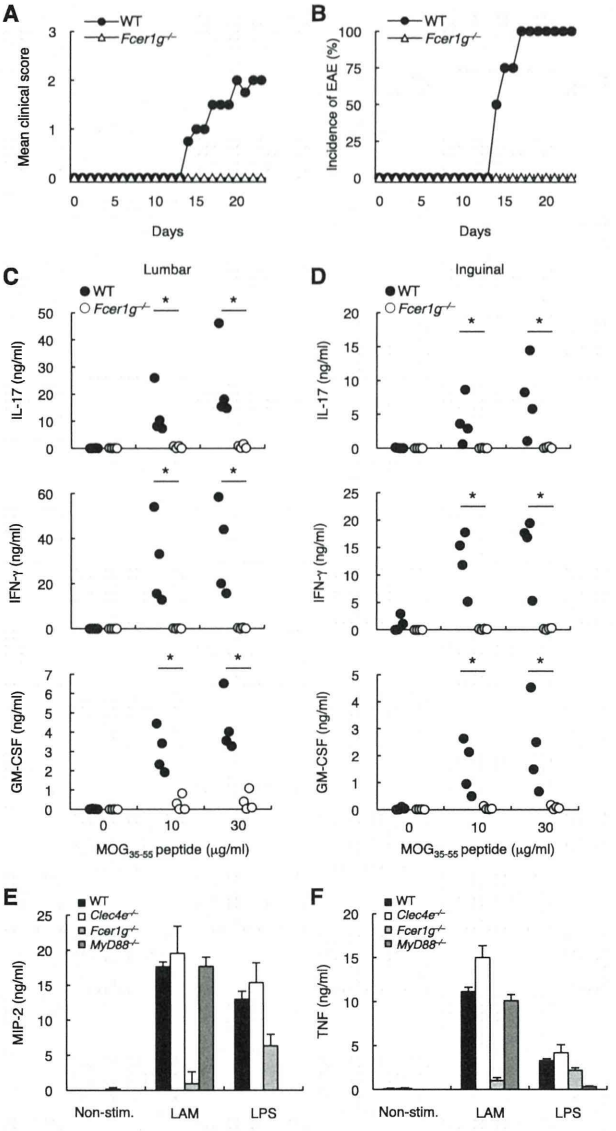

Figure 1. LAM Induces EAE through FcRγ Axis

(A and B) WT (n = 4) and *Fcer1g*$^{-/-}$ (n = 4) mice were immunized with MOG_{35-55} peptide in IFA containing LAM (500 μg), followed by i.p. injection of PT (500 ng) (day 1, 2, and 3). The disease severity of each mouse was scored, and mean clinical score (A) and disease incidence (B) at the indicated times were plotted.

(C and D) Lumbar (C) and inguinal (D) lymph nodes were collected at 23 days after immunization. Lymph node cells were stimulated with MOG_{35-55} peptide for 4 days. Concentrations of IL-17, IFN-γ, and GM-CSF were determined with ELISA.

(E and F) BMDCs obtained from WT, *Clec4e*$^{-/-}$, *Fcer1g*$^{-/-}$, or *MyD88*$^{-/-}$ mice were stimulated with plate-coated LAM (0.3 μg/well) or LPS (10 ng/ml) for 48 hr. The concentrations of MIP-2 (E) and TNF (F) were measured using ELISA.

(A–D) Data are representative of two separate experiments.

(E and F) All data are presented as the means ± SD of triplicate and are representative of three separate experiments.

See also Figure S1.

suggesting the possibility that unidentified molecules act as receptors for Man-LAM.

We recently demonstrated that CLRs Mincle (gene symbol *Clec4e*) and MCL (gene symbol *Clec4d*) are Fc receptor γ chain (FcRγ, gene symbol *Fcer1g*)-coupled activating receptors for mycobacterial glycolipids (Ishikawa et al., 2009; Miyake et al., 2013). Another CLR, DC-associated C-type lectin-2 (Dectin-2, gene symbol *Clec4n*), is located adjacent to Mincle and MCL within the gene cluster on chromosome 6. Dectin-2 is an FcRγ-coupled CLR (Sato et al., 2006) that recognizes *Candida albicans* hyphae to mediate host defense against the fungus (Robinson et al., 2009; Saijo et al., 2010). Dectin-2 and MCL seem to have arisen by gene duplication from Mincle after placentalia and are well conserved among species (Miyake et al., 2013). These findings imply that these CLRs within the gene cluster might have evolved as "mycobacterial receptors" and that Dectin-2 might also recognize mycobacteria.

In this study, we show that Dectin-2 is a direct receptor for Man-LAM. Man-LAM recognition by Dectin-2 induced the production of both pro- and anti-inflammatory cytokines in DCs. Man-LAM potently promoted T-cell-mediated acquired immunity as an adjuvant without causing detrimental inflammation. We further demonstrate, through Dectin-2-deficient mice, that Dectin-2 plays a critical role in host responses against mycobacterial infection. Collectively, these findings indicate that Dectin-2 acts as a functional PRR for mycobacterial Man-LAM.

RESULTS

LAM Promotes Experimental Autoimmune Encephalomyelitis through FcRγ

We first investigated whether LAM possess adjuvant activity in vivo. To this end, we performed a murine model of T-cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis (EAE) (Figures 1A–1D). Mice were immunized with myelin oligodendrocyte glycoprotein (MOG) peptide together with LAM derived from virulent *M. tuberculosis* strain Aoyama B. Although EAE was not induced by incomplete Freund's adjuvant (IFA) alone (data not shown), a single injection of LAM elicited EAE with 100% incidence (Figures 1A and 1B). The EAE symptoms were completely abrogated in *Fcer1g*$^{-/-}$ mice (Figures 1A and 1B), suggesting that FcRγ-coupled receptor(s) might contribute to LAM-induced EAE. Furthermore, in contrast to lymphocytes from wild-type mice, lymphocytes from *Fcer1g*$^{-/-}$ mice exhibited impaired ex vivo recall responses to MOG peptides as judged by the production of IL-17, interferon-γ (IFN-γ), and granulocyte macrophage-colony stimulating factor (GM-CSF) (Figures 1C and 1D). These results indicate that LAM could act as a potent adjuvant leading to the development of EAE through an FcRγ-dependent pathway.

Because FcRγ is mainly expressed in myeloid cells, we next treated DCs with LAM in vitro. To recapitulate uniform configuration and multivalency of LAM on the bacterial wall, LAM was coated on culture plate for the stimulation of bone-marrow-derived DCs (BMDCs). Although soluble LAM did not induce cytokine production (data not shown), plate-coated LAM could stimulate BMDCs to secrete a large amount of the proinflammatory cytokines, macrophage inflammatory protein-2 (MIP-2), and

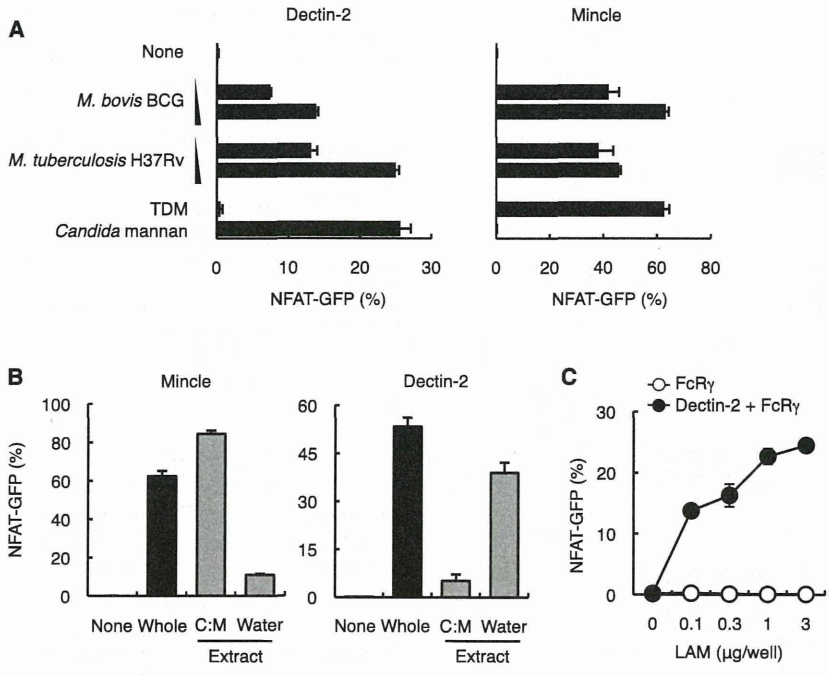

Figure 2. Dectin-2 Recognizes Pathogenic Mycobacterial Species though LAM

(A) NFAT-GFP reporter cells expressing Mincle + FcRγ (Mincle) or Dectin-2 + FcRγ (Dectin-2) were stimulated with heat-killed *M. tuberculosis* H37Rv or *M. bovis* BCG. TDM and *Candida albicans* cell wall mannan were used in plate-coated form as a positive control.

