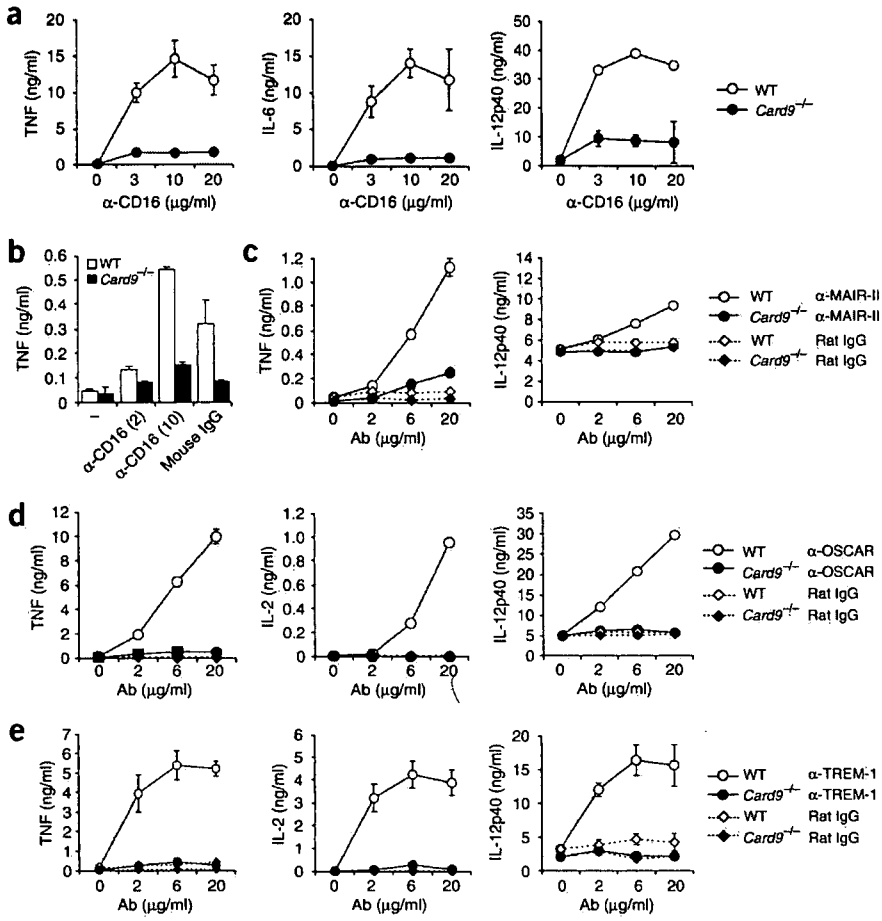

Figure 2 Impaired cytokine production by *Card9*$^{-/-}$ myeloid cells after stimulation through ITAM receptors. (**a**,**b**) ELISA of the production of TNF, IL-6 and/or IL-12p40 by wild-type or *Card9*$^{-/-}$ BMDCs (**a**) or bone marrow–derived macrophages (**b**) stimulated by crosslinking of FcγR with immobilized monoclonal anti-CD16 (α-CD16) or mouse IgG. – (**b**), no antibody; numbers in parentheses (**b**), concentration of anti-CD16 (in μg/ml). (**c**–**e**) ELISA of the production of TNF, IL-2 and IL-12p40 by wild-type and *Card9*$^{-/-}$ BMDCs after stimulation in the presence of FcR blocker (soluble mouse γ-globulin; 10 μg/ml) with immobilized monoclonal anti-MAIR-II (**c**), anti-OSCAR (**d**) or anti-TREM-1 (**e**). Rat IgG, control for stimulation. Ab (horizontal axes, **c**–**e**), antibody concentration. Data are the mean ± s.d. of triplicates and are representative of three independent experiments.

indicate that CARD9 is not involved in the development and activation of B cells.

Card9$^{-/-}$ myeloid cells have impaired ITAM-mediated signaling

Because myeloid cells had highly expression of CARD9 (**Supplementary Fig. 1a**), we next examined whether CARD9 deficiency affected the development of macrophages and DCs. The development of CD11b^{+}CD11c^{-} macrophages and CD11b^{-}CD11c^{+} DCs (**Supplementary Fig. 2g**), of subsets of CD4^{+}, CD8^{+} and CD4^{-}CD8^{-} cells among the CD11c^{+} DC populations and of CD11c^{+}CD19^{-}B220^{+} plasmacytoid DCs (**Supplementary Fig. 2h**) in the spleen was similar in *Card9*$^{-/-}$ and wild-type mice. Because it has been suggested that Bcl-10 is involved in FcR-mediated NF-κB activation in mast cells[25], we tested whether CARD9 is involved in FcR-mediated activation in myeloid cells. We prepared bone marrow–derived DCs (BMDCs) from *Card9*$^{-/-}$ mice and stimulated the cells by crosslinking of FcγRIII with monoclonal anti-CD16 or immobilized IgG. BMDCs developed normally from *Card9*$^{-/-}$ mice (**Supplementary Fig. 2i**). However, *Card9*$^{-/-}$ DCs had impaired production of TNF, IL-6 and IL-12 (**Fig. 2a**) after FcγR crosslinking. There was similar severe impairment in bone marrow–derived macrophages (**Fig. 2b**), suggesting that CARD9 is essential for FcγR signaling in myeloid cells. These results demonstrate that signals mediated through FcγR, which associates with ITAM-containing FcRγ, are impaired in *Card9*$^{-/-}$ DCs and macrophages, whereas TCR- and BCR-mediated signals in *Card9*$^{-/-}$ lymphocytes are unaffected. This suggests the possibility that CARD9 is involved in signaling through receptors associated with the ITAM-containing adaptors FcRγ or DAP12 specifically in myeloid cells.

CARD9 is involved in signaling from dectin-1, which contains an atypical ITAM in its cytoplasmic domain[30]. However, because of the unique features of dectin-1 in terms of the atypical ITAM, which requires phosphorylation of only a single tyrosine in the motif for Syk recruitment, that conclusion might be specifically restricted to dectin-1 and it may not be possible to generalize it to receptors associated with FcRγ and DAP12. To test that, we analyzed the surface expression of several receptors associated with FcRγ or DAP12 on BMDCs using specific monoclonal antibodies and verified expression of MAIR-II, OSCAR and TREM-1. Whereas MAIR-II and TREM-1 are associated with DAP12 (refs. 8,14), OSCAR is associated with FcRγ[7]. To determine whether CARD9 is involved in signaling through those ITAM-bearing receptors, we stimulated *Card9*$^{-/-}$ and wild-type BMDCs with monoclonal antibodies specific for MAIR-II (**Fig. 2c**), OSCAR (**Fig. 2d**) or TREM-1 (**Fig. 2e**). After crosslinking of MAIR-II, OSCAR and TREM-1, the production of all cytokines examined, including TNF, IL-2, IL-12 (**Fig. 2c–e**) and IL-6 (data not shown), was abrogated in *Card9*$^{-/-}$ BMDCs. Those defects were not overcome by stimulation with higher doses of antibodies, suggesting that CARD9 is crucial for cytokine production after stimulation of those FcRγ- and DAP12-associated receptors in BMDCs. In contrast to stimulation through ITAM-associated receptors, stimulation with anti-CD40 or phorbol 12-myristate 13-acetate plus calcium ionophore induced similar amounts of cytokine production in wild-type and *Card9*$^{-/-}$ BMDCs (**Supplementary Fig. 3** online), indicating that *Card9*$^{-/-}$ BMDCs are capable of producing cytokines and the defect is specific for ITAM-associated receptor–mediated activation.

