. Together, our studies provide genetic evidence that JNK1 and JNK2 are differentially regulated in the TCR-signaling pathway and play different functions.

INTRODUCTION

The c-Jun NH_2-terminal kinases (JNKs) are the classical stress-activated mitogen-activated protein kinases (MAPKs) (Davis, 2000). JNK family is encoded by three different genes: *Jnk1*, *Jnk2*, and *Jnk3*. JNK1 and JNK2 proteins are ubiquitously expressed, whereas the expression of JNK3 is restricted to the brain, heart, and testis (Gupta et al., 1996; Weiss et al., 2000; Yang et al., 1997). These isoforms differ in their interaction with transcription factors (Gupta et al., 1996; Kallunki et al., 1994). Moreover, the three different Jnk genes express two isoforms, 46 kDa (JNKp46) and 54 kDa (JNKp54), resulting from the alternative splicing on their last exons, which results

in an extra 43-residue tail at the C terminus of p54 isoforms (Gupta et al., 1996; Kallunki et al., 1994).

Because JNK1 and JNK2 share a high degree of the sequence homology, the functional redundancy of JNK1 and JNK2 has been reported. Studies with genetically inactivated JNKs have demonstrated that none of the three JNK isoforms is necessary for normal mouse development (Davis, 2000). However, a mouse with *Jnk1* and *Jnk2* double knockout dies at embryonic day (E) 10.5, suggesting that the biological functions of JNK1 and JNK2 isoforms are partially redundant during embryonic development (Kuan et al., 1999; Sabapathy et al., 1999). It has been shown that JNK2 preferentially binds to c-Jun in unstimulated cells, thereby contributing to c-Jun ubiquitination and degradation. After stimulation, JNK2 dissociates from c-Jun, and JNK1 phosphorylates c-Jun, resulting in its activation and stabilization (Sabapathy et al., 2004; Sabapathy and Wagner, 2004). Therefore, JNK2 deficiency results in elevated c-Jun phosphorylation and stability, whereas JNK1 deficiency reduces c-Jun phosphorylation and stability (Sabapathy et al., 2004).

Activation of JNK1 and JNK2 plays important roles in T cell activation and differentiation (Dong et al., 1998, 2000). Stimulation of T cell receptor (TCR) induces multiple signaling cascades and effectively activates both JNK1 and JNK2. However, the signaling pathways that lead to activation of JNK1 and JNK2 are not completely defined. In particular, it is not clear whether JNK1 and JNK2 are regulated through the same or different signaling pathways after TCR stimulation. Therefore, it is important to delineate the signaling pathway leading to the activation of different isoforms of JNKs.

CARMA1 (CARD domain and MAGUK domain-containing protein-1) is a scaffold molecule primarily expressed in the cells of lymphoid system (Bertin et al., 2001; Gaide et al., 2001), which plays an essential role in antigen receptor-induced NF-κB activation in T and B cells (Egawa et al., 2003; Hara et al., 2003; Jun et al., 2003; Newton and Dixit, 2003; Wang et al., 2002). After the stimulation of T cell receptor and CD28 coreceptor (CD3-CD28 costimulation), protein kinase C-theta (PKCθ) phosphorylates CARMA1, which induces CARMA1 to associate with the downstream scaffold/adaptor molecules, Bcl10 and

MALT1 (Matsumoto et al., 2005; Sommer et al., 2005), and recruits these molecules into the lipid rafts of the immunological synapse (Che et al., 2004; Gaide et al., 2002; Hara et al., 2004), leading to activation of NF-κB (Ruefli-Brasse et al., 2003; Ruland et al., 2001, 2003; Xue et al., 2003). Moreover, some studies suggest that CARMA1 deficiency affects JNK activation after TCR stimulation (Gaide et al., 2002; Hara et al., 2003). Although the mechanism by which CARMA1 activates the NF-κB pathway is relatively well studied, how this scaffold molecule activates the JNK pathway remains to be determined.

In this study, we found that CARMA1-deficient Jurkat T cells, as well as primary lymphocytes from CARMA1-deficient mice, were defective in JNK2 activation upon stimulation of TCR or mitogen treatment. This defect is likely due to the defect of CARMA1-dependent complex formation of Bcl10 with JNK2, MKK7, and TAK1. This complex formation is required for the activation of JNK2. In addition, the CARMA1- and Bcl10-dependent activation of JNK2 is required for the accumulation of c-Jun after the stimulation of TCR. Thus, our results demonstrate that the CARMA1-Bcl10 complex selectively regulates the activation of JNK2 and modulates the amount of c-Jun protein.

RESULTS

CARMA1-Deficient Cells Are Defective in TCR-Induced JNK2 Phosphorylation

Studies on CARMA1-deficient mice suggests that CARMA1 deficiency markedly decreases JNK activation (Hara et al., 2003). However, previous studies from our laboratory showed that JNK immunoprecipitated from activated CARMA1-deficient Jurkat T cells could still phosphorylate c-Jun substrate in an in vitro kinase assay (Wang et al., 2002). To further address the role of CARMA1 in JNK activation, we analyzed JNK phosphorylation status in CARMA1-deficient Jurkat T (JPM50.6) cells upon TCR stimulation. Because JNK1 is predominantly a 46 kDa isoform protein (54 kDa isoform is also expressed) and JNK2 is predominantly a 54 kDa isoform, we used antibodies specific for phospho-JNK, which recognizes both p46 and p54 kDa isoforms. We found that activation of JNK1, as well as ERK, was comparable in Jurkat and JPM50.6 cells after CD3-CD28 costimulation, but the activation of JNK2 was completely abolished in JPM50.6 (Figures 1A and 1B), indicating that the activation of JNK2 is regulated through a CARMA1-dependent mechanism after TCR stimulation. Moreover, we showed that JNK1 was phosphorylated much faster than JNK2 in Jurkat T cells (Figure 1A), further suggesting that the activation of JNK1 and JNK2 might be regulated by distinct signaling components. Similarly, we found that the phosphorylation of JNK2 was selectively defected in JPM50.6 cells treated with PMA plus ionomycin, which mimics CD3-CD28 costimulation (Figure 1C, lanes 1–6). To exclude the possibility that JPM50.6 cells were generally defective in JNK2 phosphorylation, the cells were treated with sorbitol that induces the osmotic stress leading to

JNK activation. With this stimulation, the activation of both JNK1 and JNK2 was comparable in Jurkat and JPM50.6 cells (Figure 1C, lanes 7–12). Finally, to confirm that observed defect was not due to limitation of used phospho-JNK antibodies, we measured the amounts of phospho-JNK2 by ELISA. As expected, CD3-CD28 and PMA-ionomycin-induced JNK2 phosphorylation was almost completely abolished in JPM50.6 cells (Figure 1D). Together, our results demonstrate that CARMA1 deficiency specifically affects JNK2 activation in T cells.

Because JPM50.6 cells were generated by chemical mutagenesis and might contain some additional mutations that affect JNK activation, we reconstituted JPM50.6 cells with wild-type CARMA1 to confirm that CARMA1 is the essential component for JNK2 activation. In these reconstituted cells, CARMA1 fully rescued CD3-CD28- or PMA-ionomycin-induced JNK2 activation (Figure 1E). Next, we investigated whether truncated forms of CARMA1 were sufficient to restore JNK2 activation in JPM50.6 cells. Because CARMA1 contains an N-terminal caspase-recruitment domain (CD) followed by a coiled-coil domain (C-C), a PDZ domain, a SH3 domain, and a guanylate kinase (GUK)-like domain (Bertin et al., 2001; Gaide et al., 2001), we constructed two truncated forms of CARMA1: CD-CC and ΔCD-CC that contained PDZ, SH3, and GUK domains (see Figure S1A in the Supplemental Data available online). The expression plasmids encoding truncated forms of CARMA1 were stably transfected to JPM50.6 cells. In contrast to wild-type CARMA1, expression of CD-CC or ΔCD-CC failed to restore JNK2 activation (Figure S1B), indicating that CARMA1 integrity is important for regulation of JNK2 activation. Together, these results demonstrate that the defect of JNK2 activation is due to CARMA1 deficiency.