(B) Reporter cells were stimulated with plate-coated water extract or C:M extract for 24 hr.

(C) Reporter cells expressing FcRγ alone or Dectin-2 + FcRγ were stimulated with the indicated amounts of LAM derived from *M. tuberculosis* strain Aoyama B in plate-coated form for 24 hr.

Induction of NFAT-GFP was analyzed using flow cytometry. All data are presented as the means ± SD of triplicate assays and representative results from three independent experiments with similar results are shown. See also Figure S2.

TNF (Figures 1E and 1F). These cytokines were still produced in *Clec4e*$^{-/-}$ (Figures 1E and 1F) and *Clec4d*$^{-/-}$ (data not shown) DCs. MyD88 was dispensable for this response, indicating that toll-like receptors (TLRs) do not play major role in LAM recognition. In contrast, LAM-induced cytokine production was abrogated in *Fcer1g*$^{-/-}$ DCs. These results suggest that some unknown FcRγ-coupled receptor(s) might function as an activating receptor for LAM in BMDCs.

Dectin-2 Recognizes Mycobacterial LAM

Mincle, MCL, and Dectin-2 are FcRγ-coupled activating receptors within the same gene cluster, and two of these receptors, Mincle and MCL, recognize mycobacteria (Ishikawa et al., 2009; Miyake et al., 2013). We therefore assumed that Dectin-2 might also be evolved as a receptor for mycobacteria. Indeed, Dectin-2 was demonstrated to recognize the virulent strain *M. tuberculosis* H37Rv and the vaccine strain *M. bovis* Bacille de Calmette et Guérin (BCG) to activate the reporter cells, in a similar manner to Mincle (Figure 2A). However, the ligand for Dectin-2 was distinct from Mincle ligand trehalose-6,6′-dimycolate (TDM) (Figure 2A).

We next fractionated components of *M. bovis* BCG using lipophilic and hydrophilic solvents, such as chloroform:methanol (C:M) and water. The Dectin-2 ligand activity for each of the extracts was assessed in a plate-coated form using reporter cells. We found that only the water phase demonstrated a stimulatory activity for Dectin-2 (Figure 2B, right), in sharp contrast to the C:M phase that activates Mincle-expressing cells (Figure 2B, left) (Ishikawa et al., 2009). These results suggest that the hydrophilic components of mycobacteria are candidates for the Dectin-2 ligand. Among mycobacterial hydrophilic components, LAM constitutes the most abundant hydrophilic lipoglycan (Figure S1 available online; Leopold and Fischer, 1993). In agreement with our prediction, LAM derived from *M. tuberculosis* activated reporter cells expressing Dectin-2 (Figure 2C). Dectin-2 directly recognized the LAM, as shown by the fact that soluble Dectin-2 protein (Dectin-2-Ig) bound to purified LAM in a dose-dependent manner (Figure S2). These findings indicate that Dectin-2 is a direct receptor for mycobacterial LAM.

Dectin-2 Recognizes Mycobacteria through Mannose-Capped LAM

The structure of LAM differs depending on the mycobacterial species, particularly with the respect to the capping moieties (Briken et al., 2004). The slow-growing strains including *M. tuberculosis* and *M. bovis* BCG possess Man-LAM, whereas rapid-growing strains of mycobacteria do not. *M. smegmatis* possesses PI-LAM. To clarify which structure of LAM is responsible for the interaction with Dectin-2, we used various strains of mycobacteria including nontuberculosis mycobacteria (NTM).

Dectin-2 recognized slow-growing strains, such as *M. intracellulare* and *M. gordonae*, which possess Man-LAM. In contrast, Dectin-2 did not recognize *M. abscessus* and *M. smegmatis* that lack mannose capping (Figure 3A). Importantly, Mincle was capable of recognizing these strains (Figure 3A). We confirmed that none of these strains activated reporter cells expressing FcRγ alone (data not shown). These results suggest that Dectin-2 preferentially recognizes the mycobacterial species that express Man-LAM. Supporting this notion, the activity of Man-LAM derived from *M. tuberculosis* (Figure 2C) was abolished by treatment with α-mannosidase that removes terminal mannose caps (Figure 3B). These results indicate that the capping structures are crucial determinants for the recognition of Man-LAM by Dectin-2.

We next investigated whether the mannose binding capacity of Dectin-2 is involved in Man-LAM recognition. To this end, we employed the Dectin-2^{QPD} mutant in which the mannose-binding activity was eliminated by substituting EPN (glutamic acid-proline-asparagine) sequence into galactose-type QPD (glutamine-proline-asparagic acid) sequence (Drickamer,

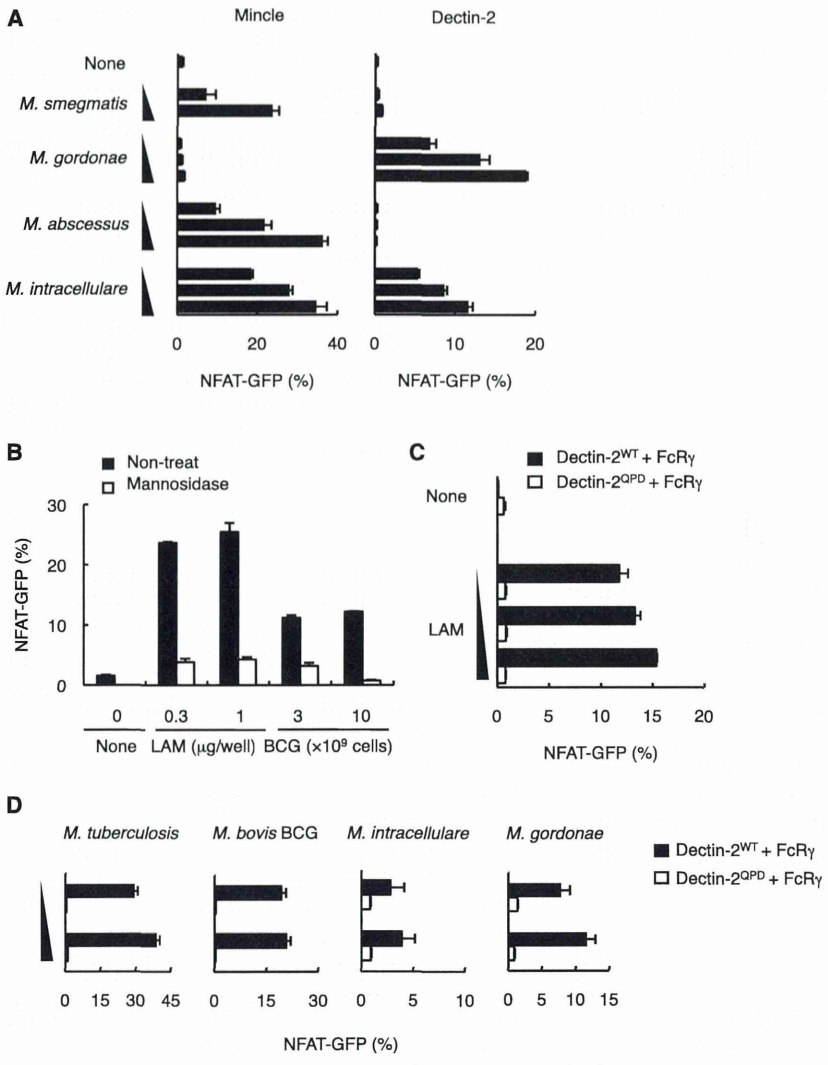

Figure 3. Dectin-2 Selectively Recognizes Mannose-Capped LAM

(A) NFAT-GFP reporter cells expressing Mincle + FcRγ or Dectin-2 + FcRγ were stimulated with the indicated strains of heat-killed NTM.