Dectin-1- and MyD88-dependent DC activation requires CARD9

Consistent with a published report[30], zymosan-induced production of TNF, IL-2 (**Fig. 3a**) and IL-6 (data not shown) was considerably impaired in *Card9*$^{-/-}$ BMDCs. However, notably, IL-12 production after stimulation with zymosan seemed to be normal (**Fig. 3a**), in contrast to the response to stimulation through FcRγ- and DAP12-associated receptors, which resulted in impaired IL-12 production similar to that of other cytokines (**Fig. 2**). Zymosan, a polysaccharide

particle from the cell walls of *Saccharomyces cerevisiae*, is the most commonly used β-glucan-containing experimental reagent, but it contains other components, including other glucans, mannans, chitins and unknown TLR2 and TLR6 ligands[33,34]. It has been reported that zymosan-induced production of TNF and IL-12 requires the 'collaboration' of dectin-1 and TLR2, followed by activation of NF-κB through MyD88 (ref. 31). Indeed, it has been suggested that production of TNF and IL-12 after zymosan stimulation is impaired by MyD88 deficiency but not by dectin-1 or Syk deficiency[21,32]. Thus, it was not apparent if the impaired cytokine responses of *Card9*$^{-/-}$ BMDCs were due to a defect in the pathway of dectin-1–Syk signaling and/or TLR-MyD88 signaling.

To clarify that point, we compared cytokine responses of *Card9*$^{-/-}$, *Myd88*$^{-/-}$ and *Clec7a*$^{-/-}$ (dectin-1-deficient) BMDCs after stimulation with untreated zymosan or NaClO-oxidized zymosan. We generated NaClO-oxidized zymosan by treating zymosan with 0.5% NaClO and 0.1 M NaOH; it represents a product composed mainly of β-glucans[35] and therefore it specifically binds to dectin-1 but not TLRs. We found that zymosan-induced production of TNF, IL-6 and IL-12 was not impaired much in *Clec7a*$^{-/-}$ BMDCs (**Fig. 3b**). In contrast, production of TNF and IL-6 was much lower in *Myd88*$^{-/-}$ BMDCs, similar to that in *Card9*$^{-/-}$ BMDCs, whereas IL-12 was slightly lower in *Myd88*$^{-/-}$ BMDCs (**Fig. 3b**). These results indicate that zymosan stimulates production of TNF and IL-6 mainly through the TLR-MyD88 signaling pathway, not through the dectin-1-mediated pathway; they also suggest that CARD9 contributes TLR-MyD88 signaling rather than to dectin-1 signaling after zymosan stimulation. In contrast, all of cytokine responses to NaClO-oxidized zymosan were abolished in *Clec7a*$^{-/-}$ and *Card9*$^{-/-}$ BMDCs but not in *Myd88*$^{-/-}$ BMDCs (**Fig. 3c**), indicating that cytokine production after β-glucan stimulation is completely dependent on the dectin-1–CARD9 signaling pathway. Our data collectively indicate that CARD9 is indispensable for myeloid cell activation through ITAM-bearing dectin-1 and that CARD9 is also involved in TLR-MyD88 signaling.

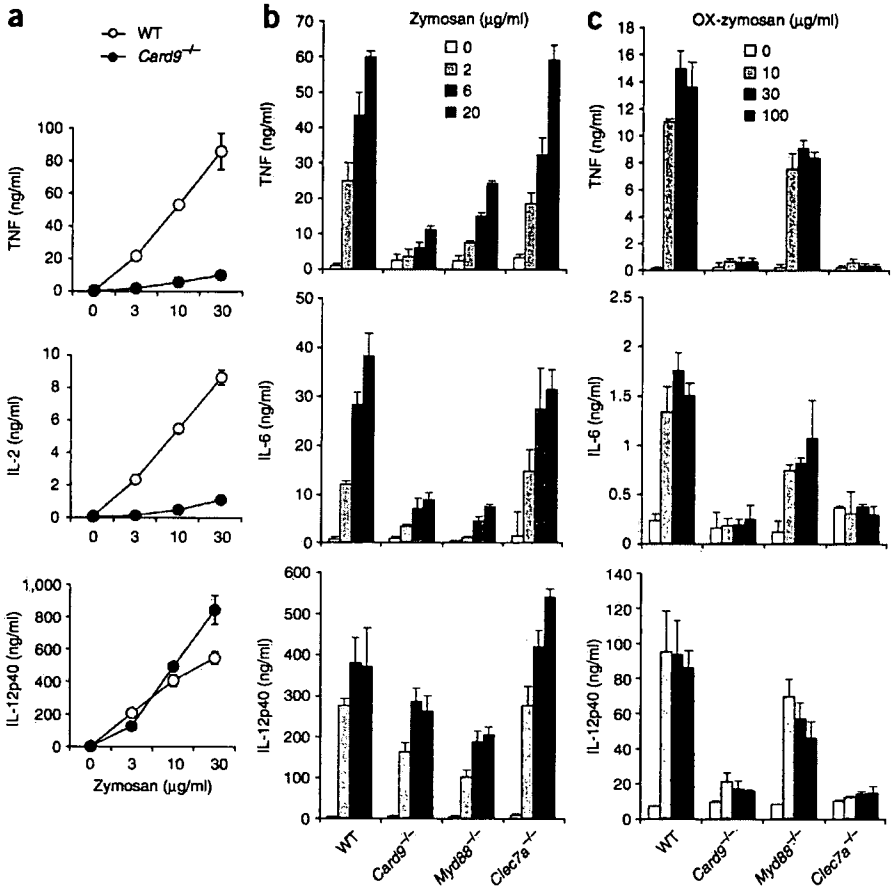

Figure 3 CARD9 is required for both MyD88- and dectin-1-mediated cytokine responses. (**a**) ELISA of the production of TNF, IL-2 and IL-12p40 by zymosan-stimulated wild-type or *Card9*$^{-/-}$ BMDCs. (**b,c**) ELISA of the production of TNF, IL-6 and IL-12p40 by wild-type, *Card9*$^{-/-}$, *Myd88*$^{-/-}$ or *Clec7a*$^{-/-}$ BMDCs in response to zymosan (**b**) or NaClO-oxidized zymosan (OX-zymosan; **c**). Data are the mean ± s.d. of triplicates and are representative of two independent experiments.

ITAM-mediated activation of NF-κB requires CARD9

We next analyzed the mechanism of impaired signaling in *Card9*$^{-/-}$ myeloid cells. As the CBM complex is essential for the coupling of antigen receptors to the activation of NF-κB and MAPK in T lymphocytes and B lymphocytes[24,26], we investigated the function of CARD9 in the ITAM-associated receptor–induced activation of NF-κB and MAPK in BMDCs through crosslinking of FcγR. Degradation of IκBα induced by FcγR crosslinking was impaired in *Card9*$^{-/-}$ BMDCs (**Fig. 4a**). Consistent with that finding, the DNA-binding activity of the NF-κB complex containing the p65 subunit was much lower in the nuclei of *Card9*$^{-/-}$ BMDCs after stimulation through ITAM-associated receptors (**Fig. 4b**). In contrast, stimulation of *Card9*$^{+/+}$ and *Card9*$^{-/-}$ BMDCs through FcγR demonstrated no apparent differences in the phosphorylation of the kinases Erk, Jnk and p38 (**Fig. 4a**). These data indicate that CARD9 is essential for the coupling of ITAM receptor signaling to activation of NF-κB but not of MAPK in myeloid cells.

