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Differential Use of CARD9 by Dectin-1 in Macrophages and Dendritic Cells[1]

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The pattern recognition receptors TLR2 and Dectin-1 play key roles in coordinating the responses of macrophages and dendritic cells (DC) to fungi. Induction of proinflammatory cytokines is instructed by signals from both TLR2 and Dectin-1. A recent report identified a role for CARD9 in innate anti-fungal responses, demonstrating CARD9-Bcl10-mediated activation of NF-κB and proinflammatory cytokine induction in murine bone marrow-derived DC stimulated via Dectin-1. We now report that Dectin-1-CARD9 signals fail to activate NF-κB and drive TNF-α induction in murine bone marrow-derived macrophages. However, priming of bone marrow-derived macrophages with GM-CSF or IFN-γ permits Dectin-1-CARD9-mediated TNF-α induction. Analysis of other macrophage/DC populations revealed further variation in the ability of Dectin-1-CARD9 signaling to drive TNF-α production. Resident peritoneal cells and alveolar macrophages produce TNF-α upon Dectin-1 ligation, while thioglycollate-elicited peritoneal macrophages and Flt3L-derived DC do not. We present data demonstrating that CARD9 is recruited to phagosomes via its CARD domain where it enhances TLR-induced cytokine production even in cells in which Dectin-1 is insufficient to drive cytokine production. In such cells, Dectin-1, CARD9, and Bcl10 levels are not limiting, and data indicate that these cells express additional factors that restrict Dectin-1-CARD9 signaling for TNF-α induction. ***The Journal of Immunology,*** **2009, 182: 1146–1154.**

The innate immune response is controlled by integrating signals from multiple receptors at the surface, in the phagosome, or in the cytosol of myeloid phagocytes, including TLRs, C-type lectins, scavenger receptors, and nucleotide-binding oligomerization domain-containing proteins (1, 2). Initiation of an immune response against pathogenic fungi by macrophages and dendritic cells (DC)[3] involves collaboration between TLR2 and the C-type lectin Dectin-1, and has been extensively characterized by us and others using zymosan (*Saccharomyces cerevisiae* yeast cell wall preparation), as well as live pathogenic fungi such as *Candida albicans*, *Aspergillus fumigatus*, and *Pneumocystis carinii* (reviewed in Ref. 3). Two groups recently demonstrated the importance of Dectin-1 for in vivo antifungal immunity using Dectin-1 knockout mice (4, 5).

Dectin-1 recognizes β-glucans in zymosan and the cell walls of live pathogenic fungi, and is a key phagocytic receptor for fungal internalization (reviewed in Ref. 3). It has an ITAM-like motif in its intracellular tail, through which it signals via Src and Syk kinases to induce reactive oxygen species production (6). TLR2 signals via the adaptor MyD88 to trigger NF-κB activation and cytokine production. Dectin-1 signaling contributes to the transcriptional response by promoting TLR-mediated NF-κB activation, as well as itself triggering activation of NFAT transcription factors (7–9). Both receptors play key roles in the induction of a variety of inflammatory mediators (reviewed in Ref. 3), thereby orchestrating the recruitment and activation of other immune cells and driving the development of adaptive immunity. For example, recent data indicated that DC matured with a Dectin-1 ligand instruct the differentiation of Th1 and Th17 responses (10), while another report demonstrated that coligation of Dectin-1 and TLR2 results in the induction of regulatory T cells and immunological tolerance (11).

In addition to its role in promoting TLR-induced NF-κB activation, a recent report indicated that Dectin-1 signals can directly activate NF-κB in DC via the caspase recruitment domain (CARD)-containing adaptor protein CARD9 (12). Gross et al. (12) reported that CARD9 knockout mice are significantly more susceptible to *Candida albicans* infection than their wild-type littermates. Furthermore, CARD9-deficient DC displayed defective NF-κB activation and impaired cytokine responses to zymosan stimulation. In addition to CARD9, activation of NF-κB in zymosan-stimulated DC required Bcl10 and MALT1. Bcl10 and MALT1 also mediate NF-κB activation downstream of lymphocyte Ag receptors by association with CARMA1/CARD11, a CARD-containing adaptor structurally related to CARD9 (13–17). Because TLR2 responses were unaffected by CARD9 deletion, the authors proposed that CARD9-Bcl10-MALT1 signaling mediates NF-κB activation downstream of Dectin-1.

In contrast, our previous studies in macrophages have indicated that while Dectin-1 signals collaborate with TLR2 signals to enhance TLR2-mediated NF-κB activation and proinflammatory cytokine

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[3] Abbreviations used in this paper: DC, dendritic cell; CARD, caspase recruitment domain; bmDC, bone marrow-derived DC; bmM, bone marrow-derived macrophage; SBPc, streptavidin binding peptide; TEPM, thioglycollate-elicited peritoneal macrophage; RPC, resident peritoneal cell; AvM, alveolar macrophage; SRE, serum response element; TRITC, tetramethylrhodamine isothiocyanate.

production, Dectin-1 signals alone do not appear to be sufficient for NF-κB activation and cytokine induction (8). In this study, we report that although Dectin-1 signals via CARD9 and Bcl10 to induce NF-κB activation and TNF-α production in bone marrow-derived DC (bmDC), this pathway does not activate NF-κB or induce TNF-α in bone marrow-derived macrophages (bmM). The data indicate that CARD9 in macrophages is recruited to phagosomes where it coordinates signaling to p38 MAPK, even in the absence of a connection to the NF-κB pathway. However, priming of bmM with GM-CSF or IFN-γ permits Dectin-1-CARD9-induced TNF-α production. Furthermore, Dectin-1 ligation directly triggers TNF-α production by resident peritoneal cells and alveolar macrophages, but not by thioglycollate-elicited peritoneal macrophages or Flt3L-derived DC. Thus, the contribution of Dectin-1 signals to proinflammatory cytokine induction is variable in different macrophage/DC populations. Finally, we present evidence that an inhibitor restricts Dectin-1-CARD9 signaling in certain myeloid cells.

Materials and Methods

Reagents

Pam_3CSK_4 and *Salmonella minnesota* LPS were from InvivoGen and zymosan was from Sigma-Aldrich. Depleted zymosan was prepared by boiling 250 μg zymosan in 1 ml 10 M sodium hydroxide for 30 min and washing three times with sterile PBS (8). Recombinant growth factors and IFN-γ were from PeproTech.

Mice and cells

HEK293 cells were maintained in complete DMEM (10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine; Mediatech). RAW264.7 murine macrophage cell lines were cultured in complete RPMI 1640. RAW264.7 cells stably expressing streptavidin binding peptide (SBPc)-tagged Dectin-1 were described previously (6).