(B) Reporter cells expressing Dectin-2 + FcRγ were stimulated with *M. bovis* BCG pretreated with or without α-mannosidase for 24 hr.

(C and D) Reporter cells expressing Dectin-2^{WT} + FcRγ or Dectin-2^{QPD} + FcRγ were stimulated with plate-coated LAM (C) and NTM strains (D) for 24 hr.

All data are presented as the means ± SD of triplicate and are representative of three separate experiments. See also Figure S3.

1992; Ishikawa et al., 2013). Man-LAM did not activate reporter cells expressing Dectin-2^{QPD} (Figure 3C) regardless of similar fluorescence intensities of wild-type Dectin-2 and Dectin-2^{QPD} on the cell surface (Figure S3). The recognition of whole mycobacteria by Dectin-2 was also dependent on this EPN motif (Figure 3D). Collectively, these results indicate that both mannose caps of Man-LAM and mannose-recognition property of Dectin-2 are required for the interaction of Man-LAM with Dectin-2.

Man-LAM Induces Cytokine Production by DCs in a Dectin-2-Dependent Manner

Among the myeloid cells, DCs most abundantly express Dectin-2 (Ariizumi et al., 2000). We therefore investigated the cytokine production in response to Man-LAM in BMDCs. Man-LAM, similar to TDM, induced the expression of inflammatory cytokines, such as MIP-2, TNF, and IL-6, in a dose-dependent manner (Figure 4A). The LAM-induced cytokine production was abolished in *Clec4n*$^{-/-}$ BMDCs, whereas the TDM-mediated cytokine production was not altered (Figure 4A). Man-LAM also slightly enhanced IL-12p40 in a Dectin-2-dependent manner (data not shown). The production of TNF and IL-6 upon *M. bovis* BCG infection was partially decreased in *Clec4n*$^{-/-}$ BMDCs as compared with WT BMDCs, although there remained Dectin-2-independent cytokine production (Figure 4B). These data indicate that Dectin-2 is critical for Man-LAM-mediated proinflammatory cytokine production in DCs.

We next focused on the anti-inflammatory potential of Man-LAM-Dectin-2 pathway, due to the fact that accumulating evidence has emphasized the importance of the immune-suppressive action of Man-LAM (Geijtenbeek et al., 2003; Wieland et al., 2007). In addition to proinflammatory cytokines, Man-LAM potently induced the production of anti-inflammatory cytokine, IL-10, in BMDCs (Figure 4C, left). Other pathogen-associated molecular patterns (PAMPs), TDM (Figure 4C, right) and LPS (data not shown), did not induce the secretion of IL-10 and IL-2, indicating that Man-LAM has a unique profile of the cytokine production. Man-LAM-induced release of these cytokines was completely suppressed in *Clec4n*$^{-/-}$ BMDCs (Figure 4C). Although TNF production during *M. bovis* BCG infection was partially dependent on Dectin-2 as described above (Figure 4B), the production of IL-10 and IL-2 was almost completely lost in *Clec4n*$^{-/-}$ DCs infected with *M. bovis* BCG (Figure 4D). Meanwhile, *M. abscessus*, which is absent from mannose caps, failed to induce the production of IL-10 and IL-2, compared to the capability of MIP-2 production (Figure 4E). These results suggest a central role of Dectin-2 in the production of IL-10 and IL-2 in response to mycobacteria.

Dectin-2-Mediated Intrinsic Signal Regulates Man-LAM-Induced Cytokine Production in DCs

It is clear that Dectin-2 is required for the cytokine production induced by Man-LAM, because such production is abrogated in *Clec4n*$^{-/-}$ cells (Figures 4A and 4C). However, it is still

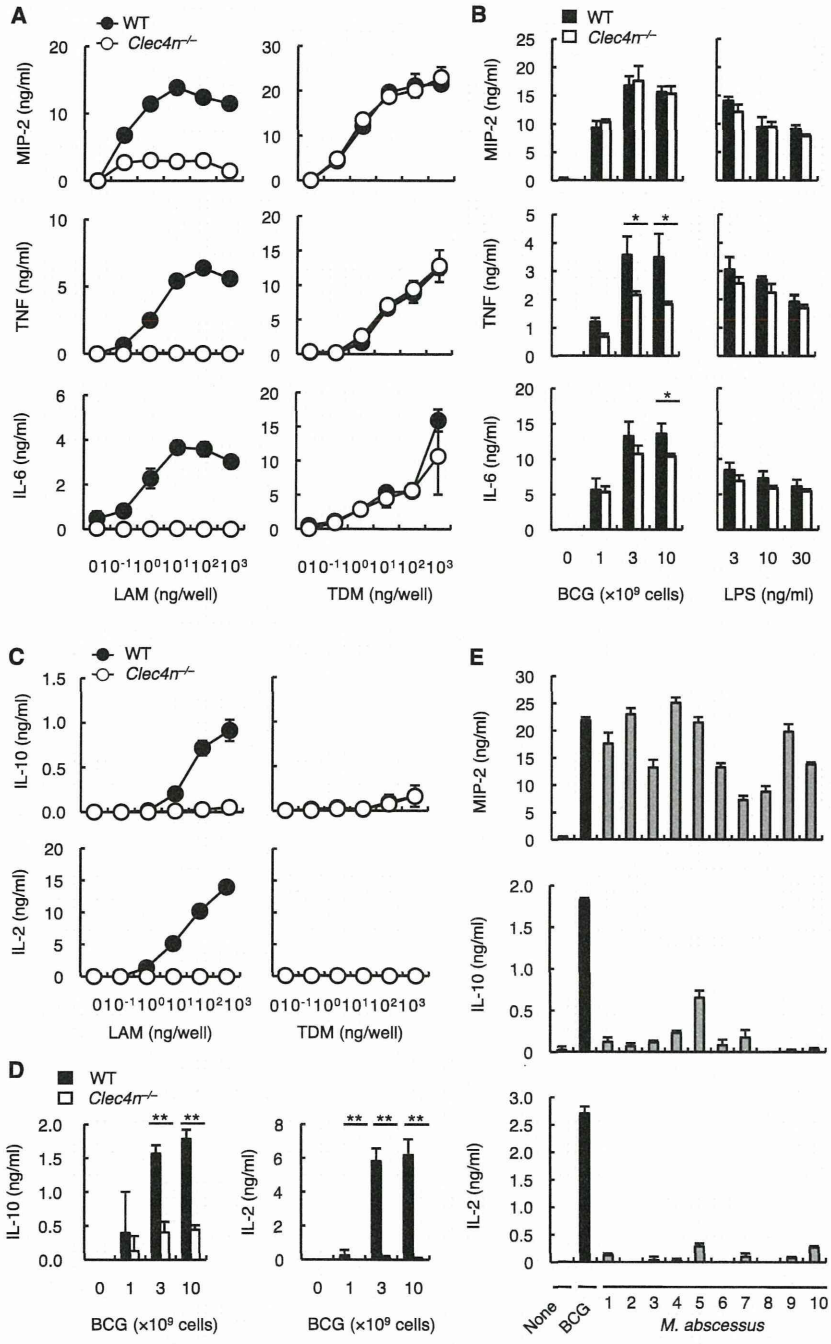

Figure 4. LAM Induces Cytokine Production in a Dectin-2-Dependent Manner

(A–D) BMDCs obtained from WT or *Clec4n*$^{-/-}$ mice were stimulated with the indicated amounts of plate-coated LAM or TDM for 48 hr (A and C). BMDCs were infected with 1 to 10 × 10^9 of *M. bovis* BCG for 48 hr (B and D). LPS was used as control. The concentrations of MIP-2, TNF, IL-6 (A and B), IL-10, and IL-2 (C and D) were measured using ELISA.

