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Dectin-1 Interaction with *Mycobacterium tuberculosis* Leads to Enhanced IL-12p40 Production by Splenic Dendritic Cells

Antonio Gigliotti Rothfuchs,2 Andre Bafica,*‡ Carl G. Feng, Jackson G. Egen,§ David L. Williams, Gordon D. Brown, and Alan Sher*

Dectin-1 is a fungal pattern recognition receptor that binds to β-glucans and triggers cytokine production by facilitating interaction with TLR2 or by directly activating spleen tyrosine kinase (Syk). To assess the possible role of Dectin-1 in the innate response to mycobacteria, we used an in vitro system in which IL-12p40 production is measured in splenic dendritic cells (SpDC) following exposure to live *Mycobacterium tuberculosis* bacilli. Treatment of SpDC with laminarin or glucan phosphate, two molecules known to block Dectin-1-dependent activity, led to a reduction in *M. tuberculosis*-induced IL-12p40 as well as IL-12p70 production. Moreover, SpDC from Dectin-1−/− chimeric mice displayed reduced IL-12p40 production in response to mycobacteria when compared with Dectin-sufficient DC. Laminarin treatment also inhibited mycobacterial-induced IL-12p40 production in DC from TLR2−/− mice, arguing that Dectin-1 functions independently of TLR2 signaling in this system. Importantly, a Dectin-1 fusion protein was found to directly bind to live mycobacteria in a laminarin-inhibitable manner indicating the presence of ligands for the receptor in the bacterium and laminarin pretreatment resulted in reduced association of mycobacteria to SpDC. In additional experiments, mycobacterial stimulation was shown to be associated with increased phosphorylation of Syk and this response was inhibited by laminarin. Furthermore, pharmacologic inhibition of Syk reduced the *M. tuberculosis*-induced IL-12p40 response. Together, these findings support a role for Dectin-1 in promoting *M. tuberculosis*-induced IL-12p40 production by DC in which the receptor augments bacterial-host cell interaction and enhances the subsequent cytokine response through an unknown mechanism involving Syk signaling. *The Journal of Immunology*, 2007, 179: 3463–3471.

The facultative intracellular, acid-fast bacillus *Mycobacterium tuberculosis* is a major human pathogen which infects a third of the world’s population and is responsible for ~2 million deaths each year (1). Host control of *M. tuberculosis* relies heavily on the activation of CD4+ T cells leading to IFN-γ-dependent mycobactericidal activity in infected macrophages (2). The heterodimeric cytokine IL-12 plays an important role in generating and maintaining this host protective response. Bioactive IL-12p70 is composed of an inducible IL-12p40 subunit and a largely constitutively produced IL-12p35 subunit (3). IL-12p40 also associates with the subunit p19 to generate bioactive IL-23, but in addition is intrinsically functional as a IL-12p40:IL-23p19 homodimer (4). Studies using mice genetically deficient in IL-12p40 have confirmed the importance of this IL-12 subunit in granuloma formation, Th1 development, and control of mycobacterial infection (5). IL-12 seems to account for most of this effect with IL-23 being protective in the absence of the former cytokine (6). Additional studies have demonstrated that IL-12p40 expression alone is sufficient to enable dendritic cells (DC) to migrate from *M. tuberculosis*-infected lungs to draining lymph nodes and to activate naive T cells (7) and that IL-12p40 administration can restore the resistance of IL-12p40−/−IL-12p35−/− mice to *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (8). Similarly, humans with congenital defects in IL-12p40 or in the β1 subunit of the IL-12R also display increased susceptibility to mycobacterial disease (9). Thus, IL-12p40 appears to play a major role in the control of mycobacterial infection in both mice and in humans.

DC are thought to provide an important source of IL-12 in the response to invading pathogens. Their production of this cytokine is induced by pattern recognition receptors (PRR) which sense conserved molecular patterns in the infecting microbes. TLR, with their ability to transmit intracellular signals for activation of NF-κB and other transcription factors, are a major class of PRR involved in the triggering of IL-12 production by DC (10, 11). In the case of murine *M. tuberculosis* as well as *M. bovis* BCG infection, both TLR2 and TLR9 have been shown to regulate DC-derived IL-12 production (12–14). For efficient activation, TLR may be complemented by and even rely on other PRR for proximal ligand binding and recognition of pathogen-derived molecules (15). In this regard, C-type lectin receptors (CLRs) have been implicated in both opsonic and nonopsonic binding and internalization of pathogens and are therefore thought to be important players in the latter process.