Wild-type C57BL/6 mice were maintained at Cedars-Sinai Medical Center, Los Angeles, CA. Wild-type and CARD9 knockout mice (18) were maintained at University of Texas, MD Anderson Cancer Center, Houston, TX. bmM and bmDC were prepared from mouse femurs by culture of bone marrow cells for 6 days in complete RPMI 1640 (10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine; Mediatech) supplemented with 50 ng/ml recombinant human M-CSF (bmM) or 10 ng/ml recombinant murine GM-CSF (bmDC). Flow cytometry of adherent cells at day 6 verified that M-CSF cultures yielded a single population of $CD11b^+$ $F4/80^+$ cells, and GM-CSF cultures yielded a single population of $CD11c^+$ cells; neither preparation was contaminated with $7/4^+$ neutrophils (data not shown).

Peritoneal macrophages were elicited by intraperitoneal injection of thioglycollate for 3 days. Thioglycollate-elicited peritoneal macrophages (TEPM) and resident peritoneal cells (RPC) were harvested by peritoneal lavage with PBS, and adherent cells were enriched by plastic adherence for 2 h. Alveolar macrophages (AvM) were obtained by bronchoalveolar lavage using a 24G catheter to wash out the lungs 10 times with 1 ml PBS plus 2 mM EDTA per wash. Flt3L-derived DC were obtained by 6-day culture of bone marrow progenitors with 10 ng/ml Flt3L.

Plasmids and cell line generation

The expression vector pEF6-CARD9-V5 used for HEK293 cell transfection was generated by cloning the coding region for murine CARD9, amplified from bmM cDNA, into the pEF6/V5-His expression vector (Invitrogen) such that it is tagged at its C terminus with the V5-His epitope. The expression vector pEF6-SBPc-Bcl10 was generated by cloning the coding region for murine Bcl10, amplified from bmM cDNA, into pEF6 such that it is tagged at its N terminus with an epitope (SBPc) combining sequences of protein C recognized by the HPC4 mAb (Amersham Biosciences) and the streptavidin binding peptide (6). The expression vector pEF6-Dectin-SBPc was generated by cloning the SBPc-tagged murine Dectin-1 (6) into pEF6. For transient expression of Dectin-1 in bmM, murine Dectin-1-SBPc was cloned into the pMAX-GFP (Amaxa) such that it is also tagged at its N terminus with GFP.

For retroviral expression of CARD9, used to generate RAW264.7 macrophages stably expressing CARD9 for immunofluorescence assays, the coding region for murine CARD9 was cloned into the retroviral expression vector pMXs-IRES-puro (pMXsip; Ref. 19) such that it is tagged at its C terminus with the SBPc tag. Shorter constructs consisting of the amino terminal CARD (aa 1–110) and the c-terminal coiled-coil domain (aa 100–536) similarly tagged were also generated. RAW264.7 macrophages stably expressing these constructs were generated by retroviral infection as previously described (6, 20).

HEK293 cell luciferase reporter assays

HEK 293 cell reporter assays were performed as described previously (8) using the indicated plasmids. In brief, HEK293 cells (American Type Culture Collection no. CRL-1573) in 96-well plates were transfected as indicated using Lipofectamine 2000 (Invitrogen) with 17 ng each of an NF-κB reporter plasmid (ELAM-luciferase) and a CMV-βgal reporter plasmid, 17 ng pEF6-SBPc-Dectin-1, 3 ng pEF6-CARD9-V5, and 3 ng pEF6-SBPc-Bcl10 per well. The following day, the cells were stimulated with the indicated ligands for 4 h, and luciferase activity was measured (Luciferase Assay System, Promega). Values were normalized for transfection efficiency according to β-galactosidase expression (β-Gal Assay Kit, Stratagene, Cedar Creek, TX). In some experiments an serum response element (SRE)-luciferase reporter plasmid (pSRE-luciferase (21), provided by Dr. Walter Born, University of Zurich, Zurich, Switzerland) was used in place of the NF-κB reporter plasmid.

Immunofluorescence microscopy

RAW264.7 macrophages stably expressing SBPc-tagged CARD9, CARD domain, or coiled-coil domain were coated on to coverslips and treated with 100 μg/ml zymosan particles for 10 min before fixing with 10% formaldehyde. Following permeabilization with ice-cold acetone, coverslips were blocked with PBS containing 5% FBS and 0.1% OVA, and stained with an Ab against the SBPc tag (HPC4, Amersham Biociences) and tetramethylrhodamine isothiocyanate (TRITC)-phalloidin to detect actin. Following staining, coverslips were washed, mounted, and viewed using a confocal microscope.

Phagocytosis assay

TRITC-labeled zymosan particles were fed to macrophages or DC for 15 min, with brief centrifugation upon zymosan addition to ensure particle contact with the cells. Cells were lifted in PBS containing 1 mM EDTA, 1 mM sodium azide, and 2.4 U/ml proteinase K (to remove bound but uninternalized zymosan particles) before analysis by flow cytometry.

ELISA

TNF-α levels in culture supernatants were assayed using ELISA kits from BD Biosciences according to the manufacturer's instructions.

Measurement of reactive oxygen production

The production of reactive oxygen species was assayed by luminol-ECL as described previously (6).

Western blotting

Whole cell lysates or phagosome preparations were probed by Western blotting using the NuPAGE system from Invitrogen according to manufacturer's instructions, and Abs against I-κB, total Erk1/2, dual phosphorylated p38 and total p38 MAP kinases from Cell Signaling Technologies, CARD9 from eBioscience, and Bcl10, LAMP1, GAPDH, and β-actin from Santa Cruz Biotechnology.

EMSA

Nuclear extracts were prepared using a kit from Active Motif. NF-κB binding to a biotinylated probe comprising an NF-κB consensus promoter sequence (5′-ATCAGGGACTTTCCGCTGGGGACTTTCCG-3′) was assessed using a LightShift Chemiluminescent EMSA kit from Pierce according to the manufacturer's instructions.

Flow cytometry

Dectin-1 surface expression was assessed by flow cytometry using FITC- and PE-conjugated monoclonal anti-Dectin-1 Abs (2A11) from AbD Serotec. PE-conjugated Abs against CD11c and TLR2, FITC-conjugated Abs against CD11b and CD11c, and FITC- and PE-conjugated isotype controls were from BD Biosciences. For measurement of intracellular TNF-α, cells were stimulated in the presence of brefeldin A (BioLegend) for 4 h. Cells were lifted with PBS containing 1 mM EDTA and 1 mM sodium azide, and Fc receptors were blocked with 2.4G2 cell supernatant for 5 min before fixing for 1 h with 2.5% formalin and 0.05% saponin in PBS. Cells were then incubated for 30 min with PE-conjugated anti-TNF-α, and FITC-conjugated anti-CD11b in permeabilization buffer (0.1% saponin, 1% FBS, 1 mM sodium azide in PBS), washed and analyzed by flow cytometry,

gating for CD11b$^+$ RPC and TEPM, and CD11b$^-$ AvM. For some experiments, bmM were transfected with pMAX-GFP or pMAX-GFP-Dectin-1-SBPc using the Nucleofector II (Amaxa) as per the manufacturer's instructions, and cells were plated into 24-well plates. The following day, the cells were given fresh medium before assessment of Dectin-1 expression and particle-induced TNF-α production.

Statistical analysis

Statistical analysis was performed using a Student's *t* test. Triplicate experimental samples were compared with unstimulated or control samples.