JNK2 Kinase Activity Is Defective in the Absence of CARMA1

To directly compare the kinase activity of JNK isoforms in Jurkat and JPM50.6 cells, we performed an in vitro kinase assay after CD3-CD28 costimulation and JNK precipitation. We used JNK2 antibodies, which recognize only p54 isoform of JNK2 (Figure S2), to precipitate JNK2p54 from Jurkat or CARMA1-deficient T cells. The immunoprecipitated kinases were used to phosphorylate the purified GST-c-Jun(1–79) protein in the presence of $[\gamma\text{-}^{32}P]ATP$. As predicted, the kinase activity of JNK2p54 was detectable in Jurkat cells (Figure 2A, lanes 5–8), but not in JPM50.6 cells (Figure 2A, lanes 13–16) after CD3-CD28 costimulation. Since commercial JNK1 antibody recognizes both p46 and p54 isoforms of JNK1 (Figure S2), we immunoprecipitated total JNK1 from Jurkat and CARMA1-deficient T cells. We found that JNK1 precipitated from Jurkat cells had relatively higher activity than JNK1 from JPM50.6 cells (Figure 2A, lanes 1–4 and 9–12), suggesting that both JNK1p46 and JNK1p54 may be activated in Jurkat cells, but only JNK1p46 in JPM50.6 cells. Therefore, to avoid limitation of used JNK antibodies, we overexpressed Flag-tagged JNK1p46, HA-tagged JNK2p54, or Myc-tagged JNK1p54 in Jurkat and JPM50.6 cells and
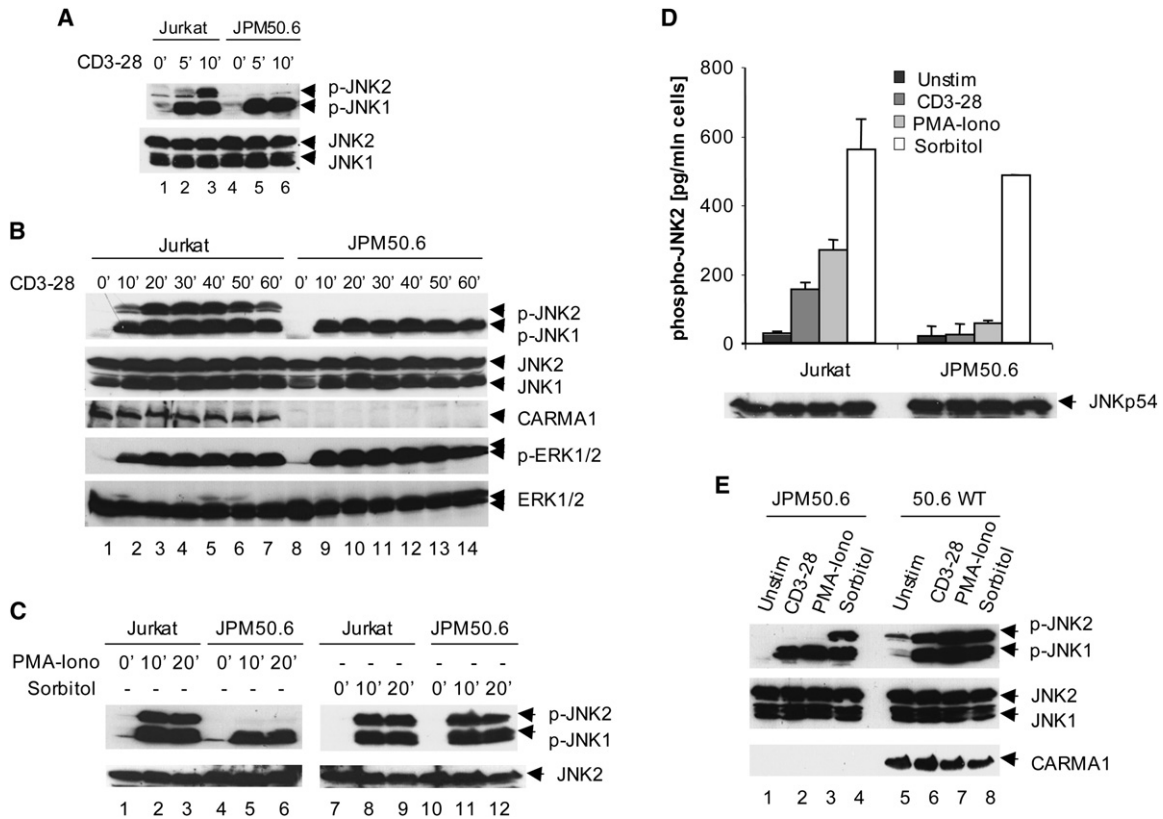
Figure 1. CARMA1-Deficient JPM50.6 Cells Are Defective in TCR-Induced JNK2 Phosphorylation

(A–C) Jurkat or JPM50.6 cells (6 × 10^6/sample) were stimulated with or without anti-CD3 plus anti-CD28 (6 and 3 μg/ml, respectively) for various time points (A, B) or with PMA (40 ng/ml) plus ionomycin (100 ng/ml) or with sorbitol (0.2 mM) (C). Whole-cell lysates were subjected to SDS-PAGE and analyzed by immunoblot with antibodies against phospho-JNK, JNK, CARMA1, phospho-ERK, or ERK.

(D) The cells (1 × 10^7/sample) were treated with different stimulators for 20 min and cell lysates were analyzed with phospho-JNK2 ELISA kit. Error bars indicate ± standard deviation in triplicate experiments.

(E) CARMA1-deficient cells (JPM50.6) or JPM50.6 cells reconstituted with CARMA1 (50.6 WT) were stimulated with or without anti-CD3 (6 μg/ml) and anti-CD28 (3 μg/ml) or PMA (40 ng) plus ionomycin (100 ng) or sorbitol (0.2 mM) for 20 min. The cell lysates were subjected to SDS-PAGE and examined by immunoblots with antibodies against phospho-JNK, JNK, or CARMA1. Results are representative of 2–3 independent experiments.

immunoprecipitated these kinases with anti-Flag, anti-HA, or anti-Myc. In this experiment, JNK1p46 was effectively activated in both Jurkat and JPM50.6 cells (Figure 2B) after CD3-CD28 costimulation. In contrast, JNK2 and long form of JNK1 (JNK1p54) were activated only in Jurkat cells (Figures 2C and 2D). These results demonstrate that JNK2 is completely defective whereas JNK1 could still be activated in the absence of CARMA1. Moreover, we found that both JNK1p46 and JNK1p54 could be activated in Jurkat cells, but only JNK1p46 in JPM50.6 cells, which is consistent with data obtained in Figure 1 with phospho-specific antibodies. It further suggests that CARMA1 deficiency specifically affects JNKp54 activation in T cells, indicating importance of the C-terminal 43-residue tail of JNK2 and JNK1. However, as mentioned previously, JNK1 is expressed mainly as the 46 kDa isoform in Jurkat T cells (Figure S2), which explains the potent activation of JNK1 in CARMA1-deficient cells.

To further confirm the requirement of CARMA1 for JNK2 activation, we decided to use T cells from CARMA1-deficient mice. Primary T cell were isolated from lymph nodes and stimulated with PMA-ionomycin. Although this stimulation effectively activated JNK1, as well as ERK, JNK2 activation was completely defective in CARMA1-deficient (Card11^−/−) cells (Figure 3A). Thus, this result further supports the conclusion that CARMA1 is required for JNK2 activation.