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2 Address correspondence and reprint requests to Dr. Antonio Gigliotti Rothfuchs, Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; 3Division of Immunology, Department of Microbiology and Parasitology, Federal University of Santa Catarina, Florianópolis, Brazil; 4Department of Surgery, James H. Quillen College of Medicine, Johnson City, TN 37614; and 5Division of Immunology, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Rondebosch, South Africa

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Abbreviations used in this paper: DC, dendritic cell; PRR, pattern recognition receptor; CLR, C-type lectin receptor; BCG, bacillus Calmette-Guérin; Syk, spleen tyrosine kinase; WT, wild type; SpDC, splenic DC; SpLOD, splenic low density; PEC, peritoneal exudate cell; BMDC, bone marrow-derived DC; MOI, multiplicity of infection; STAg, soluble tachyzoite Ag; CR, complement receptor.
DC-associated C-type lectin (Dectin-1) is a novel CLR expressed on myeloid cells that has been implicated in pattern recognition of pathogenic fungi (16, 17). Dectin-1 binds fungal β-1,3 glucan-linked carbohydrates found in abundance in fungal cell walls (18). In studies using zymosan, Dectin-1 has been shown to facilitate TLR2-mediated proinflammatory cytokine production by macrophages (19, 20). Unlike many other phagocytic receptors, Dectin-1 can directly trigger cytokine production through an ITAM-like motif in its cytoplasmic tail. Stimulation of the receptor leads to activation of spleen tyrosine kinase (Syk) which in macrophages accounts for the induction of the respiratory burst and in DC is important for IL-2 and IL-10 release and phagocytosis (21–23).

Despite the growing literature on the biology of Dectin-1 in fungal immunity, there remains a paucity of information on the function of Dectin-1 in host recognition of other pathogens. In this study, we assessed the possible involvement of Dectin-1 in promoting mycobacterial-induced IL-12 production by DC and suggest that this effect is due to enhanced bacterial association/uptake as well as increased Syk signaling.

Materials and Methods

Mice

CD11b+/− (24), TLR2−/− (25), TLR9−/− (26), MyD88−/− (27), and IL-10−/− (28) mice were originally generated by homologous recombination in embryonic stem cells as previously described. Wild-type (WT) controls were purchased from Taconic Farms which also provided CD11b+/+, IL-10+/+ mice under the National Institute of Allergy and Infectious Diseases (NIAID) Animal Supply Contract. TLR2−/− mice were crossed to TLR9−/− mice to generate TLR2−/−/TLR9−/− animals as previously described (12). CD11b+/−, TLR2−/−, TLR9−/−, TLR2−/−/TLR9+/+ and MyD88−/− mice, and CD11b+/+, IL-10+/+ mice were purchased from Taconic Farms which also provided CD11b−/−, IL-10−/− mice under the National Institute of Allergy and Infectious Diseases (NIAID) Animal Supply Contract. CD11b+/+, IL-10+/+ mice and WT controls for CD11b−/−, IL-10−/− mice were reared and maintained at the University of Cape Town, were used for the generation of Dectin-1−/− chimeric animals at the NIAID animal facility. Briefly, 129SvEv WT recipient animals were lethally irradiated with 950 rad and each animal was inoculated i.v. with 105 bone marrow cells from Dectin-1−/− or Dectin-1+/+ mice. Animals were provided with antibiotics in the drinking water for 6 wk and used for experiments after an additional period of 6 wk or more. Reconstitution was assessed by FACS analysis of blood with B cell, T cell, monocyte, and granulocyte markers and no significant differences in engraftment were observed between WT−/−→WT and Dectin-1−/−→WT chimeric mice in these assays. In addition, the WT and Dectin-1−/− chimeric mice displayed comparable percentages of I-A/I-E CD1c+ splenic cells (data not shown).

Isolation of murine DC and macrophage populations

Splenic DCs (SpDC) were isolated as previously described (29). Briefly, spleens were aseptically removed from naive mice and injected with 0.4 mg/ml Liberase CI solution (Roche Biochemicals) for 45 min at 37°C. Spleens were then gently homogenized through a 100-μm cell strainer, the homogenate was resuspended in PBS containing 5 mM EDTA and pelleted at 1200 rpm for 10 min. Erythrocytes were then lysed using ACK lysing buffer (Cambrex). Splenocytes were subjected to ultracentrifugation through a 30% BSA gradient (Sigma-Aldrich) and splenic low-density (SpLOD) cells were obtained from the resulting interface. Where indicated, SpCD11c+ cells were further enriched using Miltenyi CD11c microbeads on an AUTOMACS according to the instructions of the manufacturer (Miltenyi Biotec). This yielded a population of 90% I-A/I-E+ CD1c+ cells in some experiments. CD11bhiCD11c+ SpLOD cells were purified on a FACSAvantage flow cytometer and used in stimulation assays. To obtain peritoneal exudate cells (PEC), naive WT mice were injected i.p. with 3 ml of 3% thioglycolate broth (Sigma-Aldrich) and peritoneal lavage was performed 4 days later. To enrich for macrophages, PEC were allowed to adhere to plastic for 4 h before use. Bone marrow-derived DC (BMDC) were generated from naive WT mice using GM-CSF (BioSource International) as previously described (12).