Results

Dectin-1 signals can activate NF-κB directly in a CARD9/Bcl10-dependent manner

In their recent report, Gross et al. (12) demonstrated that reconstitution of HEK293 cells with Dectin-1 in combination with CARD9 and Bcl10 is sufficient to permit NF-κB activation following exposure to zymosan. We further investigated Dectin-1-mediated NF-κB activation in this system to rule out the possible contribution of signals derived from other receptors involved in zymosan recognition. Consistent with the reported data from us and others (8, 12), transient transfection of HEK293 cells with Dectin-1 alone was not sufficient for NF-κB activation following zymosan stimulation. This is despite the fact that Dectin-1 clearly signals in these cells because Dectin-1 transfection of HEK293 cells is sufficient to trigger phagocytosis of zymosan particles (6). However, cotransfection of Dectin-1 with CARD9 and Bcl10 was sufficient to enable robust activation of an NF-κB-driven luciferase reporter (Fig. 1, *A* and *B*). Both CARD9 and Bcl10 were necessary to permit reporter activation (Fig. 1*A*). As observed by Gross et al. (12), overexpression of CARD9 with Bcl10 was often sufficient to activate NF-κB in the absence of Dectin-1 (increased background, Fig. 1*B*). Plasmid doses were therefore titrated to minimize the background.

We also observed CARD9-Bcl10-dependent NF-κB activation when Dectin-1-transfected cells were treated with β-glucan particles prepared by boiling zymosan particles in hot alkali (depleted zymosan; Fig. 1*B*), which we have previously demonstrated destroys the TLR ligands without affecting their ability to signal via Dectin-1 (8, 9). To confirm that Dectin-1 signals alone are sufficient to activate NF-κB via CARD9-Bcl10, we directly cross-linked SBPc-tagged Dectin-1 with streptavidin-coated beads, which we have previously used extensively to study Dectin-1 signaling (6, 9), and observed NF-κB activation only in HEK293 cells transfected with SBPc-Dectin-1, CARD9, and Bcl10 (Fig. 1*B*).

Dectin-1 activates additional transcriptional responses in addition to NF-κB. We have previously reported that Dectin-1 activates NFAT (9). However, Dectin-1 signaling to NFAT cannot be reconstituted in HEK293 cells with or without CARD9/Bcl10 (data not shown), suggesting that additional macrophage/DC factors are required to enable this signaling pathway. In contrast, zymosan, depleted zymosan and streptavidin-coated beads all stimulated the activity of a luciferase reporter driven by the binding of serum response factor to the SRE-luciferase in SBPc-Dectin-1-transfected HEK293 cells, and this effect was neither dependent on nor influenced by cotransfection with CARD9 and Bcl10 (Fig. 1*C*). Taken together, the above data demonstrate that Dectin-1 signals transduced by CARD9 and Bcl10 can trigger NF-κB activation, but that CARD9 is not required for all transcriptional responses triggered by Dectin-1 ligation.

CARD9 is recruited to Dectin-1 phagosomes, but is not required for Dectin-1-mediated phagocytosis

Because CARD9 can participate in signaling to NF-κB downstream of Dectin-1, we explored whether there is evidence that

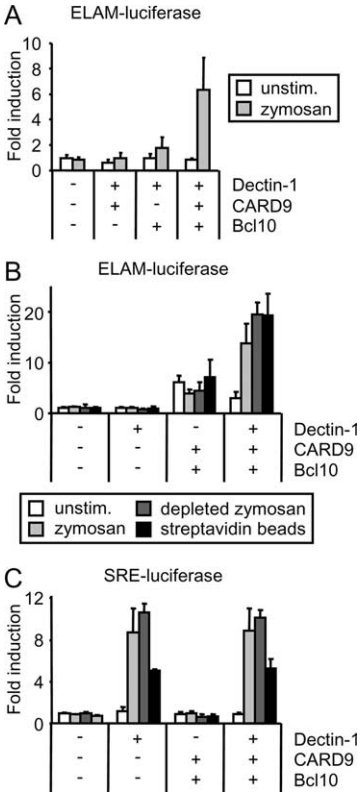

FIGURE 1. Dectin-1 signals via CARD9 and Bcl10 to activate NF-κB. HEK293 cells were transfected with ELAM- (*A* and *B*) or SRE- (*C*) firefly luciferase and CMV-β-galactosidase reporters (*A–C*), as well as plasmids encoding CARD9, Bcl10, and streptavidin binding peptide (SBPc)-tagged Dectin-1, as indicated. The following day cells were stimulated with 100 μg/ml zymosan or depleted zymosan, or 1:60 streptavidin beads for 4 h. ELAM/SRE-driven luciferase reporter activity was normalized to β-galactosidase activity and expressed as the mean plus SD of triplicate culture.

Dectin-1 signals to CARD9 in macrophages. Following binding of yeast particles by Dectin-1, actin polymerization coordinates yeast internalization by phagocytosis, a process which is dependent on Dectin-1 signaling (22). We therefore investigated whether CARD9 is recruited to Dectin-1 phagosomes. Using confocal immunofluorescence microscopy, we observed that an SBPc-tagged form of CARD9 (Fig. 2*A*) stably expressed in RAW264.7 macrophages was found diffusely in the cytosol (data not shown). Upon exposure to zymosan, CARD9 was strongly recruited to phagosomes (Fig. 2*B*, arrows). Upon complete internalization of the particles, phagosomal staining was reduced or lost completely (Fig. 2*B*, arrowheads), corresponding with the loss of actin from the maturing phagosome.

CARD9 comprises an N-terminal CARD domain and a C-terminal coiled-coil domain (Fig. 2*A*), and we therefore examined which domain is responsible for phagosome recruitment. We found that a tagged version of the CARD domain alone was sufficient to reproduce the phagosome recruitment of the full-length protein (Fig. 2*B*, arrows). In contrast, the coiled-coil domain formed aggregates in the cytosol and was not recruited to phagosomes (Fig. 2*B*). Although we cannot exclude a role for the coiled-coil domain in promoting phagosomal localization, the data suggest that the CARD domain is sufficient to target the protein to Dectin-1 phagosomes.