Bcl10 Is Required for JNK2 Activation

Previous studies indicate that Bcl10 is a signaling component downstream of CARMA1. After CD3-CD28 costimulation, CARMA1 associates with Bcl10 and recruits this adaptor protein into the immunological synapse, leading to activation of downstream signaling pathways (Gaide et al., 2002; Hara et al., 2004; Wang et al., 2004). To examine whether Bcl10 deficiency results in defective JNK2 activation, primary T cells from WT or Bcl10^−/− mice were stimulated with PMA-ionomycin. Similarly to CARMA1-deficient cells, PMA-ionomycin-induced JNK2, but not JNK1, phosphorylation was completely abolished
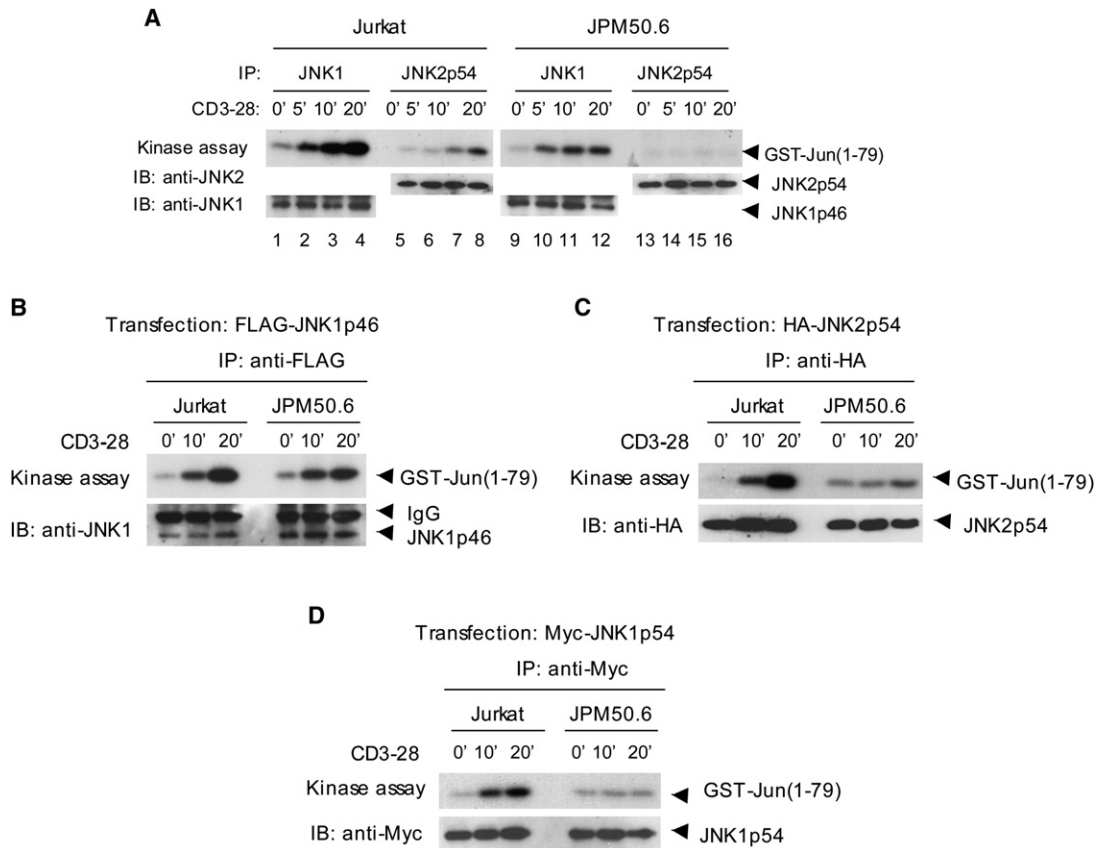
Figure 2. JNK2 Kinase Activity Is Defective in the Absence of CARMA1

(A) In vitro kinase assay. The cells (1 × 10⁷/sample) were stimulated with or without anti-CD3 plus anti-CD28, and cell lysates were precipitated with anti-JNK1 or anti-JNK2 for 4 hr. The immunocomplexes were incubated with GST-c-Jun(1-79) substrate (1 μg/sample) and [γ-³²P]ATP in kinase buffer at 30°C for 30 min. The phosphorylated proteins were resolved by SDS-PAGE, transferred to nylon membranes, and analyzed by autoradiography and immunoblotting.

(B–D) Jurkat or JPM50.6 cells were transfected with FLAG-JNK1p46, HA-JNK2p54, or Myc-JNK1p54 by electroporation. 24 hr later, cells were stimulated and overexpressed proteins were immunoprecipitated with anti-FLAG, anti-HA, or anti-Myc. Kinase assay was performed as described in (A). Results are representative of 2–3 independent experiments.

in *Bcl10*⁻/⁻ cells (Figure 3B). Together, these results suggest that CARMA1-induced JNK2 activation is mediated through Bcl10.

To determine whether CARMA1- and Bcl10-dependent JNK2 activation is a T cell-specific phenomenon, primary splenic B cells or bone marrow-derived mast cells from CARMA1- and Bcl10-deficient mice were stimulated with PMA-ionomycin. We found that JNK2 phosphorylation was also defective in both B cells and mast cells from CARMA1- and Bcl10-deficient mice (Figure 3C). Together, these results indicate that CARMA1- and Bcl10-dependent JNK2 activation is a general mechanism in the hematopoietic system.

Based on the above data, we hypothesized that Bcl10 might function as an adaptor protein for JNK2 in antigen receptor-signaling pathways. If it is true, Bcl10 might form a complex with JNK2 in activated cells. Indeed, we successfully coprecipitated JNK2 with Bcl10 from activated Jurkat cells (Figure 4A, lanes 1–3, and Figure 4B,

lanes 3–4), but this interaction was not observed in stimulated JPM50.6 cells (Figure 4A, lanes 4–6). Moreover, we were unable to detect the interaction between JNK1 and Bcl10 in the same conditions (Figure 4B, lanes 1–2). These results suggest that Bcl10 is selectively required for JNK2 activation. To test this possibility, we performed an immunoprecipitation experiment with two expression plasmids encoding JNK2p54 and JNK2p46. HEK293 cells were transfected with Myc-tagged Bcl10 expression plasmid and cotransfected with HA-JNK2p54 or HA-JNK2p46 (Figure 4C). After 24 hr, cells were harvested and the lysates were immunoprecipitated with HA antibody. We found that Bcl10 was specifically associated with JNK2p54 (Figure 4D, lane 4), but not with JNK2p46 (Figure 4D, lane 6). These results indicate that the C-terminal 43-residue tail of JNK2 is required for the association with Bcl10. To further investigate the role of Bcl10 in JNK activation, we used a Bcl10 mutant, Bcl10(L47A) (Figure 4C) that cannot oligomerize (Yan et al., 1999) to
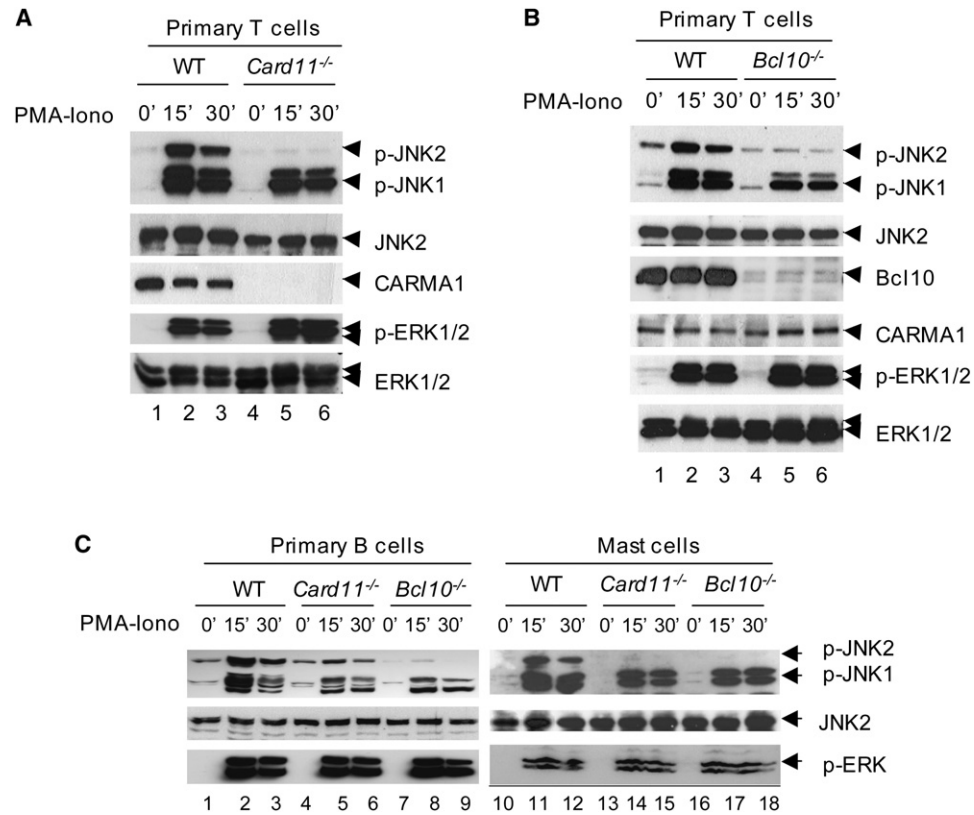
Figure 3. Primary Cells from CARMA1 or Bcl10 Null Mice Are Defective in JNK2 Phosphorylation