ITAM-mediated myeloid cell activation requires Bcl-10

Because CARD9 interacts with Bcl-10 (ref. 29), we determined whether Bcl-10 functions in the CARD9-dependent, ITAM receptor–mediated activation of myeloid cells. When we stimulated BMDCs from *Bcl10*$^{-/-}$ mice with monoclonal antibodies specific for ITAM receptors or zymosan, cytokine production was substantially impaired, similar to that in *Card9*$^{-/-}$ BMDCs (**Fig. 5a**). Thus, analogous to antigen receptor signaling, Bcl-10 is critically involved in activation signals mediated by means of ITAM-bearing receptors in myeloid cells.

The formation of the complex of Bcl-10 and CARMA1 is essential for antigen receptor signaling in lymphocytes. Splenic DCs and BMDCs have abundantly expression of CARMA1, whereas macrophages have relatively lower expression (**Supplementary Fig. 4** online). Thus, we examined whether CARMA1 also functions in the ITAM receptor–Bcl-10 signaling pathway in myeloid cells. Unlike their responses to TCR and BCR stimulation, *Card11*$^{-/-}$ BMDCs had normal production of TNF and IL-2 after stimulation through FcγR

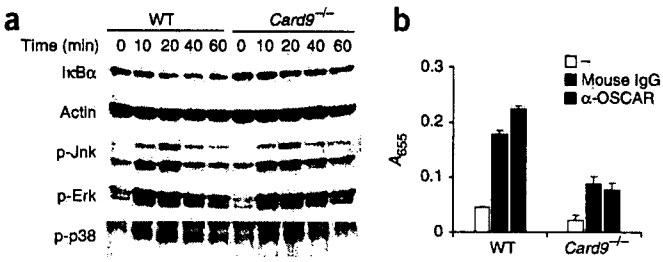

Figure 4 Impaired ITAM-associated receptor–mediated NF-κB activation in *Card9*$^{-/-}$ DCs. (**a**) Immunoblot analysis of lysates of wild-type or *Card9*$^{-/-}$ BMDCs stimulated with anti-CD16 (time, above lanes). p-, phosphorylated. (**b**) DNA-binding assay of NF-κB p65 activity in nuclear extracts from wild-type or *Card9*$^{-/-}$ BMDCs left unstimulated (–) or stimulated for 8 h with immobilized mouse IgG (10 μg/ml) or anti-OSCAR (10 μg/ml). A_{655}, absorbance at 655 nm. Data are the mean ± s.d. of triplicates and are representative of three independent experiments.

(**Fig. 5b**), OSCAR (**Fig. 5c**), TREM-1 (**Fig. 5d**) or zymosan (**Fig. 5e**), suggesting that CARMA1 is not involved in ITAM-mediated myeloid cell activation. These results collectively suggest that the CARD9–Bcl-10 complex but not the CARMA1–Bcl-10 complex relays ITAM-mediated signals for NF-κB activation in myeloid cells.

TLR signaling requires CARD9 and Bcl-10 but not CARMA1

To investigate further the possibility that CARD9 is involved in TLR-MyD88 signaling, we examined the effects of direct stimulation of *Card9*$^{-/-}$ BMDCs with various TLR ligands: Pam_3CSK_4 for TLR2, poly(I:C) for TLR3, LPS for TLR4, flagellin for TLR5, loxoribine for TLR7, and CpG DNA for TLR9. Production of both TNF (**Fig. 6a**) and IL-6 (**Fig. 6b**) was much lower in *Card9*$^{-/-}$ BMDCs than in wild-type BMDCs in response to all TLR ligands tested. In particular, the responses to the TLR7 ligand loxoribine and the TLR3 ligand poly(I:C) were severely impaired in *Card9*$^{-/-}$ BMDCs. Because the induction of *Il6* mRNA after loxoribine stimulation was much lower in *Card9*$^{-/-}$ BMDCs, the impairment in cytokine production seemed to be at the transcriptional level (**Supplementary Fig. 5** online). Notably, like the pattern of impaired cytokine production in response to zymosan stimulation (**Figs. 3b** and **6a–c**), TLR-mediated IL-12 production seemed to be similar in wild-type and *Card9*$^{-/-}$ BMDCs (**Fig. 6c**), suggesting that CARD9 is differentially required for the induction of TNF and/or IL-6 and IL-12. Notably, *Card9*$^{-/-}$ bone marrow–derived macrophages or thioglycolate-induced peritoneal macrophages did not have the defective TLR-induced cytokine production found in DCs (**Supplementary Fig. 6** online), suggesting that the contribution of CARD9 to TLR signaling differs depending on the myeloid cell type. To determine whether Bcl-10 and CARMA1 were involved in the CARD9-mediated TLR signaling pathways in BMDCs, we tested cytokine responses after stimulation with various TLR ligands in *Bcl10*$^{-/-}$ BMDCs and *Card11*$^{-/-}$ BMDCs. As with ITAM receptor signaling, *Bcl10*$^{-/-}$ BMDCs also had defective TLR-induced cytokine production (**Fig. 6d**) but *Card11*$^{-/-}$ BMDCs did not (**Fig. 6e**), suggesting that the CARD9–Bcl-10 complex but not the CARMA1–Bcl-10 complex is involved in TLR signaling.