Because CARD9 is found on Dectin-1 phagosomes, we investigated whether CARD9 recruitment and signaling are required for

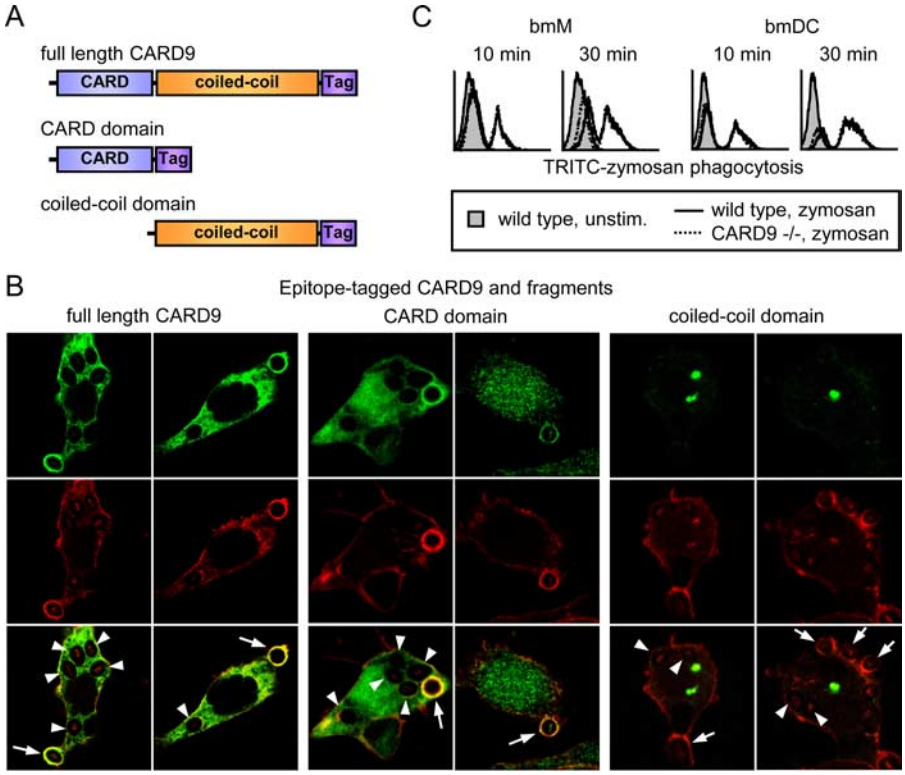

FIGURE 2. CARD9 is recruited to forming phagosomes during Dectin-1-mediated phagocytosis, but is not required for phagocytosis. *A*, Full length CARD9, the CARD domain of CARD9, and the CARD9 coiled-coil domain were tagged with streptavidin-binding peptide (SBPc). *B*, RAW264.7 macrophages stably expressing SBPc-tagged full length CARD9, CARD domain, or coiled-coil domain were fed 100 μg/ml zymosan particles for 10 min, before fixing and staining with an Ab against the SBPc tag (CARD9 constructs, green) and TRITC-phalloidin (β-actin, red). Arrows identify actin-positive early phagosomes; arrowheads identify actin-negative later phagosomes. *C*, Bone marrow-derived macrophages (bmM) and dendritic cells (bmDC) from wild-type and CARD9-deficient mice were fed 100 μg/ml TRITC-labeled zymosan for the times indicated and phagocytosis was assessed by flow cytometry.

Dectin-1-mediated phagocytosis. bmM and bmDC from CARD9-deficient mice (18) showed no defect in internalization of TRITC-labeled zymosan particles, indicating that CARD9 plays no role in phagocytosis in either cell type (Fig. 2*C*). These data are consistent with HEK293 reconstitution experiments in which Dectin-1 transfection without addition of CARD9 is sufficient for phagocytosis (6).

β-glucan particles trigger TNF-α production by bmDC, but not bmM

Although we have previously demonstrated a role for Dectin-1 signaling in promoting TLR2-driven cytokine production in zymosan-stimulated macrophages by enhancing TLR2-mediated NF-κB activation, we have thus far failed to detect direct Dectin-1-mediated NF-κB activation in macrophages (8). In contrast, Gross et al. (12) demonstrated that Dectin-1 can activate NF-κB in bmDC via CARD9, and that CARD9-deficient bmDC fail to produce TNF-α in response to zymosan stimulation. We therefore reassessed the ability of β-glucan particles to trigger TNF-α production by bmM and bmDC. We compared zymosan, which signals via both TLR2 and Dectin-1, and β-glucan particles (depleted zymosan), which do not activate TLR2.

Zymosan triggered robust TNF-α production by both bmM and bmDC (Fig. 3, *A* and *B*), although TNF-α production by bmDC was on average ~5-fold greater (Fig. 3, *A*, *B*, *E*, and *F*). Depleted zymosan triggered robust TNF-α production by bmDC but failed to elicit a response from bmM, even at a 10-fold higher dose. Similar results were obtained using RAW264.7 macrophages overexpressing SBPc-tagged Dectin-1; zymosan stimulated strong induction of TNF-α, while depleted zymosan had little effect (Fig. 3*C*), despite the equivalent abilities of zymosan and depleted zymosan to induce reactive oxygen species in these cells (Fig. 3*D*). Specific ligation of SBPc-tagged Dectin-1 with streptavidin-coated beads also failed to induce TNF-α, despite triggering a robust oxidative burst (data not shown).

Having observed that Dectin-1 signaling is insufficient to trigger TNF-α production in macrophages, but that CARD9 is nevertheless recruited to Dectin-1 phagosomes in these cells, we looked specifically at whether CARD9 signaling influences zymosan-induced TNF-α production. We stimulated bmM and bmDC from wild-type and CARD9-deficient mice (18) with either zymosan or depleted zymosan. In agreement with data published by Gross et al., TNF-α production triggered by zymosan was dramatically suppressed in CARD9$^{-/-}$ bmDC, while induction by other TLR ligands (the pure TLR2 agonist Pam_3CSK_4 and the TLR4 agonist LPS) was not affected (Fig. 3*E* and data not shown). TNF-α induction by depleted zymosan was also suppressed in bmDC lacking CARD9 (Fig. 3*E*), supporting a role for CARD9 in the Dectin-1-mediated response. TNF-α induction by zymosan was also lower in CARD9 $^{-/-}$ bmM than wild-type bmM (Fig. 3*F*). Because Dectin-1 signals do not directly trigger TNF-α production in bmM, and TLR2-induced TNF-α induction is also CARD9-independent in these cells (Fig. 3*F*), we hypothesized that Dectin-1 enhances the TLR2 signal via a CARD9-dependent mechanism, and that this collaboration effect is lost in the absence of CARD9. To verify this, we stimulated bmM with Pam_3CSK_4 in the presence of depleted zymosan, which we have previously shown enhances macrophage responses to the TLR2 ligand (8). Pam_3CSK_4-induced TNF-α production by wild-type bmM was enhanced in the presence of depleted zymosan, but this synergy was not seen in CARD9-deficient bmM (Fig. 3*G*), demonstrating that despite the inability of Dectin-1 to activate NF-κB-driven transcription via CARD9 in bmM, these cells do use CARD9 signals to enhance TLR signaling.