(A and B) Lymph nodes T cells from wild-type, CARMA1-deficient ($Card11^{-/-}$) (A), or $Bcl10^{-/-}$ (B) mice were stimulated with or without PMA (20 ng/ml) plus ionomycin (200 ng/ml).

(C) Splenic B cells and bone marrow-derived mast cells were isolated from wild-type, $Card11^{-/-}$, and $Bcl10^{-/-}$ mice and stimulated with or without PMA (20 ng/ml) plus ionomycin (200 ng/ml). Whole-cell lysates were subjected to SDS-PAGE and analyzed by immunoblot with indicated antibodies. Results are representative of 2–3 independent experiments.

perform the coimmunoprecipitation with JNK2p54. Interestingly, Bcl10(L47A) failed to associate with JNK2p54 (Figure 4E, lane 6), suggesting that JNK2 associates only with the activated and oligomerized Bcl10. Because Bcl10 constitutively associates with MALT1, we also examined whether JNK2 binds to MALT1 in activated Jurkat cells (Figure S3). Indeed, JNK2-MALT1 and JNK2-Bcl10 complexes were detectable at the same time. However, when we overexpressed JNK2 and MALT1 constructs, we were unable to detect the interaction between these proteins (data not shown), suggesting that JNK2 binds to MALT1 through Bcl10.

Bcl10 Functions as a Scaffold Molecule to Recruit TAK1 and MKK7

Previous studies indicate that JNKs are activated by sequential protein phosphorylation through a MAPK module, in which MAP kinase (MAPK) is activated by a MAP kinase kinase (MAP2K), and MAP2K is regulated by a MAP kinase kinase kinase (MAP3K) (Davis, 2000). In this cascade, JNK is connected to its MAP2K and MAP3K through the JNK-interacting protein, JIP (Morrison and Davis, 2003). Because JIP family members are not expressed in lym-

phocytes, it is likely that Bcl10 may function as the JIP-like scaffold molecule for JNK2 activation in lymphocytes. Therefore, we postulated that Bcl10 might also associate with MAP3K and MAP2K. Although several MAP3Ks has been reported to act as a MAP3K for JNK (Davis, 2000), recent studies suggest that transforming growth factor-β-activated kinase 1 (TAK1) is inducibly associated with Bcl10 in activated B cells (Sato et al., 2005) and required for JNK activation in innate and adaptive immune responses (Sato et al., 2005; Shim et al., 2005). Therefore, we examined whether Bcl10 recruits TAK1 in T cells. TAK1 was immunoprecipitated from Jurkat and JPM50.6 cells after the treatment of cells with PMA-ionomycin. We found that Bcl10 inducibly associated with TAK1, but not MEKK1, MEKK2, and MEKK3 (data not shown), in Jurkat cells but not in JPM50.6 cells (Figure 5A).

Although both MKK7 and MKK4 function as MAP2Ks for JNK (Davis, 2000), an earlier study suggests that MKK7 is likely a kinase directly phosphorylating JNK in activated T cells (Dong et al., 2000). Therefore, we also determined whether Bcl10 could associate with MKK7. We found Bcl10 inducibly associated with MKK7 in Jurkat cells, but not in JPM50.6 cells (Figure 5B). To examine
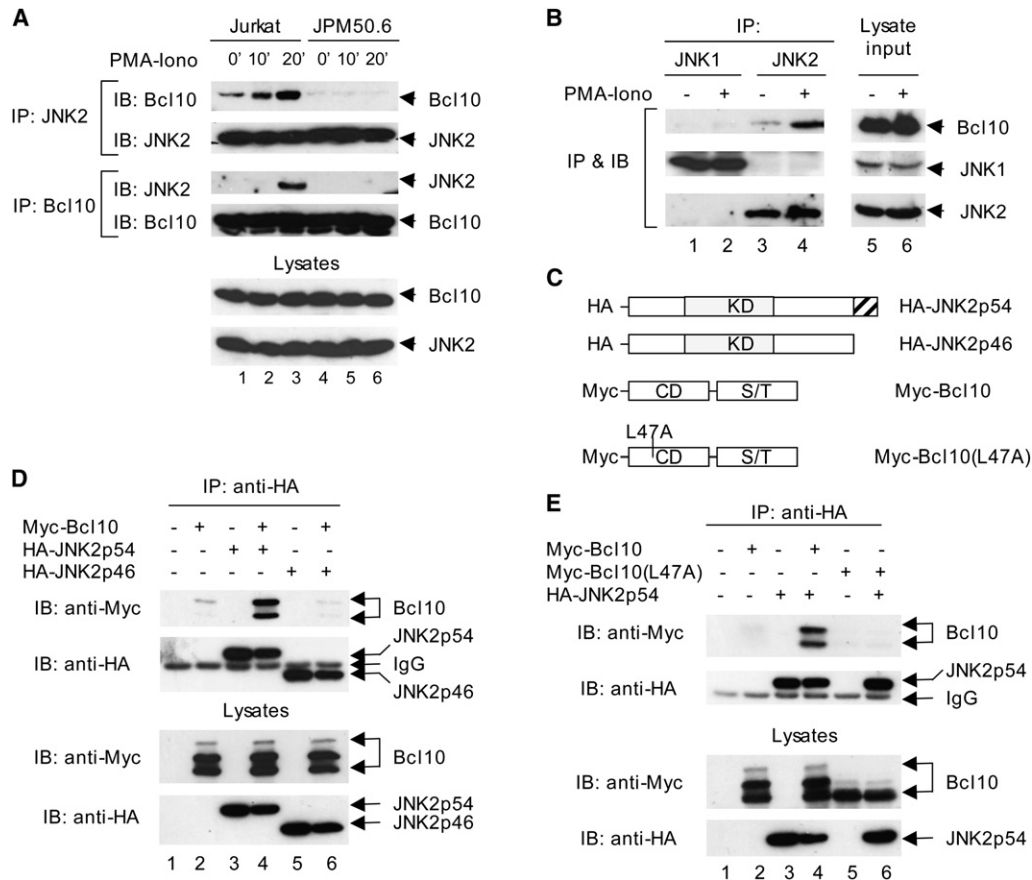
Figure 4. Bcl10 Inducibly Associates with JNK2 but Not with JNK1

(A and B) Jurkat or JPM50.6 cells (3×10^7/sample) were stimulated with or without PMA (40 ng/ml) plus ionomycin (100 ng/ml). The cells were lysed and precipitated with anti-JNK2, anti-Bcl10, or anti-JNK1 (A, B). Immunocomplexes (top and middle) or whole-cell lysates (bottom) were subjected to SDS-PAGE and analyzed by immunoblot with indicated antibodies.