Generation of mycobacterial stocks and in vitro infection of cells

M. tuberculosis strain H37Rv, M. bovis BCG strain Pasteur, DsRed-expressing BCG strain Pasteur (BCG-DsRed; provided by Dr. N. Winter, Pasteur Institute, Paris, France), GFP-expressing M. tuberculosis strain H37Rv (Mtb-GFP; provided by Dr. Clifton E. Barry, NIAID, Bethesda, MD) and Mycobacterium avium strain 2-551 SmT were expanded to log phase on Middlebrook 7H9 liquid medium supplemented with albumin-dextrose-catalase (Difco), washed, aliquotted in PBS, and stored at −80°C until further use. BCG-DsRed was expanded in the presence of hygromycin (Sigma-Aldrich) and Mtb-GFP in the presence of kanamycin (Sigma-Aldrich), respectively. Bacterial titers were quantified by plating out bacteria on 7H11 agar supplemented with oleic acid-albumin-dextrose-catalase (Difco).

Host DC and macrophages were resuspended in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS, 2 μM l-glutamine, 10 μM HEPES, and 50 μM 2-ME and seeded onto 96-well plates (0.5 × 104 cells/ml, 0.2 ml/well). Cells were infected with live mycobacteria or Toxoplasma gondii at a multiplicity of infection (MOI) of 1, prepared as previously described (29), with live Trypanosoma cruzi at a MOI of 3, prepared as previously described (30), or stimulated with 0.5 μg/ml soluble tachyzoite Ag (STAg; Ref. 31), 2.5 μM immunostimulatory CpG, 1 μg/ml Pam_Cys, 100 μg/ml zymosan, or 10 μg/ml LPS (all obtained from Invivogen). For β-glucan receptor inhibition experiments, cells were pretreated with laminarin (Sigma-Aldrich) or glucan phosphate (32) before stimulation. Cell viability as assessed by both trypan blue exclusion and MTT assay was unaffected by glucan treatment (data not shown). For pharmacological inhibition of Syk, cells were pretreated with 1 μM piceatannol (Calbiochem) or DMSO before stimulation.

Quantification of cytokine production

 Supernatants were collected for determination of cytokine production following overnight stimulation of SpDC or PEC. IL-12p40 levels were determined by sandwich ELISA as previously described (33). TNF-α and IL-12p70 were measured using the DuoSet mouse TNF-α and Quantikine mouse IL-12p70 immunoassay kits, respectively (R&D Systems). IL-23 was measured using the IL-23 Ready-Set-Go kit (eBioscience).

Bone marrow radiation chimeras

Bone marrow from Dectin-1−/− and Dectin-1−/−/mice (34), reared and maintained at the University of Cape Town, were used for the generation of Dectin-1−/− chimeric animals at the NIAID animal facility. Briefly, 129Sv/Ev WT recipient animals were lethally irradiated with 950 rad and each animal was inoculated i.v. with 105 bone marrow cells from Dectin-1−/− or Dectin-1+/+ mice. Animals were provided with antibiotics in the drinking water for 6 wk and used for experiments after an additional period of 6 wk or more. Reconstitution was assessed by FACS analysis of blood with B cell, T cell, monocyte, and granulocyte markers and no significant differences in engraftment were observed between WT−/−→WT and Dectin-1−/−→WT chimeric mice in these assays. In addition, the WT and Dectin-1−/− chimeric mice displayed comparable percentages of I-A/I-E+ CD1c+ splenic cells (data not shown).

Flow cytometric staining

SpLOD cells were incubated with a mixture of fluorochrome-conjugated rat anti-mouse mAbs specific for I-A/I-E (M5/114.15.2), CD11c (HL3), CD11b (M1/70), and MyD88 (H11002) and stained with biotinylated rat anti-mouse Fc (Ref. 35) for 45 min in FACS buffer containing 0.5 mg/ml-anti-mouse FcYII/III receptor (2,4G2; BD Pharmingen) and 5% normal mouse serum (Jackson ImmunoResearch Laboratories). Cells were then washed and incubated with PerCP-conjugated streptavidin (BD Pharmingen) for 30 min. To assess the specificity of Dectin-1 staining, cells were preincubated with unlabeled rat anti-mouse Dectin-1 (2A11) for 30 min before staining with biotin-conjugated 2A11 Ab. Data were collected using a FACSCaliber with CellQuest (BD Immunocytometry Systems) and analyzed with FlowJo (Tree Star) software.