Dectin-1 signals fail to activate NF-κB in bmM, but do trigger p38 MAPK activation

We next examined NF-κB activation in bmM and bmDC following Dectin-1 ligation. We first assessed I-κB degradation, which releases NF-κB to enter the nucleus and bind target promoters. Consistent with previous studies, stimulation of both bmM and bmDC with zymosan or the pure TLR2 agonist Pam_3CSK_4 triggered rapid degradation of I-κB (within 30 min of stimulation),

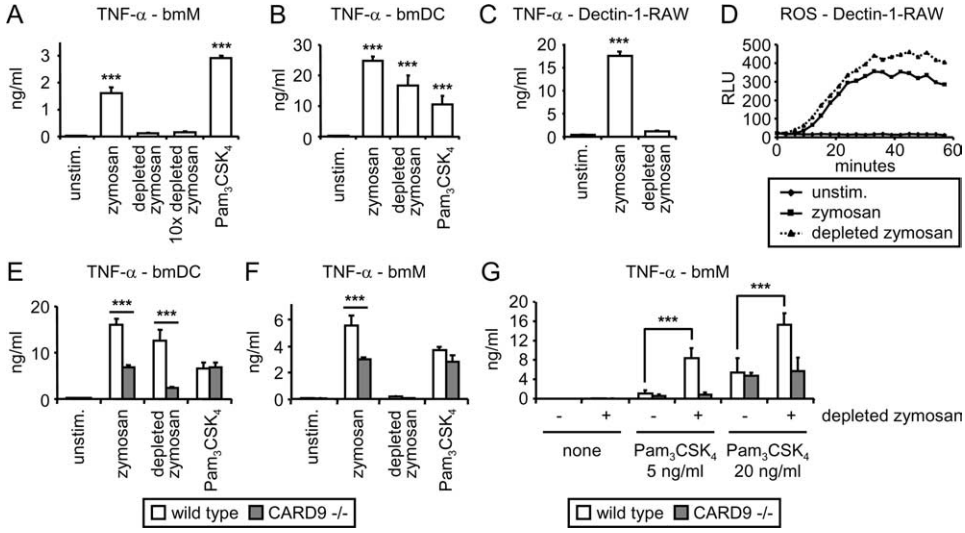

FIGURE 3. Dectin-1 signals directly trigger TNF-α production in bmDC, but not bmM. *A–C*, bmM (*A*), bmDC (*B*), and Dectin-1-expressing RAW264.7 cells (*C*) were stimulated with 100 μg/ml zymosan or depleted zymosan, 1 mg/ml depleted zymosan (10× depleted zymosan), or 100 ng/ml Pam_3CSK_4 for 24 h, and TNF-α levels in culture supernatants were assessed by ELISA. *D*, Dectin-1-expressing RAW264.7 cells were stimulated with 100 μg/ml zymosan or depleted zymosan, and reactive oxygen production was measured by luminol-ECL. *E* and *F*, bmDC (*E*) and bmM (*F*) from wild-type and CARD9-deficient mice were stimulated with 100 μg/ml zymosan or depleted zymosan, or 100 ng/ml Pam_3CSK_4 for 24 h, and TNF-α levels in culture supernatants were assessed by ELISA. *G*, bmM from wild-type and CARD9-deficient mice were stimulated with Pam_3CSK_4 at the indicated concentrations in the presence or absence of 100 μg/ml depleted zymosan for 24 h, and TNF-α levels in culture supernatants were assessed by ELISA. All stimulations were performed in triplicate, and are presented as means (plus SD for ELISA data). ***, $p < 0.001$.

followed by resynthesis at later timepoints (Fig. 4*A* and data not shown). In contrast, depleted zymosan triggered rapid I-κB degradation in bmDC, but failed to alter I-κB levels in bmM (Fig. 4*A*). Consistent with this, EMSA analysis of NF-κB binding to a consensus promoter binding site demonstrated strong NF-κB binding activity in nuclear extracts of zymosan-stimulated bmM, while depleted zymosan triggered little or no significant NF-κB binding activity (Fig. 4*B*), as reflected in the failure of depleted zymosan to trigger TNF-α production by these cells (Fig. 3, *A* and *F*). In contrast, both zymosan and depleted zymosan induced strong NF-κB binding activity in bmDC nuclear extracts (Fig. 4*B*).

In contrast to the differential activation of NF-κB, both zymosan and depleted zymosan triggered p38 MAPK phosphorylation in bmM (Fig. 4*C*). Specific ligation of SBPc-tagged Dectin-1 with streptavidin beads also induced p38 MAPK phosphorylation in RAW264.7 macrophages (data not shown). Depleted zymosan-induced p38 phosphorylation was reduced but not abolished in $CARD9^{-/-}$ bmM (Fig. 4*D*), indicating that Dectin-1-induced p38 activation is partially, but not entirely, dependent on CARD9. In contrast Pam_3CSK_4-induced p38 phosphorylation was CARD9-independent, consistent with the lack of an effect of CARD9 deletion on TLR-mediated proinflammatory cytokine production (Fig. 3, *E* and *F* and Refs. 12, 18).

Collectively, our data indicate that although Dectin-1-CARD9-Bcl10 signals directly activate NF-κB and TNF-α production in bmDC, Dectin-1 signaling alone does not activate

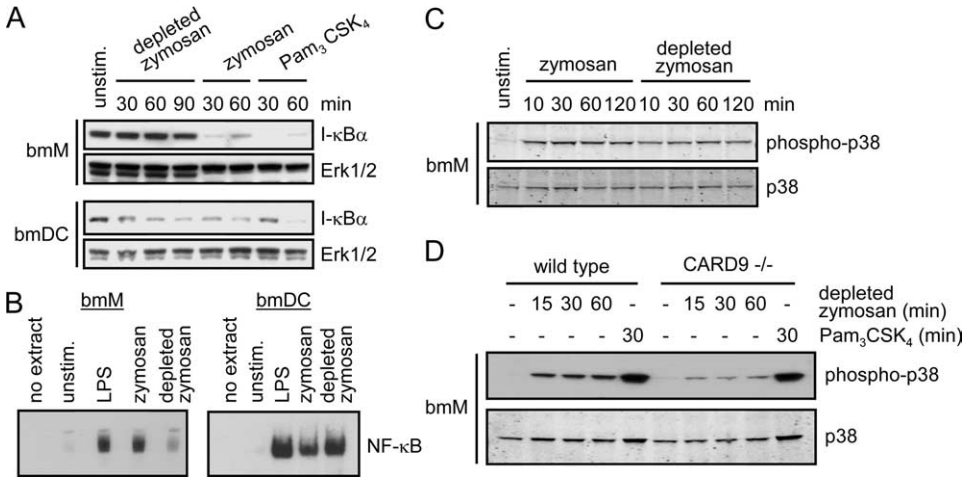

FIGURE 4. Dectin-1-CARD9 signals trigger NF-κB activation in bmDC, but not bmM, but contribute to p38 MAPK activation in bmM. *A*, bmM and bmDC were stimulated with 100 μg/ml zymosan or depleted zymosan, or 100 ng/ml Pam_3CSK_4 for the times indicated, and I-κB levels were assessed by Western blotting, with Erk1/2 as a loading control. *B*, bmM and bmDC were stimulated with 100 ng/ml LPS, or 100 μg/ml zymosan or depleted zymosan for 90 min, and nuclear translocation of NF-κB was assessed by EMSA. *C* and *D*, bmM from wild-type (*C* and *D*) and CARD9-deficient (*D*) mice were stimulated with 100 μg/ml zymosan or depleted zymosan, or 100 ng/ml Pam_3CSK_4 for the times indicated, and p38 phosphorylation was assessed by Western blotting, with total p38 as a loading control.