(C) Plasmids used in overexpression experiments.

(D and E) HEK293 cells (7×10^5) were transiently transfected with 0.5 μg of expression plasmids encoding several constructs: HA-JNK2p54, HA-JNK2p46, Myc-Bcl10 (D) or Myc-Bcl10(L47A) (E). Cells were lysed and precipitated with anti-HA. The immunocomplexes were subjected to SDS-PAGE and analyzed by western blot with indicated antibodies. The amounts of expression of the transfected products were assessed with anti-Myc and anti-HA immunoblots of total cell lysates. Results are representative of 2–3 independent experiments.

whether TAK1, MKK7, and JNK2 associate with Bcl10 at the same time, we performed timed immunoprecipitations. We found all kinases in the complex with Bcl10 after 10 and 20 min of PMA-ionomycin treatment. However, TAK1-Bcl10 was detected mostly after 10 min, MKK7-Bcl10 and JNK2-Bcl10 after 20 min (Figure 5C). Together, these data suggest that the activated Bcl10 functions as a scaffold molecule to recruit TAK1 and MKK7 for JNK2 activation.

TCR-Induced c-Jun Accumulation Is Suppressed in CARMA1-Deficient Cells

The transcription factor c-Jun is a critical regulator of cell-cycle progression and a classical JNK target (Davis, 2000). Previous study suggests that JNK activation affects the c-Jun protein amount (Sabapathy et al., 2004). Therefore, we investigated whether the c-Jun level was altered in CARMA1-deficient cells. Indeed, we found that basal

amount of c-Jun was much higher in Jurkat than in JPM50.6 cells (Figure 6A, lanes 1 and 9). Moreover, c-Jun protein was rapidly accumulated in Jurkat T cells upon CD3-CD28 costimulation, but this accumulation was defective in JPM50.6 cells (Figure 6A). To exclude the possibility that this defect of c-Jun accumulation was due to the defective NF-κB activation in JPM50.6 cells, we used a Jurkat T cell line deficient in NEMO (also known as IKKγ), a subunit of the IKK complex, which is completely defective in NF-κB activation. Because NEMO-deficient Jurkat T (JM4.5.2) cells are also defective in the expression of CD3 complex, we used PMA-CD28, instead of CD3-CD28, to stimulate NEMO-deficient and CARMA1-deficient cells. We found that JNK2 activation was intact and c-Jun amount was rapidly increased in NEMO-deficient cells upon PMA-CD28 costimulation, but PMA-CD28 costimulation failed to activate JNK2 and to induce c-Jun accumulation in CARMA1-deficient
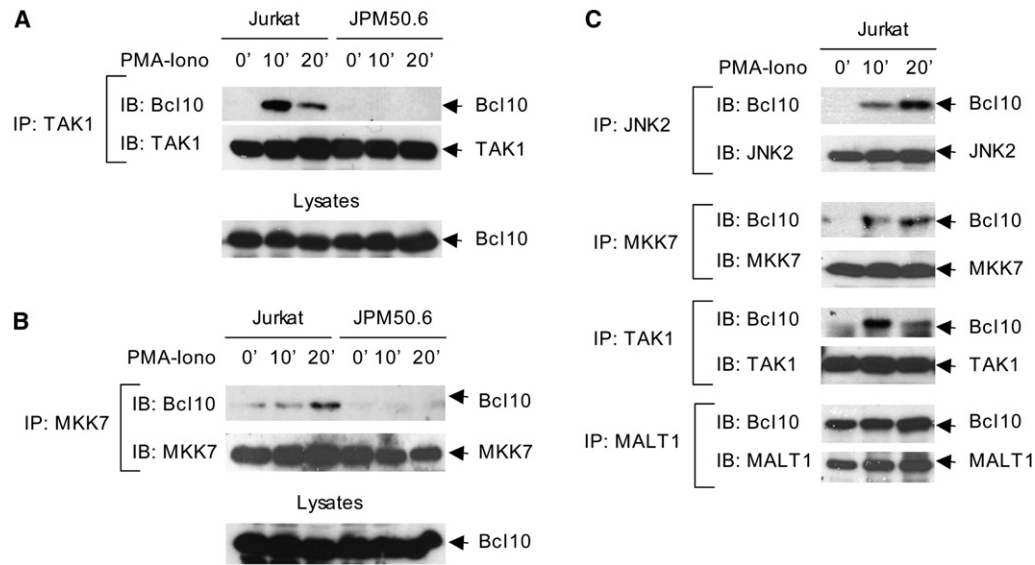
Figure 5. Bcl10 Inducibly Associates with TAK1 and MKK7 upon PMA-Ionomycin Stimulation

(A and B) Jurkat or JPM50.6 cells (3×10^7/sample) were stimulated with or without PMA (40 ng/ml) plus ionomycin (100 ng/ml). The cells were lysed and precipitated with anti-TAK1 (A) or anti-MKK7 (B). Immunocomplexes or whole-cell lysates were subjected to SDS-PAGE and analyzed by immunoblot with indicated antibodies.

(C) Timed immunoprecipitation. Jurkat cells (12×10^7/time point) were stimulated with or without PMA plus ionomycin for 10 or 20 min and lyzed in 1 ml of lysis buffer. Each lysate was divided into four portions and immunoprecipitated with anti-JNK2, anti-MKK7, anti-TAK1, and anti-MALT1. The immunocomplexes were subjected to SDS-PAGE and analyzed by western blot with indicated antibodies. Results are representative of 2–3 independent experiments.

cells (Figure 6B). Together, these results suggest that the signal-dependent c-Jun accumulation is not mainly controlled by NF-κB-dependent transcription, but is likely controlled by the activated JNK2.

To investigate the mechanism of the signal-dependent c-Jun accumulation, Jurkat T cells were treated with or without cycloheximide and MG132, which inhibits the protein synthesis and protein degradation, respectively. We found that the treatment with cycloheximide completely inhibited CD3-CD28 costimulation-induced c-Jun accumulation, whereas MG132 increased the basal and accumulated amount of c-Jun protein (Figure 6C), suggesting that CARMA1-dependent c-Jun accumulation is mainly through new protein synthesis. In contrast, proteasome-mediated degradation of c-Jun is mainly involved in maintaining the steady-state amount of c-Jun. In addition, although MG132 blocked CD3-CD28 costimulation-induced IκBα degradation (Figure 6C, lanes 9–12), which inhibited NF-κB activation, it did not inhibit CD3-CD28 costimulation-induced c-Jun accumulation, further supporting our conclusion that the c-Jun accumulation is not mainly controlled by NF-κB transcription factors (Figure 6B).

To distinguish whether newly synthesized c-Jun was controlled by the transcription or translation, Jurkat T cells were treated with or without actinomycin D, which inhibits the transcription, or actinomycin D plus MG132. We found that the treatment with actinomycin D alone (Figure 6D, lanes 5–8) or in combination with MG132 (Figure 6D, lanes

9–12) completely inhibited CD3-CD28 costimulation-induced c-Jun accumulation. These results indicate that c-Jun accumulation is mainly controlled by the transcription, but not translation or stabilizing protein. To further confirm this finding, we compared c-Jun mRNA expression in Jurkat and JPM50.6 cells activated with CD3-28 for 30 and 60 min (Figure 6E). We found that CD3-28 costimulation strongly increased c-Jun mRNA expression in Jurkat but barely in JPM50.6 cells. Interestingly, basic expression of c-Jun mRNA was also lower in JPM50.6 than in Jurkat cells. These results are consistent with published data (Jaeschke et al., 2006).