(E) BMDCs were stimulated for 48 hr with *M. bovis* BCG or clinical isolates of heat-killed *M. abscessus* derived from ten individual patients. The concentrations of MIP-2, IL-10, and IL-2 were measured using ELISA.

All data are presented as the means ± SD of triplicate and are representative of three separate experiments. See also Figure S4.

uncertain whether the Dectin-2-mediated intrinsic signal is sufficient or whether some other coreceptor is also required for these responses. The unique cytokine profiles, particularly IL-10 and IL-2 production, may be conferred by the coengagement of Dectin-2 and other Man-LAM receptors. To examine this possibility, we investigated the contribution of several candidate receptors for Man-LAM. Since SIGNR1, a putative murine homolog of human DC-SIGN, recognizes Man-LAM (Koppel et al., 2004), we evaluated the role of SIGNR1 by establishing SIGNR1-deficient mice (Figure S4A). However, BMDCs lacking SIGNR1 were still capable of producing all cytokines tested at amounts comparable to those in WT BMDCs (Figure S4B). Another DC-SIGN homolog, SIGNR3, which is expressed in a limited population of myeloid cells (Nagaoka et al., 2010), was not detected in BMDCs (Figure S4C). Furthermore, forced expression of SIGNR3 in BMDCs did not increase Man-LAM-induced cytokine production (Figure S4D). Notably, *Clec4n*$^{-/-}$ BMDCs failed to produce cytokines even with the expression of SIGNR3 (Figure S4D). These results collectively indicate that both SIGNR1 and SIGNR3 are not essential for cytokine production induced by Man-LAM in BMDCs.

Macrophage mannose receptor (MMR) could also bind to Man-LAM (Nigou et al., 2001). Because MMR expression was detected in BMDCs (Figure S4C), we assessed its role in Man-LAM-induced cytokine release by using anti-MMR blocking monoclonal antibody (mAb). However, the mAb treatment did not influence the production of IL-10 and IL-2 in BMDCs (Figure S4E).

A recent report has shown that Dectin-2 is capable of associating with MCL (Zhu et al., 2013). Meanwhile, analyses of *Clec4d*$^{-/-}$ BMDCs revealed that MCL is not required for the Man-LAM-induced cytokine production (Figure S4F). Man-LAM is weakly recognized by TLR2 and TLR4 (Mazurek et al., 2012). However, IL-10 production induced by Man-LAM was not altered in *MyD88*$^{-/-}$ BMDCs (Figure S4G), suggesting that TLR-MyD88 signaling does not play a major role in the effect of Man-LAM. Finally, we confirmed that the direct engagement of Dectin-2 alone by anti-Dectin-2 cross-linking replicated the production of IL-10 (Figure S4H).

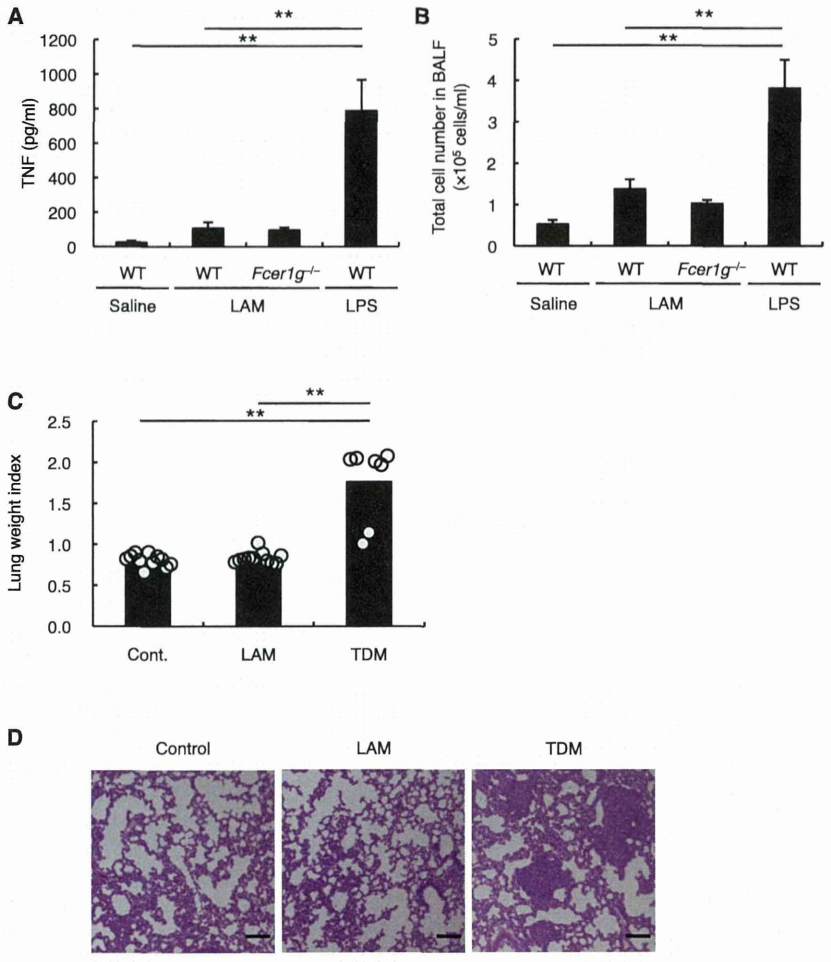

Figure 5. LAM Does Not Cause Excessive Inflammation in the Lungs

(A and B) Mice were intratracheally administered 100 μg of LAM (WT, n = 13; *Fcer1g*$^{-/-}$, n = 5), 10 μg of LPS (WT, n = 9), or 100 μl of sterile saline as a control (WT, n = 7). After 8 hr, BALF was obtained and then TNF concentrations in the BALF were determined using ELISA (A). The number of total cells was determined by hemocytometer (B). All data are presented as the means ± SD.

(C) Lungs of control mice (Cont., n = 11), 50 μg of LAM (LAM, n = 11)- or 50 μg of TDM (TDM, n = 7)-injected mice were isolated at day 7 and inflammatory intensity was evaluated by calculation of lung weight index. Each symbol represents an individual mouse. Data are representative of three separate experiments.

(D) Histology of the lungs from control, LAM (50 μg)-, or TDM (50 μg)-injected mice were was examined by hematoxylin-eosin staining at day 7. Scale bars represent 0.1 mm. Data are representative of three separate experiments.

Collectively, these results suggest that the IL-10-inducing potential of Man-LAM is likely due to the intrinsic properties of Dectin-2-mediated signaling.

Man-LAM Induces Minimal Inflammation In Vivo

To investigate whether Man-LAM triggers any inflammatory responses, we evaluated the response of mice to Man-LAM administration in vivo. The infiltration of inflammatory cells and cytokine production in bronchoalveolar lavage fluid (BALF) was examined following the intratracheal administration of LAM or LPS (Figures 5A and 5B). LPS induced a significant increase in TNF production and cell infiltration in WT mice. In contrast, Man-LAM did not induce marked inflammatory responses. Consistent with this observation, FcRγ deficiency had no apparent effect compared with Man-LAM-treated WT mice.