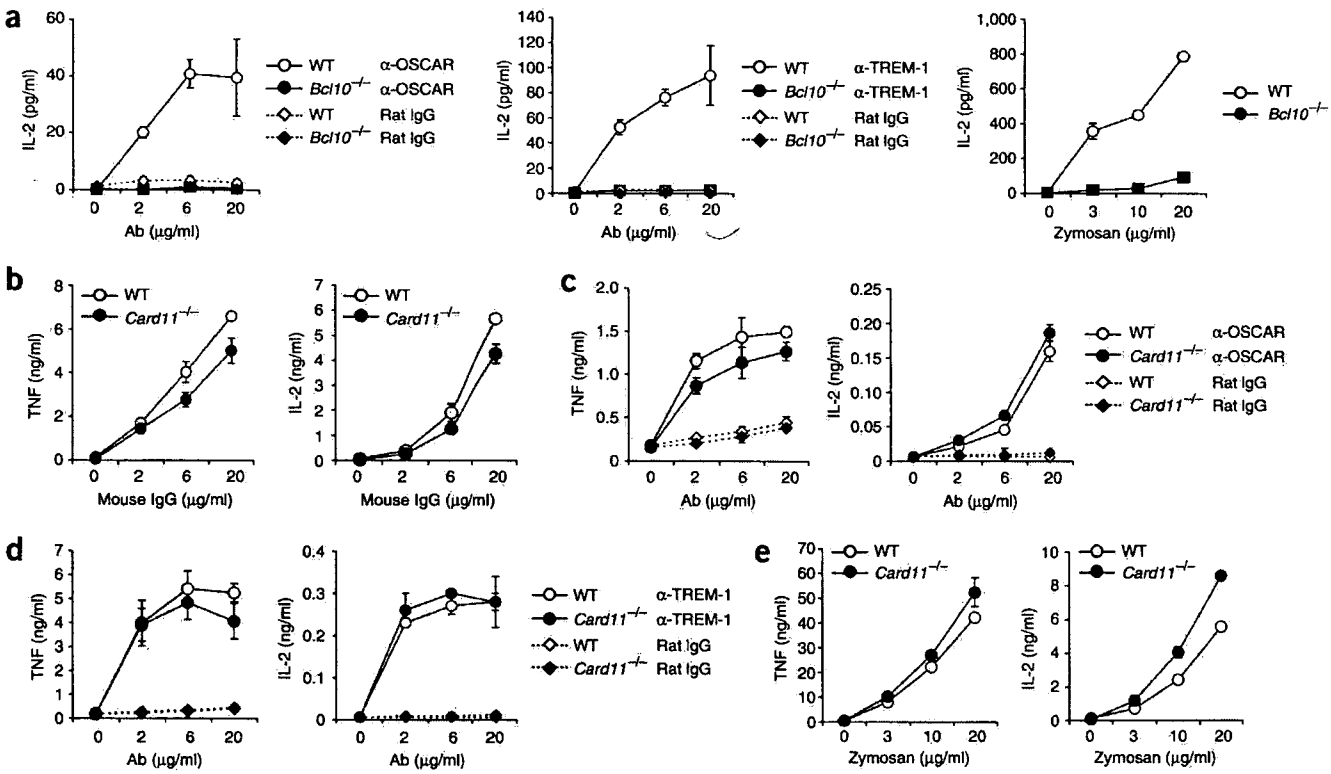

Figure 5 CARD9-dependent ITAM receptor–induced activation of DCs requires Bcl-10 but not CARMA1. (**a**) ELISA of IL-2 production by wild-type or *Bcl10*$^{-/-}$ BMDCs stimulated for 24 h with immobilized monoclonal anti-OSCAR (left) or anti-TREM-1 (middle) or control rat IgG in the presence of FcR blocker (soluble mouse γ-globulin, 10 μg/ml) or stimulated with zymosan (right). (**b–e**) ELISA of the production of TNF and IL-2 by wild-type or *Card11*$^{-/-}$ BMDCs stimulated for 24 h in the presence of FcR blocker with immobilized mouse IgG (**b**), anti-OSCAR (**c**), anti-TREM-1 (**d**) or zymosan (**e**). Rat IgG, control antibody for stimulation (**a**,**c**,**d**). Data are the mean ± s.d. of triplicates and are representative of three independent experiments.

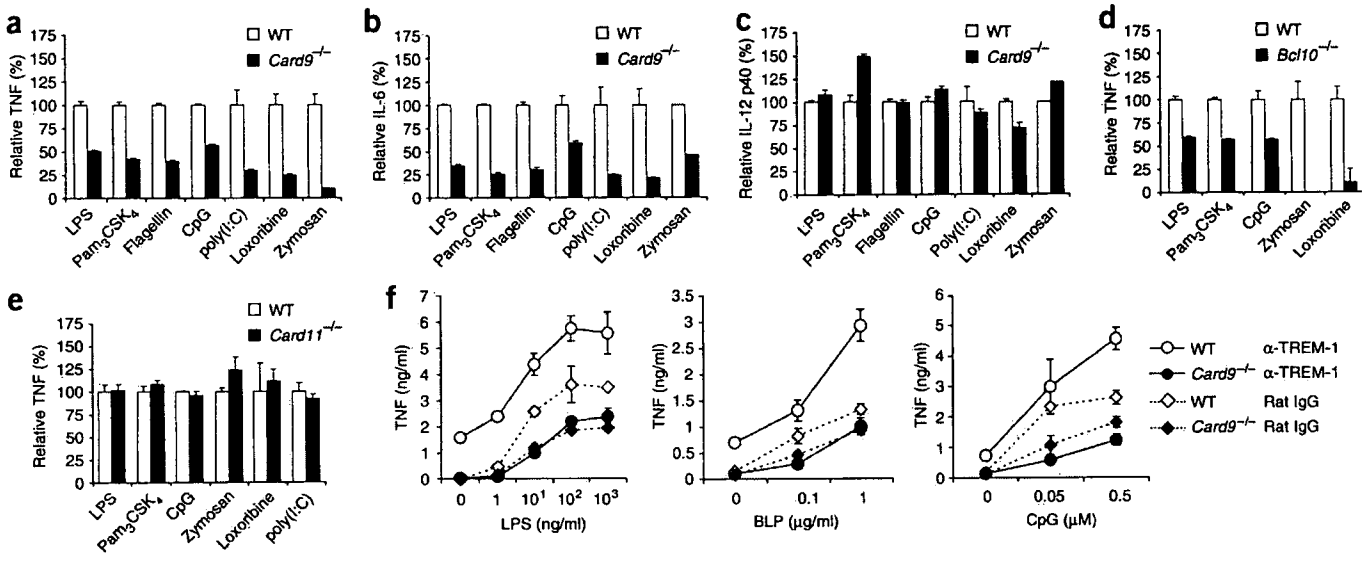

Figure 6 Impaired TLR response by *Card9*$^{-/-}$ DCs. (**a–e**) ELISA of cytokine production by BMDCs stimulated for 24 h with the TLR ligands LPS (100 ng/ml), Pam_3CSK_4 (100 ng/ml), flagellin (100 ng/ml), CpG DNA (1 μM), poly(I:C) (100 μg/ml), loxoribine (100 μM) or zymosan (20 μg/ml; **a–c**) or with all of the TLR ligands in **a–c** except flagellin (**d,e**). Results presented are relative to wild-type production (set at 100%). (**f**) ELISA of TNF production by wild-type or *Card9*$^{-/-}$ BMDCs stimulated with LPS (left), bacterial lipopeptide (BLP; middle) or CpG DNA (right) in the presence of costimulation with immobilized anti-TREM-1 or control rat IgG (10 μg/ml). Data are the mean ± s.d. of triplicates and are representative of six (**a–c**) or two (**d–f**) independent experiments.

TREM-1-mediated TLR responses require CARD9

It has been reported that TREM-1 stimulation augments LPS-induced cytokine responses both *in vitro* and *in vivo*[18]. To determine whether CARD9 is involved in the effect of TREM-1, we stimulated *Card9*$^{-/-}$ BMDCs with TLR ligands, including LPS, Pam_3CSK_4, CpG DNA, in the presence or absence of costimulation with monoclonal anti-TREM-1 and then evaluated TNF production (**Fig. 6f**). TLR-induced TNF production itself without TREM-1 stimulation was much lower in *Card9*$^{-/-}$ BMDCs. Although TNF production after stimulation with TLR ligands was enhanced in the presence of TREM-1 costimulation in wild-type BMDCs, *Card9*$^{-/-}$ BMDCs did not demonstrate such enhancement by TREM-1 costimulation. Thus, these results demonstrate that CARD9 is essential for the augmentation of TLR-mediated cytokine responses by TREM-1.