Additionally, we found that the amount of JunB was also upregulated after CD3-CD28 costimulation in Jurkat cells (Figure S4), and this accumulation of JunB was also defected in CARMA1-deficient T cells (data not shown). Although activation of JNK contributes to the activation of AP-1 transcription factors, CARMA1-dependent JNK2 activation did not affect AP-1 activation (Figures 7A and 7B; Figure S5; Hara et al., 2003; Wang et al., 2002). Together, our results indicate that CD3-CD28 costimulation-induced JNK2 activation is not involved in the regulation of AP-1 activation. Instead, CARMA1-dependent JNK2 activation is involved in the accumulation of Jun family of transcription factors through regulating the transcription of these genes in lymphocytes.

To summarize our data, we propose a model of antigen-induced activation of JNK (Figure 7C), in which PKC mediates activation of both JNK1 and JNK2. However,
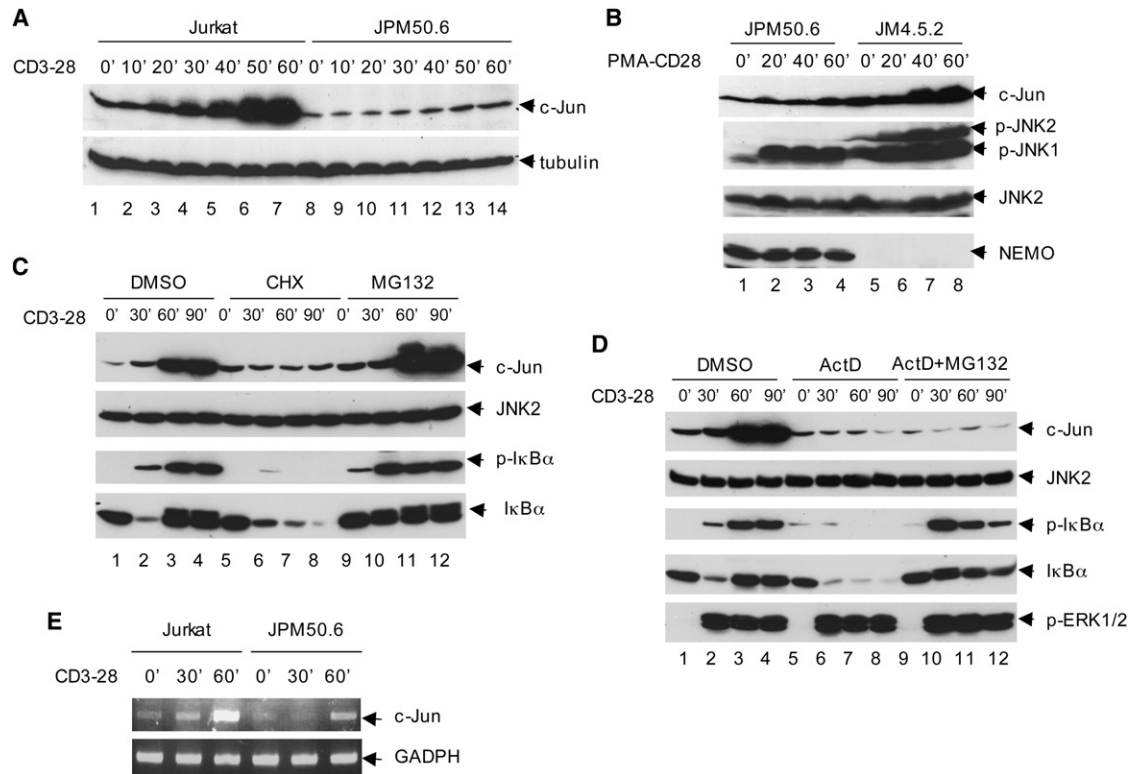
Figure 6. TCR-Induced c-Jun Accumulation Is Supressed in CARMA1-Deficient Cells
(A and B) Jurkat or JPM50.6 cells were stimulated with or without anti-CD3 and anti-CD28 antibodies (6 and 3 μg/ml, respectively) for various time points (A), or NEMO-deficient Jurkat (JM4.5.2) cells or JPM50.6 cells were stimulated with or without PMA (40 ng/ml) plus anti-CD28 (3 μg/ml) for various time points (B). Cell lysates were subjected to SDS-PAGE and immunoblot analysis with indicated antibodies.
(C and D) Jurkat T cells were preincubated with cycloheximide (CHX) (5 μg/ml), MG132 (5 μg/ml) (C), or actinomycin D (ActD) (5 μg/ml) (D) for 30 min, and then stimulated with or without anti-CD3 plus anti-CD28. Cell lysates were subjected to SDS-PAGE and immunoblot analysis with indicated antibodies.
(E) Cells were stimulated with or without anti-CD3 plus anti-CD28 (30 or 60 min), and total RNA was isolated with RNA isolation kit followed by reverse transcription polymerase chain reaction (RT-PCR). An aliquot (2 μl) of first-strand cDNA was used as a template in PCR amplifications of c-Jun and GAPDH transcripts. Amplification products were resolved on a 1% agarose gel. Results are representative of three independent experiments.

CARMA1 is required only for JNK2 activation. Activated CARMA1 induces the oligomerization of Bcl10, and the oligomerized Bcl10 functions as a scaffold molecule to recruit MKK7 and TAK1, leading to activation of JNK2. Therefore, some scaffold molecules, other than CARMA1 and Bcl10, are probably involved in assembling JNK1 with MAP2K and MAP3K.

DISCUSSION

JNK family of kinases plays pivotal roles in cell activation, differentiation, proliferation, and survival in response to environmental stresses and cytokines (Davis, 2000; Dong et al., 2002). JNK1 and JNK2 are the major forms of JNK expressed ubiquitously (Gupta et al., 1996). Although many lines of investigation have indicated that JNK1 and JNK2 have functional differences and redundancy, the upstream signaling pathway that selectively activates JNK1 or JNK2 remains unknown. In this study, we have revealed a regulatory mechanism of JNK activa-

tion, in which different isoforms of JNK are regulated by CARMA1-Bcl10-dependent and -independent mechanisms in T cells.

Our results show that CD3-CD28 costimulation-induced JNK2, but not JNK1, phosphorylation is selectively defective in CARMA1-deficient Jurkat T cells and primary T cells from CARMA1-deficient mice. In addition, we showed that the phosphorylation status of JNK was correlated with the activation of JNKs, in which the kinase activity of JNK2 was dependent on CARMA1. We have also performed the in vitro kinase assay to further confirm that JNK1 could still be activated in CARMA1-deficient cells. Therefore, our results strongly support the hypothesis that CARMA1 is mainly involved in JNK2, but not JNK1, activation. However, it is clear that JNK1 still receives signal from T cell receptor, which may involve signaling components other than CARMA1 and Bcl10.