Intravenous injection of TDM induced inflammatory lung swelling as assessed by lung weight index (LWI) (Figure 5C) and granuloma formation in lungs (Figure 5D, right) as previously reported (Ishikawa et al., 2009). In contrast, the same amount of Man-LAM induced neither lung swelling (Figure 5C) nor granuloma formation (Figure 5D, middle). These results indicate that Man-LAM does not induce strong pulmonary inflammation compared with other PAMPs such as TDM or LPS.

Man-LAM Stimulation Enhances APC Functions to Promote IL-17 Production In Vitro

We further evaluated the adjuvant activity of Man-LAM in vitro. To investigate the effect of Man-LAM on DC maturation, we examined the expression of costimulatory molecules on BMDCs after Man-LAM stimulation. Man-LAM stimulation upregulated the expression of CD40 and CD80 on WT BMDCs, which were comparable to those induced by LPS (Figure 6A). However, the induction of these costimulatory molecules was abolished in the absence of Dectin-2 and its subunit FcRγ (Figure 6A). Mincle was dispensable for the LAM-induced responses. We confirmed that the LPS-mediated responses were not altered in these mice. These results demonstrate that Man-LAM promotes DC maturation in a Dectin-2-dependent manner.

We next examined the function of antigen-presenting cells (APCs) upon Man-LAM stimulation. BMDCs were pulsed with the ovalbumin (OVA) antigen peptides and cocultured with T cells obtained from OVA-specific OT-II TCR transgenic mice in the presence or absence of Man-LAM. Because T cells do not express Dectin-2 (Ariizumi et al., 2000), this system enables us to evaluate the role of Man-LAM in APC functions toward T cell priming and activation. The antigen-specific secretion of IL-17 from CD4^{+} OT-II T cells was significantly augmented when the cells were cocultured with Man-LAM-treated APCs (Figure 6B). However, this enhancement was markedly attenuated when *Clec4n*$^{-/-}$ APCs were used. The antigen-induced T cell proliferation, as assessed by CFSE dilution, was observed regardless of Dectin-2 expression on DCs (Figure 6B, bottom). The concentration of IL-10 in coculture supernatant was increased depending on the antigen dose, suggesting the generation of IL-10-producing T cells in the presence of DCs stimulated through Man-LAM-Dectin-2 axis (Figure 6B).

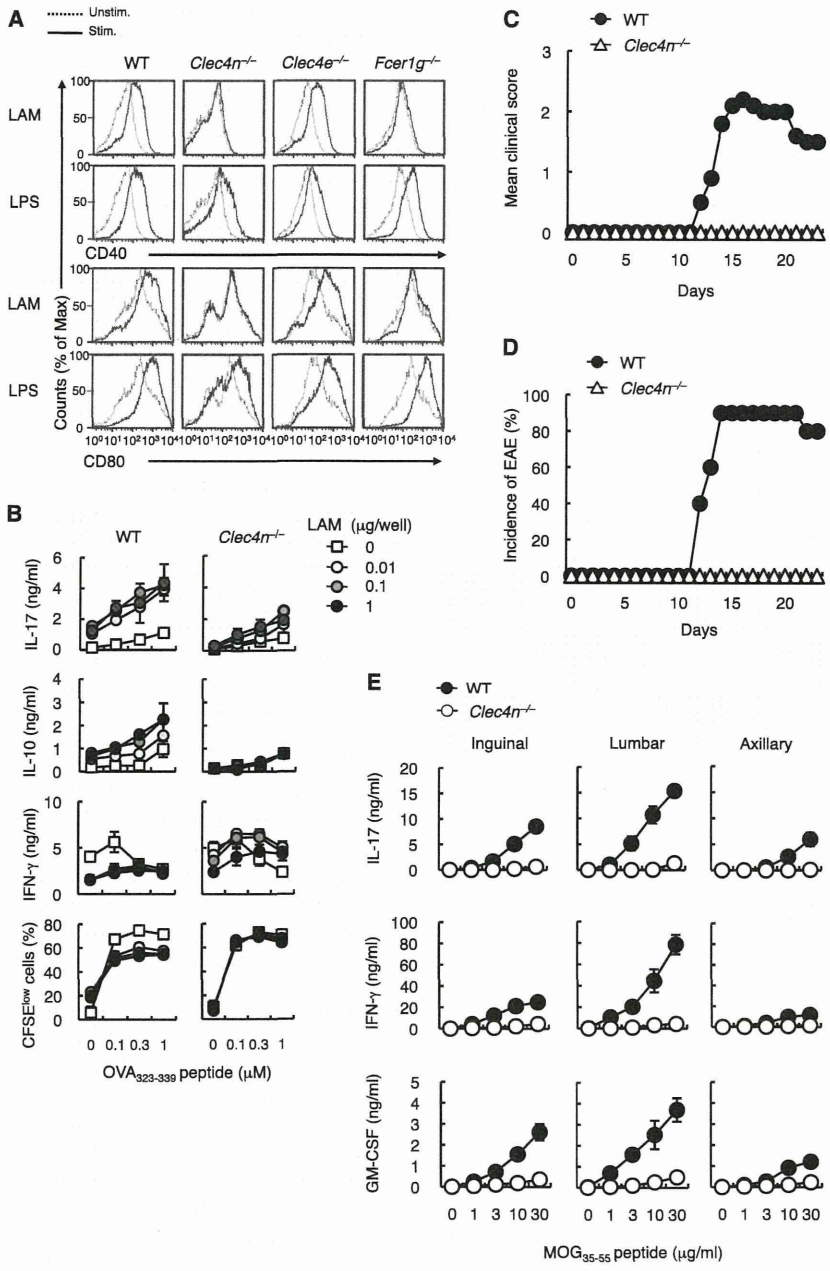

Figure 6. LAM Induces Acquired Immune Responses through Dectin-2

(A) BMDCs obtained from WT, *Clec4n*$^{-/-}$, *Clec4e*$^{-/-}$, or *Fcer1g*$^{-/-}$ mice were left untreated or stimulated with plate-coated LAM or LPS for 48 hr. The surface expressions of CD40 and CD80 were analyzed using flow cytometry.

(B) BMDCs were pulsed with $OVA_{323-339}$ peptides and cocultured with CFSE-labeled CD4$^+$ OT-II T cells for 3 days in the presence or absence of plate-coated LAM. Cytokine concentrations were determined using ELISA. Cell proliferation was analyzed using flow cytometry for dilution of CFSE within the CD4$^+$ population. The data are presented as the means ± SD of triplicate and are representative of three separate experiments.

(C and D) WT (n = 10) and *Clec4n*$^{-/-}$ (n = 10) mice were immunized with MOG_{35-55} peptide in IFA containing LAM (500 μg) as described in Figure 1A. Mean clinical score (C) and disease incidence (D) at the indicated times were plotted.

(E) Lymph nodes were collected at 23 days after immunization for EAE and stimulated with MOG_{35-55} peptide for 4 days. Cytokine concentrations were determined using ELISA. The data are presented as the means ± SD.

See also Figure S5.

Man-LAM induced weak T cell proliferation in T cell-DC coculture even in the absence of antigen, which also required Dectin-2 on DCs (Figure 6B). This "antigen-independent proliferation" might be conferred by a large amount of IL-2 secretion through Dectin-2 on DCs (Figure 4C), because the addition of anti-IL-2 neutralizing mAb ablated this response (Figure S5A).

In contrast to the enhancement of IL-17 production, Man-LAM treatment had essentially no effect on the antigen-dependent IFN-γ production (Figure 6B). IL-4 was not detected at any time point in this experiment (data not shown). Collectively, these in vitro results suggest that Man-LAM stimulation enhances APC functions to promote IL-17 production in a Dectin-2-dependent manner.