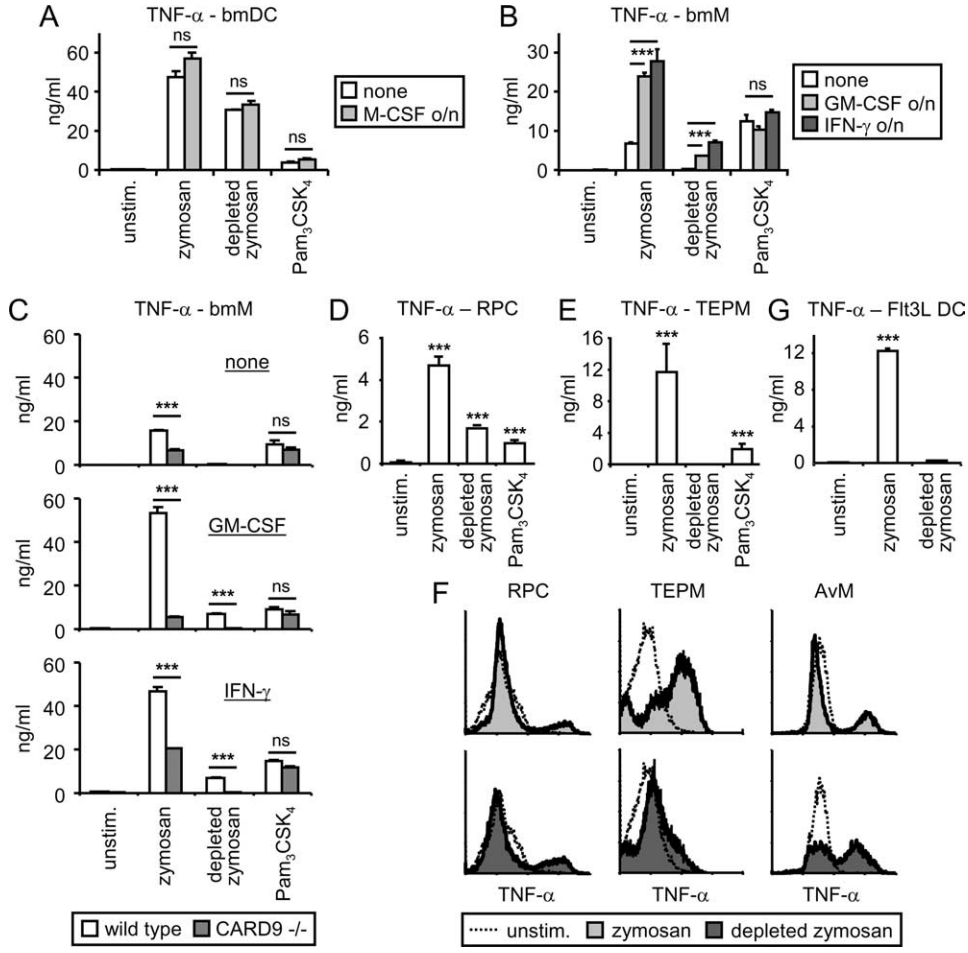

FIGURE 5. Analysis of Dectin-1-induced TNF-α production by GM-CSF- and IFN-γ-primed bmM, and other macrophage and DC populations. *A* and *B*, bmDC were pretreated overnight with 50 ng/ml M-CSF (*A*), and bmM were pretreated overnight with 10 ng/ml GM-CSF or 25 U/ml IFN-γ (*B*). bmDC and bmM were then stimulated with 100 μg/ml zymosan or depleted zymosan, or 100 ng/ml Pam_3CSK_4 for 24 h, and TNF-α in culture supernatants was assessed by ELISA. *C*, bmM from wild-type and CARD9-deficient bmM were pretreated overnight with 10 ng/ml GM-CSF or 25 U/ml IFN-γ, before stimulation with 100 μg/ml zymosan or depleted zymosan, or 100 ng/ml Pam_3CSK_4 for 24 h, and TNF-α was assessed by ELISA. *D* and *E*, Resident peritoneal cells (RPC; *D*) and thioglycollate-elicited peritoneal macrophages (TEPM; *E*) were stimulated with 100 μg/ml zymosan or depleted zymosan, or 100 ng/ml Pam_3CSK_4 for 24 h, and TNF-α was assessed by ELISA. *F*, RPC, TEPM, and alveolar macrophages (AvM) were stimulated with 100 μg/ml zymosan or depleted zymosan for 4 h in the presence of brefeldin A, and TNF-α production was assessed by intracellular flow cytometry, gating for $CD11b^+$ RPC and TEPM, and $CD11b^-$ AvM. *G*, DC derived from bone marrow with Flt3L (Flt3L DC) were stimulated with 100 μg/ml zymosan or depleted zymosan for 24 h and TNF-α in culture supernatants was assessed by ELISA. For ELISA measurements, stimulations were performed in triplicate and are presented as mean plus SD. ***, $p < 0.001$; ns, no significant difference.

NF-κB in bmM. In contrast, CARD9 signals contribute to p38 MAPK activation in bmM and can enhance TLR2-activated TNF-α production.

GM-CSF and IFN-γ permit TNF-α induction by Dectin-1 in bmM

Considering the parallel differentiation of bmM and bmDC with M-CSF and GM-CSF respectively, we hypothesized that either 1) M-CSF signals restrict the ability of Dectin-1 to activate the CARD9-NF-κB pathway in bmM, or 2) GM-CSF signals permit the direct activation of NF-κB via CARD9 in bmDC. To address the first possibility, we added M-CSF to GM-CSF-derived bmDC overnight before stimulation, and observed no effect of M-CSF on the ability of these cells to produce TNF-α upon stimulation with zymosan, depleted zymosan, or the TLR2 ligand Pam_3CSK_4 (Fig. 5*A*). In contrast, M-CSF-derived bmM exposed to GM-CSF overnight produced elevated levels of TNF-α in response to zymosan, but not upon stimulation with Pam_3CSK_4 (Fig. 5*B*). Furthermore, these cells now produced robust TNF-α following exposure to depleted zymosan (Fig. 5*B*). Overnight priming of bmM with IFN-γ had a similar effect (Fig. 5*B*). Dectin-1-induced TNF-α production by GM-CSF/IFN-γ-primed bmM was completely CARD9-dependent (Fig. 5*C*).