It has been shown that each Jnk gene is also expressed as 46 kDa (JNKp46) and 54 kDa (JNKp54) isoforms because of the alternative splicing on their last exons,
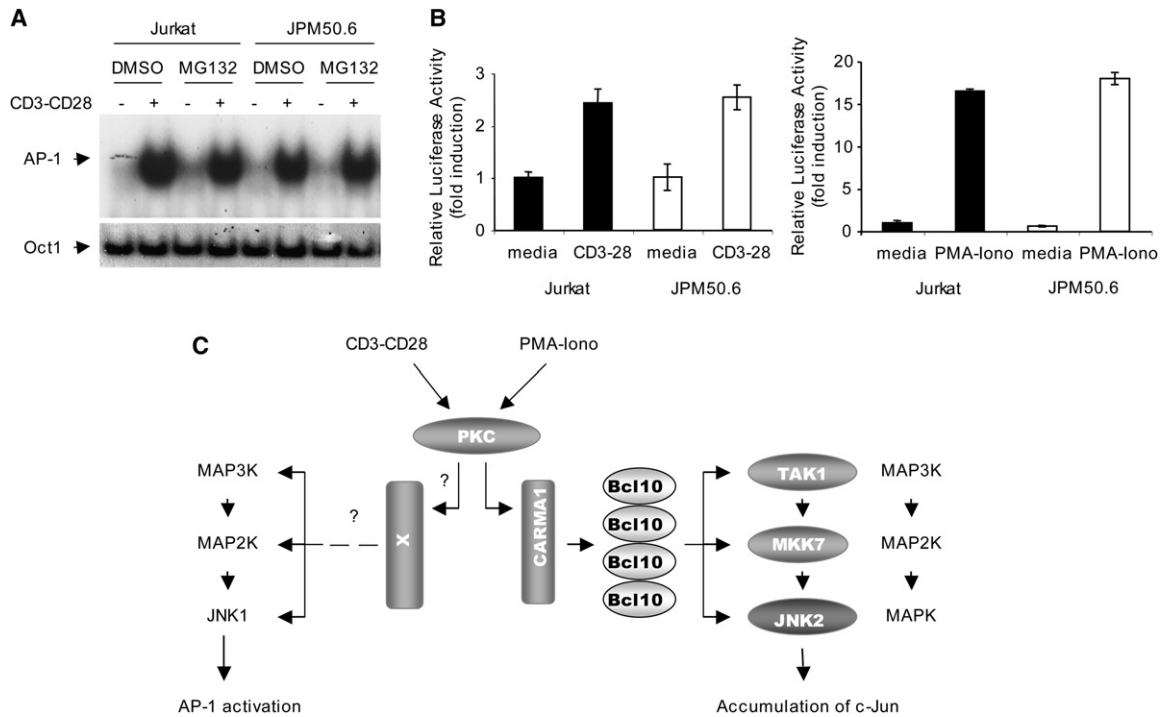
Figure 7. AP-1 Activation Is Not Affected in Activated CARMA1-Deficient Cells

(A) Jurkat or JPM50.6 cells (1×10^7/sample) were preincubated with DMSO or MG132 for 30 min and then stimulated with or without anti-CD3 plus anti-CD28 antibodies (6 and 3 μg/ml, respectively) for 30 min. Nuclear extracts were prepared and AP-1 binding activities were analyzed by electrophoretic mobility shift assay (EMSA) with ^{32}P-labeled probes containing AP-1 or Oct-1 binding sites.

(B) Cells were transfected by electroporation with reporter plasmid encoding 4xAP-1-luc (10 μg) and pEF-*Renilla*-luc (200 ng). 20 hr later, the transfected cells were either untreated or stimulated with anti-CD3 plus anti-CD28 (left) or PMA plus ionomycin (right) for 6 hr. Cell lysates were prepared and luciferase activities were measured with Dual-Luciferase assay kits. AP-1 activities were determined by normalization of AP-1-dependent luciferase to *Renilla* luciferase activity. Results are representative of two independent experiments performed in triplicate. Error bars indicate ± standard deviation in triplicate experiments.

(C) The model of TCR-induced JNK phosphorylation. CD3-CD28 costimulation activates various proximal signaling components that lead to activation of PKC and CARMA1, which, in turn, recruits Bcl10 and induces its oligomerization. The activated (oligomerized) Bcl10 serves as a template for TAK1, MKK7, and JNK2. Phosphorylated JNK2 and independently activated JNK1 led to accumulation of c-Jun and activation of AP-1.

which results in an extra 43-residue tail at the C terminus of p54 isoforms of JNK1, JNK2, and JNK3 (Gupta et al., 1996; Kallunki et al., 1994). Of note, a small portion of JNK1p54 isoform is expressed in T cells. However, this portion of JNK1 (JNK1p54) is also defective after CD3-CD28 costimulation, suggesting that the activation of JNK1p54 isoform is also controlled by the CARMA1-dependent pathway. Consistent with this possibility, ectopically expressed JNK1p54 in CARMA1-deficient Jurkat T cells failed to respond to CD3-CD28 costimulation. However, because JNK1p54 exists in a very small portion in T cells, it will require more detailed analysis, such as generating knock-in mice that express only the JNK1p54 or JNK1p46 isoform, for us to draw the more definite conclusion regarding to the requirement of CARMA1 in JNK1p54 activation.

Because CARMA1 is expressed in other hematopoietic cells, we have also examined the JNK2 activation in splenic B cells and bone marrow-derived mast cells from CARMA1-deficient mice. We show that JNK2 activation is also dependent on CARMA1 in these cells after the

stimulation with PMA plus ionomycin. Therefore, the CARMA1-dependent regulation of JNK2 is not limited to T cells, and, instead, a more general regulation in hematopietic cells. However, it remains to be determined whether JNK2 is also regulated differently from JNK1 in nonhematopietic cells that do not express CARMA1.

The CARMA1-dependent regulation of JNK2 is apparently mediated through Bcl10, because JNK2 activation is similarly defective in Bcl10-deficient cells. Previous studies indicate that JNK is regulated through a family of scaffold molecules, JIP (Morrison and Davis, 2003). Our results suggest that Bcl10 functions as a JIP-like scaffold molecule and inducibly associates with JNK2, MKK7, and TAK1 in a CARMA1-dependent manner in lymphocytes. The 43-residue tail at the C terminus of JNK2 is apparently required for its association with Bcl10, which explains why the CARMA1-Bcl10 complex specifically regulates p54, but not p46, isoform of JNK. Interestingly, we find that the oligomerization of Bcl10 through its CARD domain is required for its association with JNK2. Therefore, our current working model is that after stimulation, CARMA1

induces the oligomerization of Bcl10 and the oligomerized Bcl10 functions as a scaffold molecule to recruit MKK7 and TAK1, leading to activation of JNK2. In contrast, some scaffold molecules, other than CARMA1 and Bcl10, are probably involved in assembling JNK1 with MAP2K and MAP3K. Thus, it will be interesting to identify the signaling components specifically connecting JNK1 to antigen receptors.

Although several MAP3Ks, including MEKK1, MEKK2, and MEKK3, have been implicated to play a role in JNK activation in T cells, we can detect Bcl10 inducibly associated only with TAK1 but not other MAP3Ks (data not shown). This result is consistent with the previous findings that TAK1 plays a critical role in JNK activation in innate and adaptive immune responses (Sato et al., 2005; Shim et al., 2005). Moreover, a recent study (Wan et al., 2006) demonstrates that partial depletion of TAK1 from $CD4^+$ and $CD8^+$ T cells selectively reduces phosphorylation of JNK2. However, it is possible that other MAP3Ks may be involved in the regulation of JNK1 activation, which is consistent with the finding that JNK activation is partially defective in T cells from transgenic mice expressing a kinase-dead mutant of MEKK1 (Gao et al., 2004).

Activation of JNK has been shown to contribute to the activation of AP-1 transcription factors. However, CARMA1 deficiency does not affect AP-1 activation (Hara et al., 2003; Wang et al., 2002). Therefore, the defect of JNK2 is likely involved in the regulation of other biological events. Recent studies suggest that JNK activation controls c-Jun and JunB stability (Gao et al., 2004; Sabapathy and Wagner, 2004). Interestingly, we find that CARMA1 deficiency leads to a defect of c-Jun accumulation after CD3-CD28 costimulation. However, our results suggest that the amount of c-Jun is mainly controlled by a transcriptional regulation, instead of the protein stability, after CD3-CD28 costimulation, although the proteasome-mediated degradation is contributed to by the steady-state amount of c-Jun and JunB. Our conclusions are consistent with data demonstrating that JNK2 is a positive regulator of c-Jun transcription in mouse embryo fibroblasts (Jaeschke et al., 2006).