TLR-mediated MAPK activation requires CARD9

To explore the mechanism of CARD9- and Bcl-10-mediated TLR signaling, we analyzed signaling pathways 'downstream' of TLRs. Because *Card9*$^{-/-}$ BMDCs showed impaired NF-κB activation after stimulation of ITAM-associated receptors (**Fig. 4**), we first examined the activation of NF-κB after TLR stimulation. However, degradation by IκBα (data not shown) and the DNA-binding activity of the p65-containing NF-κB complex were not impaired much in the nuclei of *Card9*$^{-/-}$ BMDCs after stimulation with zymosan (**Fig. 7a**), LPS (**Fig. 7b**) or loxoribine (**Fig. 7c**). In contrast, *Card9*$^{-/-}$ BMDCs had apparent activation defects of the Jnk and p38 MAPKs at early time points after loxoribine stimulation (**Fig. 7d**), and throughout the time course after zymosan stimulation (**Fig. 7e**). These results suggest that CARD9 is involved in signaling for the activation of MAPKs but not of NF-κB in the 'downstream' pathway of TLRs.

A genetic study of mice deficient in the effector molecule RIP2 has shown that RIP2 regulates multiple signaling pathways 'downstream' of TLRs, including p38, Jnk, Erk and IKKs[36]. It has been shown that RIP2 is recruited to the TLR4 receptor complex and associates with the kinase IRAK1 and the adaptor molecule TRAF6 after LPS stimulation[37] and that IRAK1 also recruits Bcl-10 to the TLR4 signaling complex[38]. Moreover, it has been suggested that CARD9 directly associates with the receptor Nod2 and RIP2 and regulates Nod2-mediated activation of p38 and Jnk[39]. Given that the apparent defects in TLR-induced cytokine production by *Card9*$^{-/-}$ and *Bcl10*$^{-/-}$ BMDCs correlated with impaired activation of p38 and Jnk, we hypothesized that CARD9 may function in the signaling pathway mediated by IRAK1–RIP2–Bcl-10.

To determine whether CARD9 physically and functionally associates with RIP2 and/or IRAK1, we expressed these proteins together in HEK293T cells (**Fig. 7f**). There was direct association between RIP2 and IRAK1 (**Fig. 7f**, lane 3) and between RIP2 and Card9 (**Fig. 7f**, lane 4), as reported before[37,39]. When these three molecules were expressed together, they seemed to associate and form a complex (**Fig. 7f**, lane 5). Overexpression of IRAK1 alone did not induce the activation of MAPKs (**Fig. 7f**, lane 1). However, we detected activation of only Jnk (but not p38 and Erk) with the expression of CARD9 or RIP2 alone (**Fig. 7f**, lanes 2 and 3), which was synergistically augmented by the expression of CARD9 and RIP2 together (**Fig. 7f**, lane 4). Expression of the last two molecules together resulted in substantial activation of p38 and weak but notable Erk activation. The Erk activation was enhanced when IRAK1 was expressed together with CARD9 (**Fig. 7f**, lane 5). Notably, the synergistic activation of p38 and Jnk by RIP2 and CARD9 correlated with an increase in the phosphorylated form of Bcl-10 (**Supplementary Fig. 7** online). These collectively results suggest that after TLR stimulation, IRAK1, CARD9 and RIP2 may form a complex and thereby functionally cooperate to activate MAPKs and that Bcl-10 phosphorylation might be involved in regulating that function, which seems to be critical for TLR-induced production of TNF and IL-6 in DCs.

CARD9 is required for antibacterial defense

TLR-mediated responses of macrophages and DCs against bacterial components are crucial for antibacterial defense. It has been

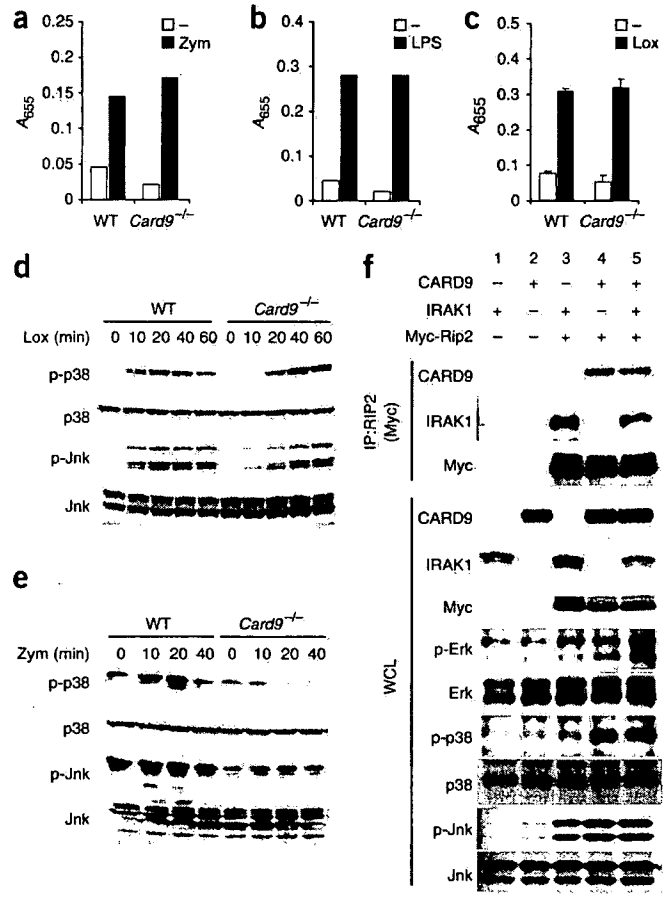

Figure 7 Impaired MAPK activation in TLR-stimulated *Card9*$^{-/-}$ DCs. (**a–c**) DNA-binding activity of NF-κB p65 in nuclear extracts from wild-type or *Card9*$^{-/-}$ BMDCs left unstimulated (–) or stimulated for 2 h with zymosan (Zym; 20 mg/ml; **a**), LPS (1 μg/ml; **b**) or loxoribine (Lox; 100 μM; **c**). (**d,e**) Immunoblot analysis of lysates of wild-type or *Card9*$^{-/-}$ BMDCs stimulated (time, above lanes) with loxoribine (100 μM; **d**) or zymosan (20 μg/ml; **e**). (**f**) Immunoblot analysis of HEK293T cells transfected with 0.5 μg of various combinations (above lanes) of expression plasmids encoding IRAK1, CARD9 or Myc-tagged RIP2; at 30 h after transfection, cell lysates were immunoprecipitated with anti-Myc and then the immunoprecipitates (IP) and lysates (WCL) were analyzed with antibodies specific for various proteins (left margin). Data are the mean + s.d. of two experiments (**a–c**) or are representative of two experiments (**d–f**).

demonstrated that TLR-mediated, MyD88-dependent signaling is essential for control of *Listeria monocytogenes* infection[40,41]. To examine the *in vivo* effect of CARD9 deficiency, we infected wild-type and *Card9*$^{-/-}$ mice with *L. monocytogenes* and then analyzed the bacterial burden of the infected organs at 3 d after the infection. We detected a significantly higher listerial burden in the infected spleens and livers of *Card9*$^{-/-}$ mice than in those from wild-type mice (**Fig. 8**), indicating that *Card9*$^{-/-}$ mice are more susceptible to *L. monocytogenes* infection than are wild-type mice. These *in vivo* results demonstrate that CARD9 is crucial for antibacterial defense, presumably through TLRs and their costimulating ITAM receptors. Our results collectively demonstrate that the activation of lymphoid and myeloid cells through ITAM receptors and TLRs are mediated by two different types of complexes containing Bcl-10–MALT1: lymphoid type (CARMA1–Bcl-10–MALT1) and myeloid type (CARD9–Bcl-10–MALT1), respectively (**Supplementary Fig. 8** online).