Dectin-1-binding assay

A soluble chimeric protein, Dectin-Fc, containing the extracellular carbohydrate recognition domain and the stalk region of mouse Dectin-1 fused to the Fc portion of human IgG1 (36), was used to assess the presence of Dectin-1-binding ligands on mycobacteria. Briefly, live BCG (105 CFU) or zymosan (100 μg/ml) was incubated for 30 min with Dectin-Fc that had been pre-exposed or not to 1 mg/ml laminarin. The samples were then washed and incubated with FITC-conjugated goat anti-human Fc (Sigma-Aldrich) for 30 min. After a final wash, fluorochrome levels were detected by flow cytometry using a FACSCaliber with CellQuest and the data were analyzed with FlowJo software.

Adherence and uptake of mycobacteria by SpDC

For CFU-based quantification of mycobacterial adherence to SpDC, SpDC were isolated from WT mice, pretreated with 1 mg/ml laminarin and infected at 4°C with live M. tuberculosis at a MOI of 1. At different times after infection, cells were extensively washed with PBS, lysed in water containing 1% saponin, and plated on 7H11 agar. CFU were counted.
3 wk later. For microscopy based quantification of mycobacterial adherence and/or uptake to SpDC, SpDC were isolated from WT mice and allowed to adhere for 1 h onto poly-L-lysine-coated glass coverslips (Molecular Probes). Cells were then pretreated with 500 μg/ml laminarin or 1 μg/ml piceatannol and exposed to live BCG-DsRed at a MOI of 5 or 50 μg/ml FITC-conjugated zymosan (Molecular Probes). SpDC were washed 4 h later and fixed with 4% paraformaldehyde and nuclei stained with 4',6-diamidino-2-phenylindole. The number of BCG-infected cells or cells containing zymosan were quantified from at least 11 different three-dimensional stacks (containing an average of 10 cells/field) using a 63/1.4NA objective on an LSM 510 confocal microscope (Carl Zeiss Micro Imaging). Data are expressed as the percentage of cells with labeled BCG or zymosan particles.

Western blotting

SpDC were stimulated with 50 μg/ml zymosan or live M. tuberculosis at a MOI of 2. After 1 h, cells were lysed in M-PER extraction buffer (Pierce) containing a phosphatase inhibitor mixture (Calbiochem). Protein extracts (10 μg) were then separated by SDS-PAGE/NuPage system (Invitrogen Life Technologies), electroblotted onto a nitrocellulose membrane, and immunoblotted with Abs specific for phospho-Syk (Y323) (1:1000) or total Syk (1:1000; Cell Signaling). Ab binding was detected with HRP-conjugated anti-IgG (1:2000), followed by ECL detection.

Statistical analyses

The significance of differences in data group means was analyzed by Student’s t test with a cutoff of \( p < 0.05 \).

Results

Blockade of the β-glucan receptor on SpDC reduces M. tuberculosis-triggered IL-12p40 and IL-12p70 production

The potential role of the β-glucan receptor in mycobacterial-triggered IL-12 production was assessed in SpLOD cells and PEC, two populations of ex vivo-derived murine phagocytes as well as in vitro-differentiated BMDC all of which are known to produce IL-12 following incubation with mycobacteria (12). These cells were pretreated with different concentrations of the soluble glucan laminarin, a β-glucan receptor-blocking reagent and specific inhibitor of Dectin-1 activity (20), and then exposed in vitro to live M. tuberculosis, M. bovis, M. avium, T. gondii tachyzoites, T. cruzi trypomastigotes, or 1 μg/ml Pam3Cys, 10 μg/ml LPS, 100 μg/ml zymosan, 2.5 μM CpG or 0.5 μg/ml STAg. Levels of IL-12p40 were measured as above. The experiment shown is representative of three performed. C, SpLOD cells were isolated from WT or IL-10−/− mice, pretreated with 100 μg/ml laminarin, exposed to live Mtb and IL-12p40 responses measured. Bars indicate SEM. *, Statistically significant differences between untreated and laminarin-treated cell groups.

FIGURE 1. Blockade of the β-glucan receptor on SpLOD reduces mycobacterial-triggered IL-12p40 production. A, PEC or SpLOD cells were isolated ex vivo from WT mice and BMDC were differentiated in vitro from WT mice using GM-CSF. Cells were pretreated with laminarin at the given concentrations and exposed in vitro to live M. tuberculosis (Mtb). Levels of IL-12p40 in overnight culture supernatants were measured by ELISA. The experiment shown is representative of three performed. B, SpLOD cells were isolated from WT mice, pretreated with 100 μg/ml laminarin, and exposed to live Mtb, M. bovis BCG, M. avium, T. gondii tachyzoites, T. cruzi trypomastigotes, or 1 μg/ml Pam,Cys, 10 μg/ml LPS, 100 μg/ml zymosan, 2.5 μM CpG or 0.5 μg/ml STAg. Levels of IL-12p40 were measured as above. The experiment shown is representative of three performed. C, SpLOD cells were isolated from WT or IL-10−/− mice, pretreated with 100 μg/ml laminarin, exposed to live Mtb and IL-12p40 responses measured. Bars indicate SEM. *, Statistically significant differences between untreated and laminarin-treated cell groups.