Man-LAM Promotes Antigen-Specific Human T Cell Responses through Human Dectin-2

We then assessed whether Man-LAM influences human T cell responses as observed in murine T cells. Importantly, Man-LAM activated reporter cells expressing hDectin-2, and this activity was blocked in the presence of anti-hDectin-2 mAb (Figure S5B). The Man-LAM-induced cytokine production in human monocytes and monocyte-derived DCs was also significantly suppressed by anti-hDectin-2 mAb (Figures S5C and S5D). Peripheral blood mononuclear cells (PBMCs) from tuberculosis patients were stimulated with C10 peptide (VVRFQEAANKQKQEL) of CFP-10 (10 kDa culture filtrate antigen) from *M. tuberculosis*. Antigen peptides alone induced a substantial amount of IFN-γ production in PBMCs, whereas it was augmented upon Man-LAM stimulation in combination with antigen peptides. Man-LAM-induced augmentation of IFN-γ production was markedly compromised in the presence of anti-hDectin-2 mAb (Figure S5E). The Dectin-2-dependent enhancement of IFN-γ production was also observed in three other individuals (Figure S5F). These results indicate that Man-LAM-hDectin-2 interaction promotes the mycobacterial antigen-specific responses of T cells from tuberculosis patients, presumably through the activation of myeloid cells in PBMCs.

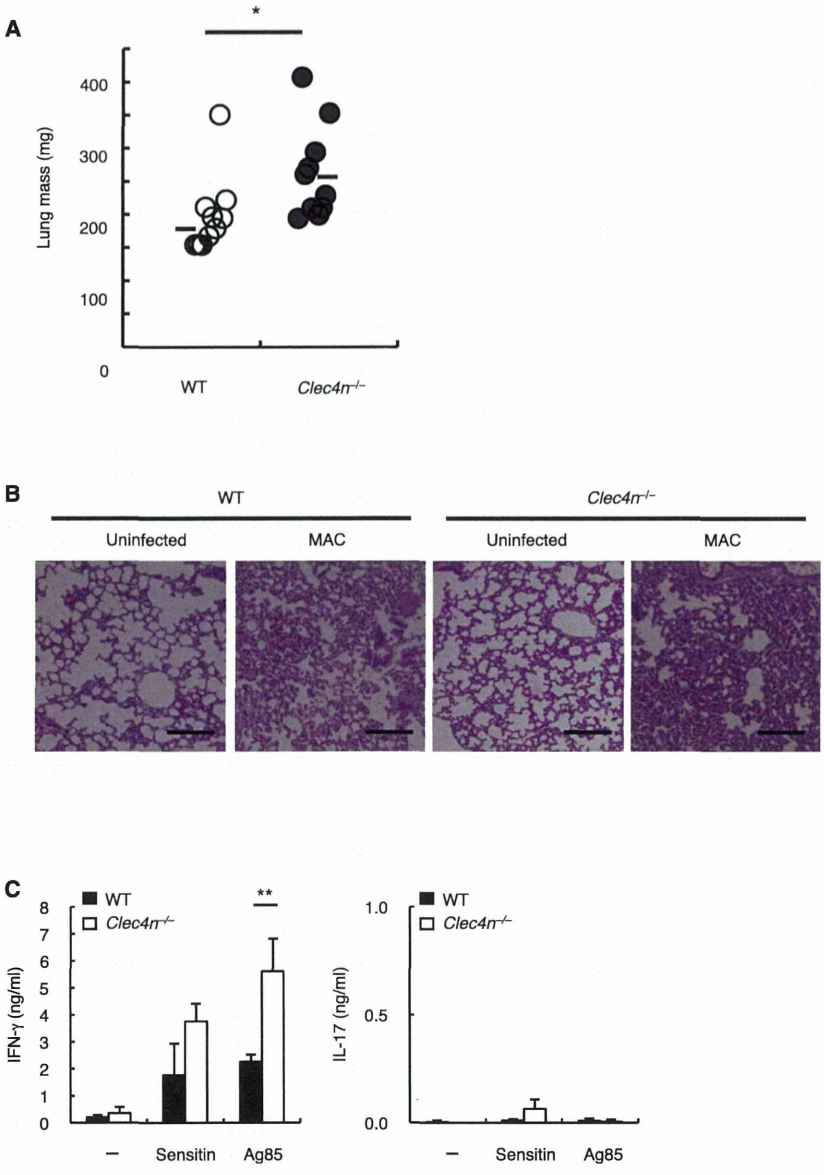

Figure 7. Immune Responses against Mycobacterial Infection in *Clec4n*$^{-/-}$ Mice

(A) Higher lung weights in *Clec4n*$^{-/-}$ mice. Lungs were isolated 23 days after infection of MAC. WT (n = 10) and *Clec4n*$^{-/-}$ (n = 10) mice were used.

(B) Histological analysis of the lung from uninfected- or MAC-infected WT or *Clec4n*$^{-/-}$ mice by HE staining. Scale bars represent 0.1 mm.

(C) Cytokine production in splenocytes after restimulation with sensitin or MACAg85 for 4 days. Splenocytes were obtained 23 days after infection of MAC and pooled from ten mice in each group. The data are presented as mean ± SD.

See also Figure S6.

Role of Dectin-2 in Mycobacterial Infection

Finally, we examined the role of Dectin-2 in mycobacterial infection in vivo. WT and *Clec4n*$^{-/-}$ mice were infected intranasally with *M. avium* complex (MAC). Although the bacterial burden in the lungs was not significantly altered at 3 weeks after infection, the average number of colony forming units (CFU) per lungs was larger in *Clec4n*$^{-/-}$ mice than in WT mice (WT, 6.54 ± 6.47; *Clec4n*$^{-/-}$, 13.1 ± 8.32 [× 10^2]). We therefore characterized the lung pathology of infected mice. Lung swelling as assessed from lung mass was significantly greater in *Clec4n*$^{-/-}$ mice (Figure 7A). In addition, *Clec4n*$^{-/-}$ mice presented with increased histopathology in the lungs after infection (Figure 7B). Chemokine concentrations were elevated in the lungs of *Clec4n*$^{-/-}$ mice compared with WT mice 3 weeks after infection (Figure S6). These chemokines seemed to be induced by bacteria residing in the lungs, because the chemokine concentration in each individual mouse was correlated with the bacterial burden in the lungs (data not shown). The production of cytokines, such as TNF, IL-6, and IL-10, were not elevated in the lungs of WT and *Clec4n*$^{-/-}$ mice at day 23 after infection (data not shown). We also examined antigen-specific T cell responses in the infected mice. Splenic T cells from *Clec4n*$^{-/-}$ mice produced a significantly larger amount of IFN-γ upon recall stimulation by mycobacterial antigens, whereas IL-17 production was not altered (Figure 7C). Thus, Dectin-2 deficiency resulted in augmented lung pathology and acquired immunity presumably due to the inefficient elimination of mycobacteria. Collectively, these results suggest that Dectin-2 is involved in host defense against mycobacteria.

Induction of EAE through Man-LAM Immunization via Dectin-2-FcRγ Axis

We next performed a murine model of T helper 17 (Th17) cell-mediated autoimmune disease, EAE, since Dectin-2 activation by Man-LAM induced IL-17 production in vitro (Figure 6B). Strikingly, *Clec4n*$^{-/-}$ mice were completely resistant to Man-LAM-induced EAE (Figures 6C and 6D), indicating that other Man-LAM receptors cannot compensate for the loss of Dectin-2 in vivo. Furthermore, ex vivo recall responses, as assessed by the production of IL-17, IFN-γ, and GM-CSF, of the lymph node cells collected from inguinal, lumbar, and axillary lymph nodes were completely abrogated in *Clec4n*$^{-/-}$ mice (Figure 6E). This indicates that Man-LAM cannot efficiently prime T cells in Dectin-2-deficient environment. Taken together, Dectin-2 is a receptor essential for the adjuvanticity of Man-LAM in vivo.

DISCUSSION

In this manuscript, we have demonstrated that Dectin-2 is a direct and functional receptor for mycobacterial Man-LAM.