DISCUSSION

In vitro studies and *in vivo* genetic analyses of deficient mice have shown that the CBM complex, composed of CARMA1, Bcl-10 and MALT1, is essential for TCR- and BCR-mediated NF-κB activation. In addition, the CBM complex is involved in TLR4 and CD40 signaling in B cells[22,23,26,28]. As CARD9 was originally identified as a protein that interacts with Bcl-10 through its CARD and triggers NF-κB activation in a synergistic way with Bcl-10, we hypothesized that CARD9 could function in antigen receptor, TLR and/or CD40 signaling by interacting with Bcl-10. However, our results here have shown that CARD9 is dispensable for those receptor-mediated lymphocyte activation responses. That might be due in part to the lower expression of CARD9 in lymphoid cells than in myeloid cells.

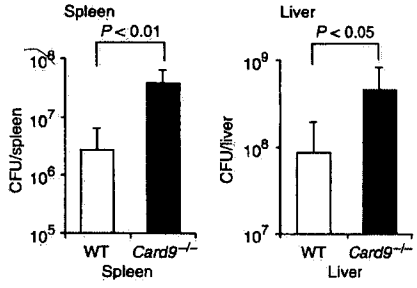

Figure 8 *Card9*$^{-/-}$ mice are more susceptible to *L. monocytogenes*. Quantification of colony-forming units (CFU) of *L. monocytogenes* in the spleens (left) and livers (right) of wild-type and *Card9*$^{-/-}$ mice infected intraperitoneally for 3 d with 5×10^5 *L. monocytogenes*. Data are the means + s.d. of groups of seven mice and are representative of two independent experiments.

Alternatively, perhaps it can be attributed to the structure of CARD9: whereas CARD9 is similar to CARMA1 in terms of the highly homologous CARD and the flanking coiled-coil domain at its amino terminus, CARMA1 but not CARD9 has the carboxy-terminal MAGUK 'signature' domains (PDZ, SH3 and Guk) required for CARMA1 signaling function[42,43]. In addition, the 'linker' region that connects the coiled-coil and PDZ domains of CARMA1 is short in CARD9 (ref. 29) and thus lacks the PKC phosphorylation sites that are critical for TCR- or BCR-mediated NF-κB activation[44,45]. Therefore, CARD9 might be incapable of coupling receptor-induced PKC activation to 'downstream' NF-κB activation despite having a Bcl-10-interacting CARD. Indeed, our data have confirmed that CARD9 deficiency did not affect DC activation induced by the direct PKC activator phorbol 12-myristate 13-acetate plus ionomycin. The unique structural characteristics of CARMA1 may generate the specificity of Bcl-10–MALT1–mediated NF-κB activation in antigen receptor signaling.

It has been established that Bcl-10–MALT1–mediated signaling is essential for NF-κB activation by TCRs and BCRs in lymphocytes and by FcεRI in mast cells. All of those receptors deliver signals by associating with signaling adaptors containing ITAMs. Our data now extend that knowledge regarding lymphocyte signaling to myeloid cells by demonstrating that Bcl-10 is also essential for NF-κB activation through various ITAM receptors expressed on myeloid cells. Our results collectively indicate a general principle that a Bcl-10–MALT1–containing complex is an essential mediator of NF-κB activation through ITAM receptors in immune cells.

Based on analyses of the function of the CBM complex in antigen receptor signaling, we assumed that the formation of a complex of

Bcl-10–MALT1 with CARMA1 would be a prerequisite for 'downstream' IKK activation even in myeloid cells. However, we found that CARMA1 was dispensable for Bcl-10-mediated ITAM receptor signaling in myeloid cells and instead found that CARD9 was the essential molecule mediating this pathway. Thus, we propose that the CARD9–Bcl10–MALT1 complex is responsible for mediating ITAM receptor signaling in myeloid cells and we further stipulate that the classical CBM (CARMA1–Bcl-10–MALT1) complex is the lymphoid complex, whereas the newly identified CARD9–Bcl-10–MALT1 complex is the myeloid complex.

The production of TNF, IL-2, IL-6 and IL-12 after the stimulation of FcRγ- or DAP12-associated receptors, including FcγR, OSCAR, TREM-1 and MAIR-II, was impaired in *Card9*$^{-/-}$ BMDCs, indicating a critical function for the myeloid CARD9–Bcl-10–MALT1 complex in ITAM-associated receptor–mediated cytokine expression. However, IL-12 production mediated by the ITAM-bearing receptor dectin-1 in response to zymosan was not affected by CARD9 deficiency, although the production of IL-2, IL-6 and TNF was impaired. Because zymosan has been shown to stimulate both the dectin-1–Syk and TLR-MyD88 signaling pathways for cytokine production, we examined in which of those pathways CARD9 is involved; to do this we simultaneously analyzed DCs from *Card9*$^{-/-}$, *Myd88*$^{-/-}$ and *Clec7a*$^{-/-}$ mice. We found that, consistent with published reports[31,32], zymosan-induced cytokine production depended mainly on the TLR-MyD88 but not the dectin-1–Syk pathway. However, stimulation of DCs from these knockout mice with NaClO-oxidized zymosan, a purified β-glucan agent that does not activate the TLR-MyD88 pathway, showed that CARD9 was essential for the production of cytokines, including IL-12, by dectin-1 signaling . Our data collectively establish the idea that CARD9 generally functions 'downstream' of ITAM-bearing adaptors and receptors and is essential for the induction of all cytokine expression by regulating NF-κB activation in myeloid cells. The data showing that CARD9 functions in the TLR-MyD88 pathway also led us to consider that CARD9 is involved in general TLR-MyD88 signaling.

Studies have shown that Bcl-10 is involved in TLR4 signaling in B cells[28] and that the RNA interference–mediated depletion of Bcl-10 in a macrophage cell line results in the impairment of LPS-induced NF-κB activation *in vitro*[46]. Our results have provided genetic evidence that Bcl-10 is involved in various other TLR signaling pathways in addition to TLR4 signaling in DCs. CARMA1 is also involved in TLR4 signaling in B cells by regulating Jnk activation[22,23,26,28]. Although DCs and B cells have similarly high expression of CARMA1, we found that CARMA1 deficiency did not affect TLR signaling in DCs, but instead that the Bcl-10-mediated TLR pathway was controlled by CARD9, similar to ITAM receptor signaling. Those findings indicate that the myeloid CARD9–Bcl-10–MALT1 complex functions not only in ITAM receptor–mediated signaling pathways but also in TLR-signaling pathways in DCs.