FIGURE 2. Blockade of the β-glucan receptor on SpDC reduces Mtb-triggered IL-12p40, IL-12p70, but not TNF-α production. A and B. SpLOD cells were isolated from WT mice and enriched for CD11c+ cells. SpDC were then pretreated with 100 μg/ml laminarin (A–D) or glucan phosphate (A and B) or 500 μg/ml laminarin (C and D) and exposed to live Mtb at a multiplicity of infection (MOI) of 1 or as indicated in the figure. Levels of IL-12p40 (A and C), IL-12p70 (B), or TNF-α (D) in overnight culture supernatants were measured by ELISA. The experiment shown is representative of three performed. For detection of IL-12p70, SpDC were exposed to live Mtb in the presence of 500 U/ml rIFN-γ. Bars indicate SEM. *, Statistically significant differences between untreated and glucan-treated cell groups.
**M. tuberculosis.** Cytokine production was then measured in culture supernatants. Although mycobacterial-induced IL-12p40 and TNF-α by PEC and BMDC was unaffected by laminarin treatment, IL-12p40 production by SpLOD cells was substantially reduced (Fig. 1A and data not shown). A similar inhibition by laminarin was also observed when the same ex vivo-derived DC-enriched population was exposed to *M. bovis* BCG or *M. avium* but not the intracellular protozoans *Toxoplasma gondii* or *T. cruzi* (Fig. 1B), implicating a preferential role for the β-glucan receptor in mycobacterial-induced IL-12p40 production by these cells. IL-12p40 production following stimulation with the TLR ligands Pam3Cys, LPS, and stimulatory CpG was also not abrogated by laminarin treatment (Fig. 1B) although a reduction in zymosan-induced IL-12p40 was detected, in line with previous data (23). Consistent with the observation with live *T. gondii* parasites, stimulation with a soluble extract of *T. gondii* tachyzoites (STAg), a potent inducer of IL-12p40 by SpDC, was also unaffected by laminarin treatment. The inhibition of mycobacterial-induced IL-12p40 production by laminarin did not appear to be due to the induction of IL-10 by the glucan because *M. tuberculosis* stimulation in the presence or absence of laminarin did not generate detectable levels of IL-10 (data not shown). More importantly, the ability of laminarin to inhibit mycobacterial-induced IL-12p40 production was unaltered when IL-10–/– SpDC were substituted for WT cells (Fig. 1C).

To confirm that the responses observed with LOD cells were mediated by DC, CD11c⁺ cells were enriched from the SpLOD population. Similar levels of IL-12p40 were detected and treatment of CD11c⁺ SpLOD cells with laminarin as well as glucan phosphate, another soluble glucan known to block Dectin-1 activity (37) both reduced *M. tuberculosis*-induced IL-12p40 (Fig. 2A) as well as IL-12p70 (Fig. 2B) production. The effect of glucan treatment on *M. tuberculosis*-triggered IL-12p40 production was observed over a wide range of bacterial doses (Fig. 2C). Because we were unable to detect IL-23 production in these cultures by ELISA, the role of the β-glucan receptor in the induction of this cytokine could not be investigated. *M. tuberculosis* does, however, trigger TNF-α production by SpDC, albeit at lower levels compared with IL-12p40. Nevertheless, TNF-α production was not affected by even high concentrations of laminarin over a wide range of bacterial MOI (Fig. 2D) supporting a specific effect of β-glucan receptor blockade on mycobacterial-induced IL-12 synthesis.
Dectin-1, but not CD11b, promotes M. tuberculosis-triggered IL-12p40 production by SpDC

Because our glucan inhibition experiments suggested Dectin-1 involvement in the DC response to mycobacteria, we measured Dectin-1 levels on SpDC. Dectin-1 was found to be expressed to a greater degree on CD11b<sup>high</sup> SpDC compared with CD11b<sup>low</sup> cells (Fig. 3). As predicted from the staining results, Dectin-1 blockade had only a minor effect on mycobacterial-induced IL-12p40 production by sorted CD11b<sup>low</sup> SpDC (data not shown). Previous studies have indicated that complement receptor (CR) 3 plays a major role in the binding of M. tuberculosis by macrophages (38). Because CD11b, one of the subunits of CR3, contains a lectin-like domain (39) that has been implicated in the binding of M. tuberculosis (40), it was possible that the observed effects of glucan treatment on mycobacterial-triggered IL-12 production by SpDC could be due to interactions with this receptor. Nevertheless, we found that laminarin blocked M. tuberculosis-triggered IL-12p40 production independently of CD11b as this effect was not abrogated in SpDC isolated from CD11b<sup>−/−</sup> mice nor was CD11b itself required for IL-12p40 release in this population (Fig. 3C). CD11b<sup>high</sup> SpDC did seem to associate to a greater extent with Mtb-GFP than CD11b<sup>low</sup> SpDC (Fig. 3D, left panel) but this effect was not directly due to CD11b itself because GFP expression was similar in WT and CD11b<sup>−/−</sup> SpDC (Fig. 3D, right panel).