The ability of Dectin-1 to directly drive TNF-α production in different macrophage and DC populations is variable

Our data have revealed an interesting difference in the ability of bmM and bmDC derived in vitro to respond to Dectin-1 ligation by β-glucan particles. To address whether in vivo populations of macrophages are similarly restricted in their ability to respond to β-glucan particles, we measured TNF-α production by RPC, TEPM, and AvM. Like bmM, all of these macrophage subtypes produced robust TNF-α following zymosan stimulation (Fig. 5, *D–F*). However, we observed variable responsiveness of the different cell types to depleted zymosan (Fig. 5, *D–F*). Depleted zymosan stimulated TNF-α production by RPC, but failed to induce TEPM to produce TNF-α (Fig. 5, *D–F*). AvM also produced TNF-α in response to depleted zymosan exposure (Fig. 5*F*). Thus, it appears that there is considerable variation

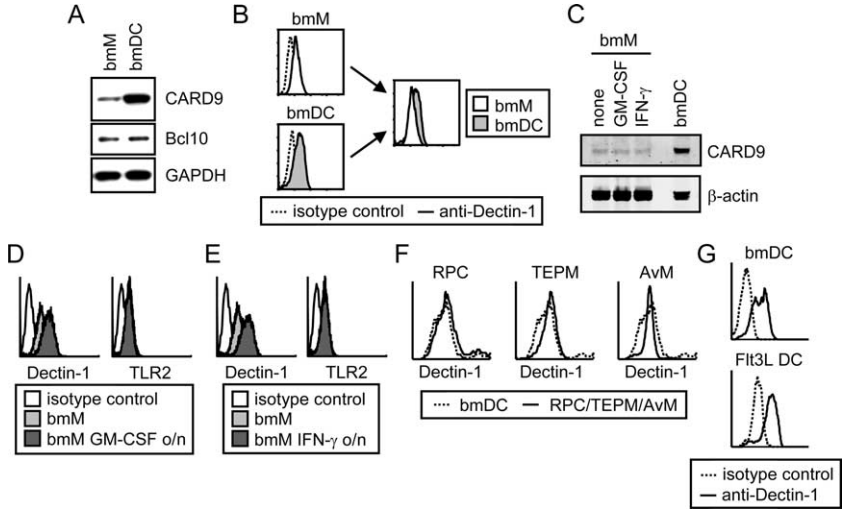

FIGURE 6. There is no correlation between Dectin-1, CARD9, and Bcl10 expression levels and the ability of Dectin-1 to directly induce TNF-α. *A*, CARD9 and Bcl10 levels in bmM and bmDC were compared by Western blotting, with GAPDH as a loading control. *B*, Surface expression of Dectin-1 on bmM and bmDC was assessed by flow cytometry. *C*, CARD9 expression by bmM and bmM pretreated overnight with 10 ng/ml GM-CSF or 25 U/ml IFN-γ was assessed by Western blotting, with β-actin as a loading control. *D* and *E*, Surface expression of Dectin-1 and TLR2 on bmM and bmM pretreated overnight with 10 ng/ml GM-CSF (*D*) or 25 U/ml IFN-γ (*E*) was assessed by flow cytometry. *F* and *G*, Dectin-1 surface expression by RPC, TEPM, AvM, bmDC, and Flt3L DC was assessed by flow cytometry.

between in vivo-derived macrophage populations in the contribution of Dectin-1 signals to TNF-α induction.

We also measured TNF-α production by DC derived from bone marrow using Flt3L (Flt3L DC). These cells are thought to represent steady-state resident tissue DC (23). Zymosan stimulated robust TNF-α production, but in contrast to GM-CSF-derived bmDC, Flt3L DC failed to produce TNF-α upon exposure to depleted zymosan (Fig. 5*G*).

Differential responsiveness to β-glucan particles is not due to variation in Dectin-1 and CARD9 expression

We hypothesized that the inability of β-glucan particles to directly trigger TNF-α production in certain macrophage and DC populations (unprimed bmM, TEPM, and Flt3L-derived bmDC) indicates that these cells either lack a key component of the Dectin-1-CARD9-NF-κB signaling pathway, or that an inhibitor active in these cells blocks the signal.

To address whether bmM are deficient in any of the key components of this pathway, we first compared the expression of CARD9 and Bcl10, as well as Dectin-1 itself, in bmM, and in bmDC. Consistent with our demonstration above that CARD9 is recruited to Dectin-1 phagosomes in macrophages (Fig. 2, *B* and *C*) and that CARD9 is responsible for Dectin-1 collaboration with TLR2 in bmM (Fig. 3*G*), we observed CARD9 expression in bmM as well as GM-CSF-derived bmDC, although the DC had much higher levels of CARD9 than bmM (Fig. 6*A*). Surface expression of Dectin-1 was also slightly higher on bmDC than bmM (Fig. 6*B*). Bcl10 was detected in both cell types at similar levels and hence its expression level does not appear to correlate with the presence or absence of a response (Fig. 6*A*).

We next examined whether GM-CSF and IFN-γ priming alter CARD9 levels or the surface expression of Dectin-1 on bmM. Neither GM-CSF nor IFN-γ had any effect on CARD9 expression (Fig. 6*C*). Because these primed bmM respond robustly to depleted zymosan, we can conclude that CARD9 is not limiting in unprimed bmM. In contrast, both GM-CSF and IFN-γ enhanced Dectin-1 surface expression to levels comparable to those of bmDC, without affecting surface expression of TLR2 (Fig. 6, *D* and *E*). However, we also found that levels of Dectin-1 on RPC, TEPM, AvM, and Flt3L DC were comparable to those on bmDC (Fig. 6, *F* and *G*), despite the varied responsiveness of these cells (Fig. 5, *F* and *G*). Hence, there was no clear correlation between Dectin-1 surface expression levels and the ability of the cells to respond to depleted zymosan.

Macrophage unresponsiveness to β-glucan particles is likely due to the action of an inhibitor of the Dectin-1-CARD9 pathway

Although Dectin-1, CARD9, and Bcl10 levels do not appear to correlate with the differential responsiveness of the myeloid populations, it remains possible that these cells lack some other intrinsic component of the CARD9 pathway. Alternatively the signaling pathway may be fully intact, but expression of additional proteins may restrict its activation. To distinguish between these possibilities, we overexpressed Dectin-1 in bmM. If these cells lack an intrinsic signaling component, no amount of enhanced Dectin-1 signaling would be sufficient to permit activation of the pathway. In contrast, if an inhibitor is blocking CARD9-mediated NF-κB activation, increased Dectin-1 signaling might be expected to overcome this blockade.

We therefore transfected bmM with a Dectin-1 expression vector, and verified Dectin-1 overexpression at the cell surface by flow cytometry (Fig. 7*A*). We observed dramatically enhanced Dectin-1 surface expression following Dectin-1-GFP transfection compared with expression of endogenous Dectin-1 expression by control GFP transfected cells (which was poorly detected by the PE-conjugated anti-Dectin-1 Ab, in contrast to the FITC-conjugated Ab used in Fig. 6). We then assessed the ability of zymosan and depleted zymosan to induce TNF-α production by control and Dectin-1-overexpressing bmM by intracellular flow cytometry. Although bmM overexpressing either Dectin-1 or a control plasmid

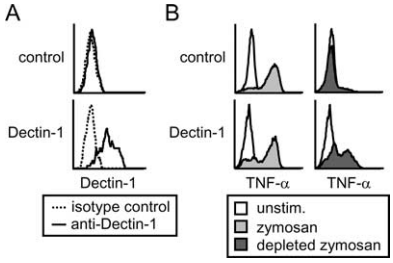

FIGURE 7. Overexpression of Dectin-1 in bmM overcomes blockade of direct Dectin-1-mediated TNF-α induction. *A*, bmM were transfected with either Dectin-1-GFP or a GFP control plasmid, and Dectin-1 surface expression was assessed by flow cytometry of unpermeabilized cells. *B*, Control or Dectin-1-overexpressing bmM were stimulated for 4 h with 100 μg/ml zymosan or depleted zymosan in the presence of brefeldin A, and TNF-α production by GFP$^+$ cells (control and Dectin-1 plasmids) was assessed by intracellular flow cytometry.

responded robustly to zymosan stimulation, only bmM overexpressing Dectin-1 produced TNF-α following exposure to depleted zymosan (Fig. 7*B*). Thus, it appears possible to activate CARD9 signaling directly downstream of Dectin-1 in these cells with a sufficiently large signal, supporting the notion that this pathway is intact but is held in check by expression of additional factors.

