Requirement for CARMA1 in Antigen Receptor-Induced NF-κB Activation and Lymphocyte Proliferation

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Summary

Ligation of antigen receptors (TCR, BCR) on T and B lymphocytes leads to the activation of new transcriptional programs and cell cycle progression. Antigen receptor-mediated activation of NF-κB, required for proliferation of B and T cells, is disrupted in T cells lacking PKCδ and in B and T cells lacking Bcl10, a caspase recruitment domain (CARD)-containing adaptor protein [1, 2]. CARMA1 (also called CARD11 and Bimp3), the only lymphocyte-specific member in a family of membrane-associated guanylate kinase (MAGUK) scaffolding proteins that interact with Bcl10 by way of CARD-CARD interactions [3, 4], is required for TCR-induced NF-κB activation in Jurkat T lymphoma cells [5–7]. Here we show that T cells from mice lacking CARMA1 expression were defective in recruitment of Bcl10 to clustered TCR complexes and lipid rafts, in activation of NF-κB, and in induction of IL-2 production. Development of CD5−/−peritoneal B cells was disrupted in these mice, as was B cell proliferation in response to both BCR and CD40 ligation. Serum immunoglobulin levels were also markedly reduced in the mutant mice. Together, these results show that CARMA1 has a central role in antigen receptor signaling that results in activation and proliferation of both B and T lymphocytes.

Results and Discussion

Antigen receptor stimulation in B and T lymphocytes results in rapid activation of membrane-proximal tyrosine kinases of the Src and Syk families, phosphorylation of adaptor proteins, and binding of various cytosolic proteins to the newly phosphorylated adaptors [8]. In T cells, these events are required for recruitment of PKCδ to the immunological synapse (IS) and for subsequent activation of NF-κB [9]. In B cells, PKCβ is thought to serve a similar role [10]. Many different signaling path-
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Figure 1. Targeted Disruption of the carma1 Locus

(A) The wild-type carma1 locus and the targeting vector (middle) are shown with EcoRI restriction enzyme sites (RI). The targeting vector was designed to knock-in the loxP-flanked neomycin-resistant cassette (neo) into intron 4 and another loxP site into intron 2. The loxP sites and exons are indicated with triangles and open rectangles, respectively. Real-time PCR analysis with cDNA from thymus and spleen showed that expression levels of 3' adjacent genes Lfng and Gna12 and a 5' adjacent gene, Foxk1, were not affected by insertion of the neomycin resistance cassette (data not shown). These loci are 260 kbp, 44 kbp, and 1.5 Mbp away from the carma1 gene, respectively, and all of these genes are expressed in thymus and spleen.

(B) Southern blot analysis of genomic DNA from carma1+/+/, FN/+ and FN/FN mice. Genomic DNA was digested with EcoRI and probed with the 3' external probe shown as a shaded box in (A).

(C) Western blot analysis for CARMA1 and Bcl10 expression in splenocytes from carma1+/+, FN/+ and FN/FN mice. A monoclonal antibody specific for residues 804–959 (a kind gift from V. Dixit, Genentech) was used.

serum immunoglobulin levels, particularly levels of IgM, IgG1, IgG2b, IgG3, and IgA isotypes (Figure 2D). A similar reduction in basal immunoglobulins was reported in Bcl10-deficient mice [1]. Selective loss of B1 cells accompanied by reduced immunoglobulin levels has also been described in mice deficient for proximal B cell signaling components required for induction of NF-kB and proliferative responses, including Btk and PKCδ [15].

We next compared responses of mature T cells from mutant mice and wild-type littermates to various stimuli. There was reduced upregulation of cell-surface CD25 and CD69 in cells from carma1 FN/FN mice compared to control mice after stimulation with anti-CD3 or anti-CD3 plus anti-CD28 (Figure 3A). This defect was considerably more severe in cells from mutant mice after stimulation with the phorbol ester PMA with or without ionomycin (Figure 3A). Proliferation in response to anti-CD3/anti-CD28 was also significantly impaired in T cells from the carma1 FN/FN mice (Figure 3B). This defect was chiefly due to the reduced production of IL-2 (Figure 3C); proliferation in response to anti-CD3 was restored when IL-2 was added back (Figure 3B). There was no difference in IL-2 production when CARMA1-deficient and wild-type cells were activated with PMA plus ionomycin, although there was a blunting of the proliferative response, probably due to the poor upregulation of CD25, a component of the high-affinity IL-2 receptor (Figures 3A–3C). Very similar results were observed with T cells from pkcδ−/− mice, consistent with our previous findings [2].