To confirm the glucan inhibition experiments, we assessed the ability of SpDC from Dectin-1<sup>−/−</sup> chimeric animals to respond to mycobacterial stimulation. These animals were generated by reconstituting irradiated WT animals with bone marrow from Dectin-1<sup>−/−</sup> mice. Importantly, total SpDC as well as the CD11b<sup>high</sup> subpopulation of SpDC from Dectin-1<sup>−/−</sup> chimeric animals displayed reduced IL-12p40 production relative to cells from control WT→WT chimeras following stimulation with live M. bovis BCG or live M. tuberculosis, respectively. In contrast, Dectin-1<sup>−/−</sup> chimeric cells mounted an unaltered IL-12p40 response to STAg (Fig. 3E).

**TLR2 deficiency does not affect Dectin-1-dependent M. tuberculosis-triggered IL-12p40 production by SpDC**

Because Dectin-1 has been shown to play a role in TLR2-driven cytokine production (19, 22, 41), the effect of laminarin inhibition on M. tuberculosis-induced IL-12p40 release by SpDC isolated from different TLR-deficient mice was investigated. Confirming our previous findings (12), mycobacterial-induced IL-12p40 production was only partially reduced in TLR2<sup>−/−</sup> SpDC while this reduction was more profound in TLR9<sup>−/−</sup> cells and totally ablated in MyD88<sup>−/−</sup> or TLR2<sup>−/−</sup>/TLR9<sup>−/−</sup> cells (Fig. 4). Significantly, Dectin-1 blockade in the absence of TLR2 lead to a further reduction in IL-12p40 production, indicating that interaction with the

**FIGURE 4.** Dectin-1 blockade in the absence of TLR2 but not TLR9 reduces Mtb-triggered IL-12p40 production by SpDC. Splenic CD11c<sup>+</sup> cells were isolated from WT, TLR2<sup>−/−</sup>, TLR9<sup>−/−</sup>, TLR2<sup>−/−</sup>/TLR9<sup>−/−</sup>, and MyD88<sup>−/−</sup> mice, pretreated with 100 µg/ml laminarin, and exposed to live M. tuberculosis. IL-12p40 levels were then measured. Bars indicate SEM. The experiment shown is representative of three performed. #, Statistically significant differences between untreated and laminarin-treated WT cells and untreated and laminarin-treated TLR2<sup>−/−</sup> cells.

**FIGURE 5.** Syk regulates Mtb-triggered IL-12p40 but not TNF-α production by SpDC. A, Splenic CD11c<sup>+</sup> cells were isolated from WT mice, pretreated with 500 µg/ml laminarin, and exposed to 50 µg/ml zymosan or live Mtb for 1 h. Protein extracts were then prepared, separated by SDS-PAGE, electroblotted onto nitrocellulose membranes, and immunoblotted with Abs against total or phosphorylated Syk. Relative band intensities were measured using Photoshop and displayed as bars under each sample. The experiment shown is representative of two performed. B and C, SpLOD cells were isolated from WT mice and enriched for CD11c<sup>+</sup> cells, pretreated with 1 µM piceatannol or DMSO, exposed to live Mtb at the indicated MOI, or 100 µg/ml zymosan or 2.5 µM CpG. Levels of IL-12p40 (B) or TNF-α (C) in overnight culture supernatants were measured by ELISA. Bars indicate SEM. The experiment shown is representative of three performed. *, Statistically significant differences between DMSO- and piceatannol-treated, Mtb-infected cells.
TLR2-triggered pathway of IL-12p40 production is not required for Dectin-1-dependent activity in SpDC. In contrast, Dectin-1 blockade in the absence of TLR9 had no effect on IL-12p40 synthesis, suggesting that Dectin-1 may function upstream of TLR9 signaling.

Pharmacological inhibition of Syk leads to impaired M. tuberculosis-triggered IL-12p40 production by SpDC

The role of Syk during M. tuberculosis infection of SpDC was also investigated because Dectin-1, through its ITAM-like motif, has been shown to activate Syk for the induction of cytokine production in DC (23). Phosphorylation of Syk was detectable but weak in M. tuberculosis-exposed SpDC and this response was reduced following laminarin treatment (Fig. 5A). Moreover, inhibition of Syk with piceatannol led to a reduction in M. tuberculosis-triggered IL-12p40 production over a wide range of MOI (Fig. 5B) but did not affect CpG-induced production of the cytokine, and as reported elsewhere (23), zymosan-stimulated IL-12p40 release. Piceatannol treatment did not affect TNF-α release following stimulation with M. tuberculosis, zymosan, or CpG (Fig. 5C). The inability of piceatannol in reducing mycobacterial-triggered TNF-α was independent of the dose of mycobacteria used (Fig. 5C).