Mincle, MCL, and Dectin-2 are located in the same gene cluster, and we found that all these CLRs recognize mycobacteria (Ishikawa et al., 2009; Miyake et al., 2013). The acquisition of different CLRs recognizing distinct mycobacterial components during evolution would enable the host to exert stable immune responses against this life-threatening bacteria.

Dectin-2 has been reported to recognize high-mannose structures of fungi (McGreal et al., 2006), such as α-1,2-mannan derived from *Candida albicans* (Robinson et al., 2009; Saijo et al., 2010) and mannoprotein from *Malassezia* fungus (Ishikawa et al., 2013). Glycan array analysis confirms that Dectin-2 preferentially binds to high-mannose structures, similar to DC-SIGN, SIGNR1, SIGNR3, and MMR (McGreal et al., 2006), all of which possess the mannose-binding EPN sequence within their carbohydrate recognition domains (CRDs) (Drickamer, 1992). Man-LAM possesses polysaccharide chains that terminates in a α-1,2-mannose cap (Mishra et al., 2011). It is highly likely that the α-1,2-linked mannose residues of Man-LAM are the direct determinant recognized by Dectin-2.

Man-LAM is densely distributed in the envelopes of mycobacteria with the specific configuration, and thereby their polar mannose caps are exposed on the bacterial surface with oligomeric valency. The multivalent α-1,2-mannose residue is not inconsistent with the characteristics of PAMPs recognized by Dectin-2 (Ishikawa et al., 2013; Saijo et al., 2010). To replicate this configuration in vitro, Man-LAM was used as plate-coated form to evaluate Dectin-2-mediated responses in this study. Previous studies show that soluble Man-LAM alone does not induce cytokine production in myeloid cells (Geijtenbeek et al., 2003; Gringhuis et al., 2009; Nigou et al., 2001) and we confirmed this with BMDCs (data not shown). These different outcomes depending on the stimuli might be attributed to the difference in the receptor engagement caused by the nature of the ligand—monovalent (soluble) versus multivalent (immobilized). It has been demonstrated that soluble Man-LAM influences myeloid cell function in the presence of other stimuli such as TLR ligands (Geijtenbeek et al., 2003; Gringhuis et al., 2007; Nigou et al., 2001). One possible explanation for these findings is that TLR-bound lipoprotein might provide a scaffold for soluble Man-LAM through hydrophilic interaction in aqueous media, which leads to ligand multimerization sufficient for the engagement of Dectin-2. Consistent with these ideas, multimerized Man-LAM in oil emulsion exhibited potent adjuvant activity in vivo. Collectively, Dectin-2 might discriminate multivalent PAMPs presented on "real" pathogens, presumably to prevent false recognition of their targets.

The α-1,2-linked mannose residues are also present in phosphatidyl-*myo*-inositol mannosides (PIMs). Because PIMs have been shown to be potentially associated with MMR and DC-SIGN (Torrelles et al., 2006), Dectin-2 might recognize PIMs. However, *M. abscessus*, which lacks Man-LAM but possesses PIMs, did not activate reporter cells expressing Dectin-2, implying that PIMs might not be a potent ligand for Dectin-2. Alternatively, Dectin-2 may not be able to access "short" PIMs within "tall" cell wall components, such as long-chain mycolic acids, glycolipids, lipoglycans, and polysaccharides (Mishra et al., 2011; Torrelles et al., 2006).

SIGNR1, SIGNR3, and MMR have been reported as murine receptors for Man-LAM (Koppel et al., 2004; Schlesinger et al., 1994; Tanne et al., 2009). Peritoneal macrophages from *Cd209b*$^{-/-}$ mice produce less, but detectable, IL-10 in response to Man-LAM (Wieland et al., 2007). However, gene ablation of SIGNR1 and anti-MMR blocking mAb did not influence the LAM-induced cytokine production in BMDCs. SIGNR3 is not expressed on BMDCs and forced expression of SIGNR3 did not rescue cytokine production in *Clec4n*$^{-/-}$ BMDCs. Thus, the characteristic cytokine production induced by Man-LAM seems to be determined by intrinsic signaling via Dectin-2 in DCs. Although Man-LAM-induced in vivo responses were also completely abolished in *Clec4n*$^{-/-}$ mice, it is possible that SIGNR3 plays a role in Man-LAM responses in particular cells, such as dermal DCs, that express SIGNR3 (Nagaoka et al., 2010). Alternatively, these Man-LAM-binding molecules might promote the binding of myeloid cells to Man-LAM-bearing bacteria (Tanne et al., 2009), thereby leading to the efficient phagocytosis of mycobacteria.

Our data demonstrate that Man-LAM is a sufficient component to trigger IL-10 production. However, we cannot exclude the possibility that Man-LAM is not necessarily required for IL-10 production induced by whole mycobacteria, although NTM strain *M. abscessus* lacking Man-LAM did not induce IL-10 production. The results from mutant *M. bovis* BCG, which lacks the mannose cap of LAM, suggest its redundant role in IL-10 production in LPS-primed human DCs (Appelmelk et al., 2008). It has been reported that mycobacteria may possess other possible unidentified Dectin-2 ligands, such as mannosylated proteins (Pitarque et al., 2005). These components might account, at least in part, for the IL-10 production induced by whole bacteria.

It has been reported that the IL-10 production during infection correlates with susceptibility to *M. tuberculosis*. Large amounts of IL-10 can be detected in the serum of active tuberculosis patients, particularly in response to hypervirulent strains of *M. tuberculosis* (O'Garra et al., 2013). In line with these observations, increased susceptibility to mycobacteria has been shown in mice that constitutively overexpressed IL-10 (Feng et al., 2002). In addition, secretion of another immune-regulatory cytokine transforming growth factor-β (TGF-β) was also slightly enhanced by Man-LAM through Dectin-2 (data not shown). However, the precise contribution of the mannose cap of LAM to the virulency of mycobacteria still remains controversial in vivo (Afonso-Barroso et al., 2012; Appelmelk et al., 2008).

On the other hand, it has been proposed that IL-10 might limit excessive damage to the host tissue (Redford et al., 2011). The mutant mice lacking CARD9, a downstream adaptor of FcRγ, exhibited severe lung pathology and enhanced lethality in response to *M. tuberculosis*, which is correlated with abolished secretion of IL-10 (Dorhoi et al., 2010). Although CARD9 also mediates signaling through Mincle, the effect of Mincle deficiency on the pathologies during mycobacterial infection was modest compared with those of *CARD9*$^{-/-}$ mice (Behler et al., 2012; Heitmann et al., 2013; Lee et al., 2012). Given the augmented lung inflammation in *Clec4n*$^{-/-}$ mice, the Dectin-2-FcRγ-CARD9 axis appears to be involved in the control of mycobacterial infection. Indeed, *Fcer1g*$^{-/-}$ mice showed increased immunopathology in the lungs during mycobacterial infection (Maglione et al., 2008).

How does Man-LAM efficiently promote Th17 cell responses in mice? A previous study demonstrates that Dectin-2 ligand is

capable of inducing the Th17 cell differentiation through the release of soluble factors (Saijo et al., 2010). Man-LAM stimulation also induced the production of IL-6, TNF, and TGF-β, all of which are Th17-cell-inducing cytokines. In addition, we observed that the transcription of IL-23p19 was upregulated in BMDCs upon Man-LAM in a Dectin-2-dependent fashion (data not shown), as previously reported in BMDCs stimulated with *C. albicans* (Robinson et al., 2009). A recent report has demonstrated that a NTM strain *M. avium* bearing Man-LAM could induce IL-23 production. This activity is lost in the lipophilic extract of the strain (Jönsson et al., 2012), supporting the idea that hydrophilic Man-LAM is involved in the promotion of Th17 cell differentiation.