B cells from CARMA1-deficient mice also exhibited defects in activation. Proliferation in response to anti-IgM, which stimulates the BCR, to anti-CD40, or to a
Figure 2. Analysis of B and T Cell Subsets and Serum Immunoglobulin Levels in carma1<sup>−/−</sup> Mice

(A) Flow-cytometric analysis of CD4 and CD8 expression on thymocytes (top) and CD25 and CD44 expression in the Lin<sup>−</sup> (CD4<sup>−</sup> CD8<sup>−</sup> B220<sup>−</sup> TCR<sup>+</sup> Gr1<sup>−</sup> Mac1<sup>−</sup>) thymocyte subpopulation (bottom). Percentages of cells within each quadrant are indicated.

(B) TCR<sup>β</sup> and TCR<sup>δ</sup> expression and AnnexinV staining in Lin<sup>−</sup> thymocytes.

(C) CD5 and B220 expression in Mac1<sup>−/−</sup>-gated peritoneal cells.

Results in (A)–(C) are representative data from four different experiments.

(D) Decreased baseline serum immunoglobulin levels in carma1<sup>−/−</sup> mice. Concentration of serum immunoglobulin of each isotype was determined by ELISA in serum samples from 6- to 8-week-old carma1<sup>−/−</sup> mice (M, shaded circles, n = 4), and littermate wild-type mice (W, open circles, n = 4). Mean values are shown by horizontal bars.

combination of the two was markedly reduced (Figure 3D). However, the Tlr4 signaling pathway induced by LPS was intact in the mutant B cells. These results are very similar to those previously observed in Bcl10-deficient mice [1]. To determine whether CARMA1 is required for NF-κB wild-type mice (open bars), carma1<sup>−/−</sup> mice (filled bars), and pκcα<sup>−/−</sup> mice (hatched bars) and stimulated for 72 hr with 5 μg/ml of plate bound anti-CD3 alone or with 5 μg/ml of anti-CD28 or 10 U/ml of recombinant IL-2. Alternatively, cells were stimulated with 100 ng/ml of PMA with or without 200 ng/ml of ionomycin. Proliferative responses were measured by H thymidine incorporation in (B). For measurement of IL-2 production (C), culture supernatants were harvested 30 hr after stimulation, and IL-2 levels were determined by examination of proliferation of the IL-2 dependent cell line CTLL-2 for 48 hr. Representative results from four independent experiments are shown as mean values and standard deviations.

(D) Defective B cell proliferation induced by BCR and CD40 crosslinking in the absence of CARMA1. Purified B cells were stimulated for 48 hr with anti-IgM (10 μg/ml) antibodies, anti-CD40 (10 μg/ml) antibodies, anti-IgM + anti-CD40 antibodies, anti-IgM antibody + IL-4 (5 U/ml), or LPS (1 μg/ml), and proliferation was measured by H thymidine incorporation.

(E) TCR-induced NF-κB activation in CARMA1-deficient thymocytes and CD4<sup>+</sup> T cells. Thymocytes and purified CD4<sup>+</sup> T cells were stimulated for 8 hr with 10 μg/ml of plate bound anti-CD3 with or without 10 μg/ml of anti-CD28 antibody or were stimulated with 100 ng/ml of PMA with or without 200 ng/ml of ionomycin (Iono). NF-κB activation was examined by electrophoretic mobility shift assay. Nuclear extract from thymocytes and CD4<sup>+</sup> T cells treated with 20 μg/ml of TNFα was used as a control.

(F) Defective IκB degradation induced by TCR stimulation in carma1<sup>−/−</sup> CD4<sup>+</sup> T cells. Purified CD4<sup>+</sup> T cells were stimulated with 10 μg/ml of anti-CD3 or 20 μg/ml of TNFα for different durations as indicated. Cytosolic extracts were subjected to Western blotting with antibodies against IκBα. The same blots were probed with an anti-p56<sup>κcα</sup> antibody for a loading control.
Figure 3. Impaired Lymphocyte Activation in CARMA1-Deficient Mice

(A) Surface expression of CD25 and CD69 after activation of the TCR signaling pathway. CD4+ T cells from mutant mice (open histogram) or control littermates (filled histogram) were stained with anti-CD25 and anti-CD69 antibodies 16 hr after stimulation with 10 μg/ml of plate bound anti-CD3 with or without 10 μg/ml of anti-CD28 antibody or after stimulation with 50 ng/ml of PMA with or without 200 ng/ml of ionomycin and then analyzed by flow cytometry. Representative data from three independent experiments with similar results are shown.

(B and C) Impaired proliferation and IL-2 production of CD4+ T cells in the absence of CARMA1 expression. CD4+ T cells were purified from

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Figure 4. Defective Bcl10 but Normal PKCθ Recruitment to Clustered TCR Complexes and Lipid Rafts in the Absence of CARMA1

(A) Purified CD4⁺ T cells labeled with Alexa488-conjugated cholera toxin B subunit (CTX, green) were treated with anti-CTX antibody for 30 min at 37°C and stained for Bcl10 (Cy5, red) after fixation.