However, we also found that 'downstream' signaling events controlled by the myeloid CARD9–Bcl-10–MALT1 complex were different for ITAM receptors and TLRs. Whereas the myeloid CARD9–Bcl-10–MALT1 complex controlled the activation of NF-κB but not MAPKs in ITAM-receptor signaling, in TLR signaling it controlled the activation of MAPKs but not NF-κB. Although zymosan-induced activation of p38 and Jnk was lower in *Card9*$^{-/-}$ mice, loxoribine-induced activation of these kinases were not impaired much, which correlated with delayed onset of activation. Such an activation delay might affect proper induction of cytokine genes, as *Myd88*$^{-/-}$ macrophages, which show signaling abnormality only with delayed activation of NF-κB and MAPKs in response to LPS, have an almost complete lack of cytokine production[47].

Our finding that *Card9*$^{-/-}$ BMDCs had impaired production of TNF and IL-6 but not of IL-12 led to our model that two different pathways operate 'downstream' of TLRs in DCs: the myeloid CARD9–Bcl-10–MALT1 complex–dependent pathway we have outlined here, which contributes mainly to IL-6 and TNF gene expression through MAPK activation; and a distinct myeloid CARD9–Bcl-10–MALT1 complex–independent pathway, which probably depends on MyD88 and/or the adaptor TRIF[48]. The latter pathway may trigger *Il12* expression through NF-κB activation but is not enough to induce TNF and IL-6 production. Similar signal dependency for the expression of a different set of cytokine genes has been reported for CD40 signaling in DCs[49]. After CD40 stimulation, activation of NF-κB but not p38 is required for IL-12 production, whereas activation of p38 but not NF-κB is required for IL-6 production. These data suggest that there is epigenetically differential regulation of TNF and IL-6 versus IL-12 through the use of MAPK or NF-κB activation.

Our *in vitro* overexpression study suggested that the signaling pathway dependent on the myeloid CARD9–Bcl-10–MALT1 complex seems to be mediated by IRAK1- and RIP2-mediated Bcl-10 phosphorylation and that it induces 'downstream' MAPK activation. Support for the idea of a functional IRAK1–RIP2–CARD9–Bcl-10–dependent pathway is found in the similar phenotypes of mice deficient in CARD9 or RIP2, in which myeloid cells are hyporesponsive to various TLR stimuli[36] and hence the mice become susceptible to *L. monocytogenes* infection[50]. The finding that *Card9*$^{-/-}$ DCs had impaired cytokine responses to stimulation by a variety of MyD88-mediated TLR ligands may be explained by the molecular 'linkage' of IRAK1 and CARD9, as IRAK1 is recruited to the TLR complex through MyD88–adaptor molecule TIRAP after TLR stimulation, after which IRAK1 associates with TRAF6 to further activate 'downstream' IKK complexes and MAPKs[38]. Our data have shown that *Card9*$^{-/-}$ BMDCs had impaired cytokine response after stimulation with poly(I:C), a ligand for TLR3 that transduces signals by association with TRIF independently of MyD88. It has been reported that *Card9*$^{-/-}$ macrophages have impaired MAPK activation in response to *in vitro* infection by the double-stranded RNA virus vascular stomatitis virus, suggesting that CARD9 controls signaling through RIG-I, which senses intracellular double-stranded RNA[51]. Thus, the impaired cytokine response to poly(I:C) in *Card9*$^{-/-}$ BMDCs might be attributed to impairment of RIG-I-mediated signaling. Alternatively, a common 'downstream' molecule may couple both MyD88-mediated and TRIF-mediated signaling to CARD9-regulated MAPK activation. However, the exact molecular connection leading to MAPK activation 'downstream' of CARD9 remains to be addressed.

The finding that DCs but not macrophages from *Card9*$^{-/-}$ mice had impaired TLR signaling suggests that cytokine production mediated by the myeloid CARD9–Bcl-10–MALT1 complex is induced in a cell type–specific way. Similar cell type specificity of pattern-recognition receptor signaling has been reported for RIG-I-mediated production of type I interferon[52]. The exact mechanistic basis for the function of the myeloid CARD9–Bcl-10–MALT1 complex 'downstream' of specific TLRs in DCs remains to be determined.

In conclusion, we have shown here that the CARD9–Bcl-10 complex was critical for ITAM receptor–mediated myeloid cell activation and was also required for TLR-induced cytokine responses in DCs. In contrast, CARMA1 was dispensable for those receptor-mediated myeloid cell activation responses. Our results have provided genetic evidence of essential and nonredundant functions for the CBM complex in lymphoid cells and the CARD9–Bcl-10–MALT1 complex in myeloid cells in the ITAM receptor–mediated and TLR-mediated cellular activation and immune responses. Based on our findings,

therapeutic approaches targeting either the lymphoid CBM complex or the myeloid CARD9–Bcl-10–MALT1 complex might provide a strategy for specifically modulating lymphoid versus myeloid cells for the activation or inhibition of their functional responses.

METHODS

Mice and gene targeting of *Card9*. *Card11*$^{-/-}$, *Bcl10*$^{-/-}$, *Myd88*$^{-/-}$ and *Clec7a*$^{-/-}$ mice were generated before[26,28,47]. C57BL/6 and BALB/c mice were from Clea Japan. All mice were maintained at the animal facility of the RIKEN Research Center for Allergy and Immunology according to institutional guidelines.

Card9 was isolated from genomic DNA extracted from embryonic stem cells (R1) by PCR. The targeting vector was constructed by replacement of exon 2, including the ATG start codon and the CARD-encoding region, with a neomycin-resistance gene cassette, and insertion of a fragment of the gene encoding diphtheria toxin A driven by the phosphoglycerate kinase promoter into the genomic fragment (for negative selection). After the targeting vector was transfected into embryonic stem cells, G418-resistant colonies were selected, screened by PCR and further confirmed by Southern blot analysis. Germline chimeras were generated by aggregation. The resulting male chimeras were backcrossed with C57BL/6J females, and germline transmission in F_1 *Card9*$^{+/-}$ mice was verified by Southern blot analysis. *Card9*$^{+/-}$ mice that were backcrossed for at least five generations with C57BL/6J mice were intercrossed to obtain *Card9*$^{-/-}$ mice with the C57BL/6 genetic background. *Card9*$^{-/-}$ and C57BL/6J control (wild-type) mice were used throughout the experiments.

Antibodies, transfection, plasmids and reagents. Antibodies specific for MAIR-II (ref. 14) and OSCAR[53] have been described. Antibodies specific for Erk (9102), phosphorylated Erk (9101), p38 (9212), phosphorylated p38 (9102), Jnk (9258), phosphorylated Jnk (9251) and IκBα (9242) were from Cell Signaling Technology; anti-actin (sc-8432), anti-c-Myc (sc-789), anti-IRAK1 (sc-7883) and anti-Bcl-10 (331.3) were from Santa Cruz Biotechnology; anti-RIP2 (PX092) was from Cell Science; anti-TREM-1 (174031) was from R&D Systems; anti-CD40 (3/23) and anti-CD16 (2.4G2) were from BD Bioscience; and anti-Flag (M2) was from Sigma. The expression plasmid pCMV-flag–Card9 was created by PCR. Expression plasmids encoding IRAK-1 (pCMV-SPORT–IRAK1), CARD9 (pCMV-Flag–Card9), and Myc-tagged RIP2 (pcDNA3-Myc–RIP2) were also used. Rabbit anti-CARD9 serum was raised against a synthetic peptide corresponding to amino acids 520–536 of mouse CARD9 (CGDRGNTTGSDNTDTEGS). Zymosan, LPS and poly(I:C) (polyinosinic-polycytidylic acid) were from Sigma; phosphorothioate-stabilized CpG DNA (ODN 1668; TCCATGACGTTCCTGATGCT) was from Hokkaido System Science; and Pam_3CSK_4 (*N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-[*R*]-Cys-[*S*]-Ser$_1$-[*S*]-Lys4 trihydrochloride), flagellin, loxoribine, R837 (imiquimod), R848 (resiquimod) and zymosan were from Invivogen. NaClO-oxidized zymosan was prepared as described[35].