Our study demonstrates that the defect of c-Jun accumulation is not due to the failure of CARMA1-dependent NF-κB activation, because c-Jun is still rapidly accumulated after the stimulation in NEMO (IKKγ)-deficient cells that are defected in NF-κB activation. In addition, although the treatment of cells with MG132, a proteasome inhibitor, blocked the degradation of IκBα and NF-κB activation, CD3-CD28 costimulation could still effectively induce the accumulation of c-Jun. Therefore, the accumulation of c-Jun after CD3-CD28 costimulation is likely through a JNK2-dependent, but NF-κB- and AP-1-independent, mechanism.

In summary, our results provide genetic and biochemical evidence that JNK1 and JNK2 are differentially regulated. The CARMA1-Bcl10 complex selectively regulates JNK2 activation after TCR stimulation. Future studies are required for determining how JNK2 activation affects c-Jun accumulation and whether this type of differential

regulation of JNK1 and JNK2 occurs in nonhematopoietic cells. Finally, it will be interesting to determine how JNK1 activation is regulated by other MAP3Ks, such as MEKK1 after TCR stimulation.

EXPERIMENTAL PROCEDURES

Antibodies and Expression Plasmids
Antibodies specific for phospho-JNK (rabbit, cat. no. 9251) and antibodies for total JNK, JNK2 (cat. no. 4672), c-Jun (60A8), phospho-ERK1/2, and MKK7 were purchased from Cell Signaling Tech. (Beverly, MA). Antibodies specific for JNK1 (C-17), ERK2 (C-14), Myc (A14), HA (F-7), TAK1 (C-9), Bcl10 (H-197), MEKK2 (N-19), NEMO (FL-419), and β-tubulin (D-10) were obtained from Santa Cruz Biotech. (Santa Cruz, CA). Monoclonal antibodies against the C termini of MALT1 were generated in the Genentech central production facility. Rabbit and mouse TrueBlot HRP were purchased from Bioscience. CD3 antibodies were from BD Biotechnology and CD28 from Caltag Biotechnology (Burlingam, CA). CARMA1 antibodies were kindly provided by M. Thome (University of Lausanne, Switzerland). Expression plasmids encoding Bcl10, Bcl10 (L47A), JNK2p54, and CARMA1 were described previously (Kallunki et al., 1994; Wang et al., 2004; Yan et al., 1999). CARMA1 was cloned into a mammalian expression vector, pCMV-Tag2 (Stratagene), at BamHI and EcoRI sites. JNK2p46, a JNK2p54 deletion mutant, was constructed by inserting a stop codon that truncates the last 39 amino acids at the COOH terminus of JNK2p54. Long form of JNK1 (JNK1p54) was cloned from Jurkat T cells and inserted into the pRK6-Myc vector.

Cell Cultures and Stable Transfection
Jurkat T cells deficient in CARMA1 (JPM50.6) and cells reconstituted with WT CARMA1 (50.6WT) were described earlier (Wang et al., 2002, 2004). Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Human embryo kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and the antibiotics described above. Cells were grown in 5% CO_2 at 37°C and passed every 3 days. Stable transfection of JPM50.6 cells with truncated forms of CARMA1 was established by lentiviral infection. Plasmids encoding FLAG-JNK1p46, HA-JNK2p54 and Myc-JNK1p54, were transfected into Jurkat and JPM50.6 cells by electroporation with a gene pulser (Bio-Rad, Hercules, CA) at 250 V, 950 μF. HEK293 cells were transfected by the calcium phosphate coprecipitation method.

Western Blot and Coimmunoprecipitation
The cells were lysed in a buffer containing 50 mM HEPES (pH 7.4), 250 mM NaCl, 1% nonidet P-40, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 mM dithiothreitol, and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The cell lysates were subjected to SDS-PAGE and western blot or immunoprecipitated with various antibodies (anti-JNK1, anti-JNK2, anti-Bcl10, anti-TAK1, anti-MEKK2, anti-MKK7, or anti-HA). The immunoprecipitates were washed with lysis buffer four times and eluted with 2× SDS loading buffer. After boiling (4 min), the samples were fractionated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were incubated with specific primary antibodies followed by HRP-conjugated secondary antibodies and were developed by the enhanced chemiluminescence method according to the manufacturer's protocol (Pierce, Rockford, IL). TrueBlot HRP (anti-rabbit and anti-mouse IgG) was used to detect immunoprecipitated JNK2. This reagent reduces the interference of 55 kDa IgG heavy chain in the immunoprecititated complex.

In Vitro Kinase Assay
Endogenous or overexpressed JNK1 and JNK2 were immunoprecipitated from cells treated with CD3 and CD28 antibodies for various time

points. Resulting immunoprecipitates were washed 3 times with lysis buffer and once with kinase buffer containing 10 mM HEPES (pH 7.4), 1 mM $MnCl_2$, 5 mM $MgCl_2$, 12.5 mM glycerol-2-phosphate, 0.1 mM Na_3VO_4, 4 mM NaF, and 1 mM dithiothreitol. The reactions with GST-c-Jun(1-79) were performed with 20 mM of cold ATP and 5 mCi of $[\gamma\text{-}^{32}P]$ATP in the kinase buffer at 30°C for 30 min. The reactions were stopped by adding 2× SDS loading buffer and the samples were boiled for 4 min. The eluted proteins were fractionated on 10% SDS-PAGE and transferred to nitrocellulose membranes, followed by autoradiography.

ELISA

DuoSet IC Human Phospho-JNK2 (T183/Y185) ELISA kit was obtained from R&D Systems (Minneapolis, MN). The amounts of phosphorylated JNK2 were measured in cell lysates according to the manufacturer's protocol.

Purification and Stimulation of Murine T and B Cells

T cells were purified from total lymph node cells by mouse pan-T cell purification kit (Miltenyi Biotech, Auburn, CA) according to the manufacturer's recommendations. The purity of T cells was more than 95% based on anti-CD3 staining. B cells were purified from RBC-lysed splenocytes from WT, CARMA1 KO, and Bcl10 KO mice with anti-CD43 magnetic beads according to the manufacturer's recommendations (Miltenyi Biotech). The purity of B cells was more than 95% based on B220/IgM staining. Purified cells were either unstimulated or stimulated with PMA (20 ng/ml) plus ionomycin (200 ng/ml) for indicated times. Cell lysates were subjected to SDS-PAGE and western blotting analysis with indicated antibodies.

Generation and Stimulation of Bone Marrow-Derived Mast Cells

Bone marrow was harvested from the femur and tibia of WT and KO mice and cultured in the complete RPMI-1640 medium supplemented with IL-3 for 4–5 weeks to obtain bone marrow-derived mast cells (BMMC). During the BMMC culture, nonadherent cells were recultured in fresh medium containing IL-3 every week. Mast cell development was confirmed by staining for FcRI and c-Kit receptor expression and flow cytometric analysis. More than 95% cells obtained under this culture condition were mast cells. These cells were either unstimulated or stimulated with PMA (20 ng/ml) plus ionomycin (200 ng/ml) for indicated time points. Cell lysates were subjected to SDS-PAGE and western blotting analysis with indicated antibodies.

Mice

CARMA1 and Bcl10 null mice were described previously (Hara et al., 2003; Xue et al., 2003). C57BL/6 mice were obtained from Harlan Laboratory (Indianapolis, IN). All mice were used at 10–16 weeks of age. Animals were housed under specific pathogen-free conditions at the institutional animal facility. All 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 M.D. Anderson Cancer Center.

Supplemental Data

Five Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/26/1/55/DC1/.

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