In human PBMCs from tuberculosis patients, we found that Man-LAM enhanced IFN-γ production induced by mycobacterial antigen. The undetectable concentration of IL-17 secretion (data not shown) is consistent with previous observation (Yamashita et al., 2013), although the underlying mechanism is currently unknown. One possible explanation is that T cells in tuberculosis patients might have already skewed to a Th1 cell phenotype upon repetitive antigen exposure during infection.

In sharp contrast to Mincle ligand TDM, Dectin-2 ligand Man-LAM uniquely induces the production of IL-10 and IL-2, despite the fact that both CLRs share the same signaling subunit FcRγ. IL-2 production from DCs might contribute to the adjuvanticity by promoting T cell priming (Granucci et al., 2001). Previous studies have highlighted the role of Syk-CARD9 pathway in the production of IL-10 and IL-2 in DCs (LeibundGut-Landmann et al., 2007; Robinson et al., 2009; Saijo et al., 2010), because the TLR-MyD88 or TRIF pathway does not lead to secretion of these cytokines (LeibundGut-Landmann et al., 2007). However, the Syk-CARD9 pathway is not sufficient to induce these cytokines, because the Mincle ligand TDM did not allow the production of either IL-10 or IL-2. It remains unclear how distinct CLRs lead to different cellular responses through a common signaling subunit. We previously reported that the quantity and duration of FcRγ signals can determine the quality of cellular responses (Yamasaki et al., 2004). It is intriguing to hypothesize that the kinetics, affinity, or valency of receptor engagement potentially generates distinct signaling through FcRγ.

In addition to the functions of Man-LAM described above, Man-LAM is known to have pleiotropic functions during mycobacterial infection. Mycobacteria limit phagosome-lysosome fusion to survive in macrophages, which allow mycobacteria to establish latent and persistent infection (Pieters, 2008). Man-LAM is one of the candidate involved in this process (Fratti et al., 2003; Vergne et al., 2004), although more detailed studies are needed to determine whether Dectin-2-mediated signaling affects phagosome-lysosome fusion. A recent study has demonstrated that Man-LAM treatment inhibits T cell migration from the draining lymph nodes (Richmond et al., 2012). This effect seems to occur independently of Dectin-2, as its expression was not detected in any subsets of T cells (Ariizumi et al., 2000).

In the present study, we have shown that Dectin-2 recognizes Man-LAM to mediate its adjuvanticity. In addition, the simultaneous induction of both immunostimulatory and inhibitory responses by Man-LAM-Dectin-2 axis might be beneficial for host organisms to maintain balanced immune responses. During EAE development, skin inflammation is observed at the injection site when TDM was used as an adjuvant (Miyake et al., 2013). However, no such inflammation was observed in the skin of Man-LAM-injected mice (data not shown), implying that the anti-inflammatory cytokines induced by Man-LAM might control excessive inflammation at the injection site. The limited inflammatory responses induced by Man-LAM could thus be beneficial as an adjuvant for therapeutic vaccines for infectious diseases and cancer. It is therefore proposed that Man-LAM analogs might represent unique hydrophilic "regulatory" adjuvants that promote the development of acquired immunity, with minimal detrimental inflammation.

EXPERIMENTAL PROCEDURES

Lipids Extract

M. bovis BCG was fractionated by distilled water with repeated washing five times. After centrifugation, the soluble fraction was collected. The insoluble fraction was further delipidated with C:M (2:1, vol/vol). Each fraction was resuspended in a volume of isopropanol at equivalent amount of 0.1 mg as the original *M. bovis* BCG weight.

Cells

2B4-NFAT-GFP reporter cells expressing FcRγ alone, Mincle, Dectin-2, and Dectin-2^{QPD} were prepared as previously described (Yamasaki et al., 2009). BMDCs were prepared as previously described (Miyake et al., 2013).

In Vitro Stimulation

Mycobacterial lipid extracts, LAM in aqueous solution (1 mg/ml), TDM dissolved in C:M at 1 mg/ml, and *Candida albicans* cell wall mannan (5 mg/ml) were diluted in isopropanol and added to 96-well plates at 20 μl/well, followed by evaporation of the solvents as previously described (Ishikawa et al., 2009). Reporter cells were stimulated for 24 hr and the activation of NFAT-GFP was monitored using flow cytometry. BMDCs were stimulated for 2 days, then the culture supernatants were collected. The concentrations of each cytokine were determined by ELISA. Activation was determined using surface staining of the costimulatory molecules CD40 and CD80 by flow cytometry.

OVA-Specific CD4$^+$ T Cell Responses

BMDCs were generated from WT and *Clec4n*$^{-/-}$ mice as described above. BMDCs were left untreated or stimulated with indicated amount of plate-coated LAM in the presence of $OVA_{323-339}$ peptides (ABGENT). CD4$^+$ T cells from OT-II Tg mice were purified with anti-CD4-conjugated magnetic beads (MACS, Miltenyi) and then labeled with CFSE (DOJINDO) and cocultured with OVA-pulsed DCs in 96-well plates. On day 3, the supernatants were harvested and determined the concentration of IFN-γ, IL-17, and IL-10 using ELISA. CFSE-labeled T cells were analyzed for dilution of CFSE within the CD4$^+$ T cell population using flow cytometry.

Experimental Autoimmune Encephalomyelitis

Mice were immunized via subcutaneous administration of 200 μg of MOG_{35-55} peptide (Invitrogen) emulsified in IFA (Difco) containing 500 μg of LAM on day 0. The mice received three daily intraperitoneal (i.p.) administrations of 500 ng of pertussis toxin (PT) (List Biological Laboratories) starting on day 1. The disease severity was scored as previously described (Miyake et al., 2013). For the in vitro restimulation analysis, cells were collected from the axillary, inguinal, and lumbar (paraaortic) lymph nodes on day 23. Lymphocytes (5×10^5 cells/well) were stimulated with MOG_{35-55} peptides at the indicated concentrations for 4 days. The concentrations of IL-17, IFN-γ, and GM-SCF in culture supernatants were determined by ELISA. All animal protocols were approved by the committee of Ethics on Animal Experiment, Faculty of Medical Sciences, Kyushu University, Chiba University, or Tokyo University of Pharmacy and Life Sciences.

Mycobacterial Infection

For in vitro infection, BMDCs were infected with 1 to 10×10^9 CFU of *M. bovis* BCG. After 48 hr, the culture supernatants were collected and cytokine

concentration was determined by ELISA. For in vivo infection, WT mice and *Clec4n*$^{-/-}$ mice were anesthetized with isoflurane, and each mouse was subsequently infected intranasally with 2.5 × 10^6 CFU *M. avium* complex (MAC) per mouse. At 3 weeks after infection, the lungs were isolated and homogenized with a Physcotron handy microhomogenizer (Microtec). Serial dilutions of the homogenates were subjected to the determination of CFU on 7H11 agar plates supplemented with OADC and penicillin (100 U/ml). The homogenates were also subjected to the determination of chemokines with a Cytometric Bead Array System (BD Biosciences). Lungs from other infected mice were fixed with 10% formaldehyde for hematoxylin-eosin staining. Single-cell suspensions of splenocytes (5 × 10^5 cells) were stimulated with *M. avium* sensitin PPD (5 μg/ml) or MAC Ag85A (10 μg/ml) for 4 days, and the concentrations of cytokines and chemokines in culture supernatants were determined by ELISA. Four patients of National Tokyo Hospital in Tokyo, Japan, were enrolled in this study after giving informed consent. The research protocol was approved by the Institutional Review Board of National Tokyo Hospital and by the ethical committee of the National Institute of Infectious Diseases for medical research using human subjects.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.08.005.

AUTHOR CONTRIBUTIONS

A.Y., S.S., and S.Y. designed the research; A.Y., S.S., Y.H., Y.M., E.I., M.S., and M.Y. did the experiments; H.I., M.T., and K.A. provided the materials; and A.Y., S.S., M.O., and S.Y. wrote the manuscript.

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