Dectin-1 binds to mycobacteria and mediates enhanced association of bacilli with SpDC

To test whether there is direct interaction between mycobacteria and Dectin-1, a recently characterized Dectin-Fc fusion protein (36) was used. As expected, Dectin-Fc bound directly to zymosan (Fig. 6A). Interestingly, this fusion protein also interacted with live M. bovis BCG (Fig. 6B). Although this binding was not as dramatic as that observed with zymosan, preincubation with laminarin blocked the interaction of Dectin-Fc with mycobacteria to the same extent as with zymosan. In a second assay involving measurement of adherent bacteria by CFU, laminarin treatment led to a reduction in the binding of M. tuberculosis to SpDC (Fig. 6C). Furthermore, in a third microscopy-based assay using BCG-DsRed and FITC-conjugated zymosan, laminarin treatment also resulted in reduced association of bacteria and zymosan with SpDC (Fig. 6D). Thus, through its ability to directly interact with mycobacteria, Dectin-1 appears to promote the binding and/or uptake of bacilli by SpDC. Interestingly, inhibition of Syk with piceatannol did not affect association of BCG to DC although it did partially reduce the association of zymosan to these cells (Fig. 6D).

Discussion

DCs are thought to play an important role in the induction of host resistance to mycobacteria. Direct evidence for this function has
been obtained in experiments in which elevated bacterial counts and reduced mycobacterial-specific IFN-γ production by CD4+ T cells were observed in *M. tuberculosis*-infected CD11c-DTR-transgenic mice following depletion of SpDC (42). An important question concerns the nature of the PRR(s) on DC that recognize mycobacterial bacilli and trigger their function. Previous studies have indicated a major role for TLR signaling in this process and have implicated the specific involvement of TLR2 and TLR9 in mediating the response of these cells to live *M. tuberculosis* infection (12, 43). That non-TLR PRR are also involved in innate recognition of mycobacteria was first revealed in studies demonstrating the importance of CR3, scavenger receptors, and CLR such as mannose receptor in the uptake of mycobacteria by macrophages (44, 45). The findings that ligation of mannose receptor or DC-SIGN on DC inhibits TLR-induced IL-12 (46) indicate that CLR can additionally play a role in regulating mycobacterial-induced cytokine production by these cells. The observations reported here on the role of the β-glucan receptor in IL-12 induction by DC support these earlier findings but argue that the role of this CLR in mycobacterial recognition is stimulatory rather than inhibitory.

Based on its blockade by either laminarin or glucan phosphate, the β-glucan receptor involved in stimulation of IL-12p40 production by DC appears to be Dectin-1. Dectin-1−/− mice (34, 47) have only recently been generated and were not available for isolation of SpDC in our studies. However, experiments with chimeras constructed with bone marrow from Dectin-1-deficient mice confirmed the involvement of the receptor in promoting mycobacterial-induced IL-12p40 production by SpDC. Nevertheless, in both the glucan inhibition experiments and Dectin-1−/−/chimeras, a residual Dectin-1-independent IL-12p40 response was observed. This finding could be explained by a requirement for Dectin-1 to cooperate with other PRR (e.g., TLR2) for full activation of DC or alternatively an indirect facilitatory role of Dectin-1 in promoting the interaction of mycobacterial bacilli with other receptor(s) that directly trigger production of the cytokine. An additional possibility that is difficult to test is that the residual Dectin-1-independent response is due to the compensatory function of a different PRR up-regulated in the absence of Dectin-1 signaling.

The hypothesis that Dectin-1 functions by cooperating with other cytokine-inducing PPR is supported by the observation that optimal zymosan induced TNF-α and IL-12p40 production by macrophages depends on dual ligation of Dectin-1 and TLR2 (19, 20). Interestingly, TLR2-dependent, *Mycobacterium smegmatis*-induced TNF-α production by bone marrow-derived macrophages has recently been shown to involve Dectin-1 but this interaction was not observed when the same cells were stimulated with *M. tuberculosis* (41). Our findings with PEC as well as unpublished observations with a Dectin-1-transfected macrophage (RAW) cell line also support the notion that Dectin-1 is not required for *M. tuberculosis*-triggered cytokine responses in macrophages (Fig. 1A and data not shown). Nevertheless, we did observe a major contribution of Dectin-1 in promoting *M. tuberculosis*-induced IL-12p40 production by SpDC, thus extending the involvement of this PRR from the recognition of avirulent mycobacteria to this important human pathogen. Significantly, in contrast to the aforementioned studies on the recognition of zymosan and *M. smegmatis*, our findings failed to reveal a major cooperation between Dectin-1 and TLR2 in *M. tuberculosis*-induced IL-12p40 production as evidenced by the clear-cut effect of Dectin-1 blockade on the response of TLR2−/− DC to the pathogen.