For transfection, HEK293T cells (1×10^5) were plated on 24-well culture dishes and were transfected with expression plasmids by lipofection with Lipofectamine LTX according to the manufacturer's instructions (Invitrogen). Lysates of transfected cells were separated by SDS-PAGE at 30 h after transfection and then exogeneously expressed and endogeneous proteins were analyzed by immunoblot.

Cell preparation and flow cytometry. Whole T cells from lymph nodes or spleen were purified by magnetic-activated cell sorting (MACS; Miltenyi Biotec) for removal of B220$^+$, NK1.1$^+$, Mac-1$^+$ and Gr-1$^+$ cells; the purity was over 95% CD3$^+$ by flow cytometry. CD4$^+$ or CD8$^+$ T cells were isolated from purified whole T cell samples by positive sorting with MACS. Splenic B cells were purified by MACS for the removal of CD3$^+$, NK1.1$^+$, Mac-1$^+$ and Gr-1$^+$ cells; the purity was over 95% B220$^+$ by flow cytometry. BMDCs or bone marrow–derived macrophages were prepared by culture of bone marrow cells for 5–10 d with granulocyte-macrophage colony-stimulating factor (20 ng/ml; Peprotech) or for 3–5 d with macrophage colony-stimulating factor (25 ng/ml; Peprotech), respectively. Thioglycolate-induced peritoneal macrophages were prepared as described[32].

For flow cytometry, single-cell suspensions of thymus, lymph node, bone marrow and spleen were stained with fluorescein isothiocyanate–, phycoerythrin–, allophycocyanin- or biotin-conjugated antibodies. Biotinylated antibodies were visualized with streptavidin–peridinine chlorophyll protein (BD Pharmingen). Antibodies used were to B220, IgM, IgD, CD21, CD23, CD4, CD8, CD3, CD25, CD44 and CD69. Stained cells were analyzed with a FACSCalibur (Becton Dickinson) and CellQuest software (BD Biosciences).

Immunoassays, real-time PCR and NF-κB DNA binding. BMDCs were stimulated with anti-CD16 (2.4G2; Pharmingen) followed by crosslinking with anti–rat IgG F(ab')$_2$ (112-006-072; Jackson ImmunoResearch) or with 100 μM loxoribine (Invivogen). After incubation at 37 °C for various periods of time, cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% (vol/vol) Triton X-100, 20 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitors). Cell lysates were separated by SDS-PAGE and proteins were transferred onto polyvinyldifluoride membranes. Membranes were incubated with antibodies to Erk, phosphorylated Erk, p38, phosphorylated p-38, Jnk, phosphorylated Jnk, IκBα, actin, c-Myc, Flag and Bcl-10 and then with horseradish peroxidase–conjugated secondary antibodies, followed by development with the ECL detection system (Amersham Pharmacia). Band intensities were quantified with a LAS-3000 imaging system (FUJIFILM).

For real-time PCR, total RNA was extracted from cells with TRIzol reagent (Invitrogen). For cDNA synthesis, total RNA was reverse-transcribed with SuperScript II and random hexamers according to the manufacturer's instructions (Life Technologies). The cDNA was analyzed quantitatively for expression of genes with the quantitative PCR system iCycler (BIO-RAD). Primer sequences were as follows: mouse *Card9*, forward, 5'-CCCGATGATGAGGAG CAG-3', and reverse, 5'-AAGCCACGTAGCCCTTGT-3'; and *Il6* forward, 5'-ACAACCACGGCCTTCCCTACTT-3', and reverse, 5'-CACGATTTCCCAGA GAACATGTG-3'.

For analysis of NF-κB activity, BMDCs were stimulated for 8 h with immobilized anti-OSCAR (10 μg/ml), mouse γ-globulin (10 μg/ml) or LPS (1 μg/ml). Nuclear extracts were prepared from the stimulated cells and the binding activity of NF-κB subunit p65 in the extracts was measured with a Mercury TransFactor kit (Clontech).

***In vitro* functional analyses of T cells, B cells, DCs and macrophages.** For proliferation assays, T cells were stimulated with anti-CD3ε (145-2C11; Pharmingen), anti-CD28 (37.51; Pharmingen), phorbol 12-myristate 13-acetate (10 ng/ml) plus ionomycin (1 μM), and staphylococcal enterotoxin B (5–500 ng/ml; SC BioScience) together with irradiated syngeneic spleen cells. Purified B cells were stimulated with anti–IgM F(ab')$_2$ (115-006-020; Jackson ImmunoResearch), anti-CD40, recombinant mouse IL-4 (R&D Systems), LPS and CpG DNA. After 2 or 3 d, cultures were pulsed for 8 h with 1 μCi [^{3}H]thymidine (Amersham) and collected. [^{3}H]thymidine incorporation was measured with a Microbeta (Perkin-Elmer) or Matrix 96 (Packard). For cytokine production assays, supernatants of CD4$^+$ and CD8$^+$ T cells were assayed in triplicate by ELISA (R&D Systems) for IFN-γ production. For allogenic T cell responses, purified T cells (5×10^4) from C57BL/6 mice were cultured for 48 h with irradiated (30 Gy) spleen cells (2.5×10^5) from BALB/c mice and [^{3}H]thymidine incorporation was measured in a similar way.

BMDCs or macrophages were stimulated with immobilized anti-CD16, anti-TREM-1, anti-OSCAR, anti-MAIR-II or isotype control rat IgG in the presence of soluble mouse γ-globulin (10 μg/ml) as an FcR blocker or with zymosan. IL-2, TNF, IL-6 and IL-12 in culture supernatants were analyzed with ELISA kits (BD Bioscience).

***In vivo* antibody responses.** Mice were immunized intraperitoneally with 100 μg DNP-KLH (LSL) adsorbed to alum (Sigma). At 24 d after immunization, DNP-specific serum titers were determined by ELISA (Southern Biotechnology Associates) in plates coated with DNP–human serum albumin (LSL).

Listeria infection. *L. monocytogenes* strain EGD was used. C57BL/6 mice were infected intraperitoneally with one fifth of the 50% lethal dose (5×10^5 colony-forming units per mouse) of *L. monocytogenes* in 0.2 ml of PBS. Bacterial burdens in the spleen and liver were determined 3 d after infection by plating of tenfold serial dilutions of organ homogenates on tryptic soy agar plates. Colonies

were counted after 24 h of incubation at 37 °C. Experiments were done according to institutional guidelines and were approved by institutional committees.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

H.H. designed and did experiments and wrote the paper; C.I., A.T. and H.K. made knockout mice; T.I. did experiments; L.X., S.W.M., S.S. and Y.I. provided knockout mice; M.I., T.T., A.S. and N.O. provided antibodies or reagents; H.Y. and J.M.P. contributed to discussions; and T.S. designed experiments and wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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