Discussion

Integration of signals derived from Dectin-1 and TLR2 directs the coordinated response of myeloid phagocytes to fungal particles. Previous studies indicated that the transcriptional induction of proinflammatory mediators is controlled by TLR2-MyD88-induced NF-κB activation, a signal that can be amplified by Dectin-1 and Dectin-1-mediated ITAM-like signaling, which activates NFAT transcription factors (8, 9, 24, 25). A recent report also indicated that Dectin-1 signals can directly activate NF-κB in DC via the adaptor protein CARD9 (12). In our current study, we have revealed an additional layer of regulation of Dectin-1/TLR2-induced proinflammatory cytokine production.

Our data show that Dectin-1 signaling via CARD9 is differentially regulated in myeloid cells, resulting in the ability of only certain macrophage/DC populations to produce TNF-α upon Dectin-1 ligation alone. bmM, TEPM, and Flt3L-derived DC failed to produce TNF-α in response to Dectin-1 ligation, whereas bmDC, GM-CSF/IFN-γ-primed bmM, resident peritoneal cells, and alveolar macrophages all displayed robust TNF-α production following Dectin-1 ligation (Figs. 3 and 5). Expression of Dectin-1, CARD9 and its interacting partner Bcl10 did not correlate with the ability of the cells to produce TNF-α in response to Dectin-1 ligation (Fig. 6). However, we demonstrated that overexpression of Dectin-1 in bmM is sufficient to permit CARD9-dependent TNF-α induction (Fig. 7), indicating that these cells possess an inhibitor of Dectin-1-CARD9 signaling.

Meyer-Wentrup et al. (26) recently demonstrated that the tetraspanin CD37 associates with Dectin-1 and limits proinflammatory cytokine induction. Induction of IL-6 by zymosan was 10-fold higher in TEPM from CD37 knockout mice compared with wild-type TEPM. Furthermore, although wild-type TEPM failed to produce IL-6 in response to the β-glucan curdlan, IL-6 was induced by curdlan treatment of CD37-deficient TEPM. Our data suggest that CD37 may block Dectin-1-CARD9-mediated cytokine induction in macrophage/DC populations unresponsive to depleted zymosan. Consistent with this, we observed higher CD37 expression by bmM than bmDC, and a reduction in CD37 expression following treatment of bmM with GM-CSF and IFN-γ (data not shown). Meyer-Wentrup et al. also observed a reduction in CD37 expression upon differentiation of DC from human monocytes (26).

Tetraspanins are small transmembrane scaffold proteins that anchor proteins in membrane microdomains (27). Hence, CD37 may sequester Dectin-1 into a microdomain that selectively modulates the recruitment of key signaling molecules downstream of CARD9. Lower CD37 expression in bmDC/primed bmM, coupled with higher Dectin-1 expression in these cells, would be predicted to result in a larger pool of Dectin-1 that is not associated with and limited by CD37. Further studies are required to determine whether a CD37-based mechanism is responsible and sufficient for the differential CARD9 signaling we have observed in this study.

Dectin-1 has also been reported to associate with another tetraspanin, CD63, in human monocyte-derived DC, although the function of this interaction is unknown (28). Hence, the relative levels of different tetraspanins may also determine the type of signal generated. Detailed investigation of the regulation of Dectin-1 signaling by CD37 and CD63 is required to further assess the ability of tetraspanins to differentially regulate Dectin-1 signals.

Gross et al. (12) had demonstrated that Dectin-1-CARD9 signaling results in NF-κB activation in bmDC, which contrasted with our previous failure to observe NF-κB activation in RAW264.7 macrophages and bmM (8). We have now shown that Dectin-1 can indeed activate NF-κB directly in certain myeloid cells, but that this signaling connection is not always made. This is in contrast to other Dectin-1 signals, including Src-Syk activation (6), p38 phosphorylation (Fig. 4*C*), and NFAT activation (9), which are triggered robustly in bmM (and RAW264.7 macrophages) as well as bmDC. Whether other signals downstream of CARD9 are also absent in the "unresponsive" macrophages, contributing to the inability of Dectin-1 signals to directly induce TNF-α, remains to be established.

The cell type-specific variability of CARD9-mediated NF-κB activation downstream of Dectin-1 ligation stands in sharp contrast to MyD88-mediated NF-κB activation downstream of TLRs. As far as we are aware, TLR signaling via MyD88 always results in NF-κB activation, regardless of cell type. In contrast, we have shown that Dectin-1 signaling via CARD9 does not always result in NF-κB activation. Variability in Dectin-1 signaling in different cell types represents a novel mechanism by which myeloid cells can be fine-tuned to regulate antifungal immunity, with different myeloid populations having distinct inflammatory and antimicrobial properties. Whereas alveolar macrophages are inherently ready to release cytokines and initiate an inflammatory response to β-glucan particles, other macrophages may require information about an ongoing infection, such as IFN-γ production, to become primed for this response. Furthermore, as we have observed fundamentally different proinflammatory signaling in GM-CSF- and Flt3L-derived bmDC, there may be significant further heterogeneity in how different DC subsets prime adaptive immunity. Future studies will define the consequences of this variability on cell function and the contribution of differentially responsive cells to antifungal immunity.

Despite the apparent restricted ability of CARD9 to activate NF-κB in bmM, CARD9 is not completely silent in these cells, as indicated by the defect in Dectin-1-induced p38 phosphorylation in CARD9-deficient bmM (Fig. 4*D*). Furthermore, CARD9 appears to be responsible for the collaboration of Dectin-1 signals with TLR2 signals, as evidenced by the defect in TNF-α production by zymosan-stimulated CARD9-deficient bmM (Fig. 3, *F* and *G*). These data are consistent with our observations of CARD9 recruitment to Dectin-1 phagosomes even in macrophages where Dectin-1 signaling is insufficient to drive TNF-α production (Fig. 2).

Hara et al. (29) recently showed that CARD9 mediates signaling downstream of the FcγR, which, like Dectin-1, is an ITAM-containing phagocytic receptor. Hence, we predict that CARD9 is similarly recruited to FcγR phagosomes. That the CARD domain of CARD9 is enough to drive the protein to the Dectin-1 phagosome suggests that phagosomes might be an important scaffold for coordinating CARD-CARD signaling interactions. Future studies will have to define how the presence or absence of additional signaling molecules on the phagosomes of different cell types regulates inflammation and immunity.

Disclosures

The authors have no financial conflict of interest.

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