(B and C) Purified CD4⁺ T cells from wild-type, carma1FN/FN and pkcθ/θ mice were incubated with latex beads coated with anti-CD3 and anti-CD28 antibodies or BSA for 30 min at 37°C and stained for (B) Bcl10 (FITC, green) and Carma1 (Cy5, blue) or (C) for PKCθ (Alexa488, green) after fixation.

activation in mouse lymphocytes, we analyzed nuclear extracts from thymocytes and CD4⁺ T cells in electrophoretic mobility shift assays. NF-κB activation following stimulation with anti-CD3/CD28 and PMA with or without ionomycin was only slightly reduced in thymocytes from mutant and wild-type mice (Figure 3E). In contrast, there was a marked reduction in NF-κB activation in CARMA1-deficient mature T cells after TCR stimulation or phorbol treatment. However, NF-κB activation in response to TNF treatment was intact in these cells. Monitoring the level of IκBα after various stimuli can also serve as a gauge for activation of the IKK signaling pathway. After anti-CD3 treatment, IκBα was reduced in wild-type T cells but remained unchanged in T cells from mutant mice (Figure 3F). However, a reduction was observed in cells from both sets of mice after treatment with TNF. Together, these results indicate that NF-κB activation following TCR ligation is compromised in mice lacking CARMA1 and confirm that this pathway for IKK activation is distinct from that downstream of the TNF receptor. These results are consistent with phenotypes reported for T cells from mice lacking c-Rel or both c-Rel and p50, which had no NF-κB activation and IL-2 production after TCR stimulation [16, 17].

The similarity of the B and T cell phenotypes observed in the absence of CARMA1 and Bcl10 supports the notion that these molecules are closely associated in a signaling complex involved in the activation of NF-κB.
Studies in Jurkat cells have demonstrated that CARMA1 is constitutively associated with lipid rafts within membranes, whereas a fraction of cellular Bcl10 is recruited to lipid rafts and is coprecipitated with CARMA1 only after T cell activation [5, 6], suggesting that CARMA1 functions upstream of Bcl10. To determine whether the murine orthologous proteins have the same properties, we treated primary T cells from mutant and wild-type mice with fluorescent cholera toxin (CTX), which binds to GM1 ganglioside within lipid rafts. Crosslinking of CTX activates signaling pathways similar to those activated by TCR ligation [18]. In the absence of crosslinking, Bcl10 was localized to the cytoplasm in both wild-type and CARMA1-deficient T cells (Figure 4A). After crosslinking of CTX, Bcl10 was recruited to the plasma membrane and was colocalized with the patched CTX in wild-type cells. However, in cells from mutant mice, Bcl10 remained localized throughout the cytoplasm. We also used beads coated with antibodies against CD3 and CD28 to mimic stimulation of T cells by antigen-presenting cells. In wild-type T cells, CARMA1 staining could be observed at the plasma membrane, and there was recruitment of Bcl10 to the interface between beads and the T cell surface. In cells from carma1F0/F0 mice, no CARMA1 staining was observed, and Bcl10 remained distributed throughout the cytoplasm (Figure 4B). Similarly, in pcki−/− T cells, there was no recruitment of Bcl10 to the cell/bead interface, although CARMA1 staining at the plasma membrane was intact (Figure 4B).

The relationship between PKCi and CARMA1 in the NF-κB activation pathway remains unresolved. PKCi is rapidly recruited to the IS during T cell activation [19]. Upon stimulation with anti-CD3/CD28-coated beads, PKCi was recruited to the cell/bead interface even in cells lacking CARMA1 (Figure 4C). Together, these results suggest that PKCi functions either upstream of or in parallel to CARMA1 to induce recruitment of Bcl10 to the IS. It is possible that PKCi acts directly or indirectly on CARMA1 and that its action results in exposure of the CARMA1 CARD, which can then bind to the CARD of Bcl10. However, such an event may not be sufficient to account for the relocalization of Bcl10 from the cytoplasm to the IS. After its recruitment and activation at the site of the IS, PKCi could thus function independently of CARMA1 in signaling pathways that influence cytoskeletal reorganization and result in recruitment of other signaling complexes, including Bcl10/MALT1/IKK.

Although mice lacking CARMA1 and Bcl10 have similar defects in B and T cell development and function, 30% of Bcl10-deficient embryos die because of exencephaly caused by a neural tube closure defect [1]. This most likely reflects the lymphoid cell-specific expression of CARMA1. In addition to CARMA1, the widely expressed Bcl10 protein has multiple potential CARD-CARD interaction partners, including CARD10 (CARMA3/Bimp1) and CARD14 (CARMA2/Bimp2), which are expressed in non-lymphoid tissues [3, 20]. The key interactions of Bcl10 with these molecules in cells other than B and T lymphocytes are therefore predicted to remain intact in the carma1 mutant mice.

Supplemental Data
Supplemental Data including detailed Experimental Procedures are available online at http://www.current-biology.com/cgi/content/full/13/14/1252/DC1.

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