The above observation suggested that Dectin-1 interaction with mycobacteria might instead play an indirect, facilitatory role in triggering of IL-12p40 production by SpDC by promoting the association of mycobacteria with these cells. Consistent with this interpretation, inhibition of internalization of *M. tuberculosis* through cytochalasin D treatment has been previously shown to reduce IL-12p40 release by DC (12, 48). In a similar fashion, reduced internalization of mycobacteria in the absence of Dectin-1 engagement would limit the interaction of intracellular bacteria with TLR9, an endosomal receptor (49, 50) that has been shown to be critically involved in *M. tuberculosis*-induced IL-12p40 production by SpDC (12). That Dectin-1 functions upstream of TLR9 triggering is consistent with the observation that laminarin fails to further reduce IL-12p40 secretion in TLR9−/− DC stimulated with mycobacteria. Whether Dectin-1 specifically promotes trafficking of bacteria into TLR9-containing endosomes remains to be determined. Alternatively, Dectin-1 might facilitate internalization and TLR9 interaction by simply enhancing the binding of mycobacteria to the cell surface. This interpretation is supported by experiments (data not shown) in which laminarin treatment was found to reduce the association of BCG with BMDC at 4°C, a temperature at which particle binding can be studied in the absence of internalization (19, 35, 51).

In addition to its functions in cellular uptake, Dectin-1 is known to directly trigger cytokine production by a signaling pathway involving the tyrosine kinase Syk. In the case of zymosan-stimulated BMDC, this pathway also regulates particle uptake (23). Although *M. tuberculosis*-induced phosphorylation of Syk in SpDC was not as pronounced as that induced by zymosan, this response was strongly inhibited by laminarin indicating a role for Dectin-1 in Syk activation. In addition, inhibition of Syk with piceatannol lead to a reduction in mycobacterial-induced IL-12p40 production. Nevertheless, using the assays available to us involving drug treatment, we were unable to establish (or for that matter formally exclude) a role for Syk in bacterial uptake, as has been previously demonstrated in experiments on zymosan internalization using Syk−/− BMDC (23). Studies on mycobacterial uptake and cytokine production which compare piceatannol-treated with Syk−/− SpDC should help resolve this discrepancy. Interestingly, Syk has been shown to immunoprecipitate with TLR9 in the monocytic cell line THP-1 following stimulation with CpG (52). Therefore, one possible mechanism by which Syk could promote IL-12 production is by mediating the intracellular trafficking of mycobacteria-bound Dectin-1 to TLR9 in endosomal compartments.

The binding of Dectin-Fc to mycobacteria was unexpected because β-glucans, while previously detected in bacteria such as *Brucella*, *Agrobacterium*, and *Rhizobium* (53, 54), have not been described in *Mycobacterium* sp. Although the mycobacterial genome does not appear to contain genes encoding the enzymes traditionally associated with β-glucan synthesis this does not exclude the presence of potential β-glucan synthase ortholog(s) in mycobacteria. Alternatively, components of the mycobacterial cell wall may mimic carbohydrate structures present in fungal β-glucans. Indeed, serological cross-reactivities have been noted between β-glucans and both *M. tuberculosis* cell walls and live *M. bovis* BCG (55). In addition, Dectin-1 has been described to interact with an endogenous protease-sensitive ligand on T cells (56), suggesting that this receptor may have the potential to bind to noncarbohydrate ligands as well.

An important issue not addressed in this study because of the shortage of knockout mice concerns the role of Dectin-1 in host resistance to mycobacterial infection. As noted previously in this report, IL-12p40 plays an essential role in immune control of *M. tuberculosis* through its associations with p35 to form IL-12, p19 to form IL-23, and as a homodimer. Thus, one might expect Dectin-1−/− mice to show decreased resistance associated with impaired production of these cytokines. Dectin-1 has
been shown to be expressed in DC in the T cell areas of the spleen and lymph nodes (57) and targeting of Ag to Dectin-1-expressing SpDC promotes Ag-specific CD4+ T cell-derived IFN-γ production in vivo (58). Therefore, it is logical to propose that the association of mycobacteria with Dectin-1 on DC and other APC in vivo might promote both cytokine production and effector T cell activity in hosts exposed to the pathogen. If such a role can be confirmed, these observations would establish a function for Dectin-1 in host defense that extends beyond its original description as a fungal PRR.

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Disclosures

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References


