A murine DC-SIGN homologue contributes to early host defense against *Mycobacterium tuberculosis*

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The C-type lectin dendritic cell–specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) mediates the innate immune recognition of microbial carbohydrates. We investigated the function of this molecule in the host response to pathogens in vivo, by generating mouse lines lacking the DC-SIGN homologues SIGNR1, SIGNR3, and SIGNR5. Resistance to *Mycobacterium tuberculosis* was impaired only in SIGNR3-deficient animals. SIGNR3 was expressed in lung phagocytes during infection, and interacted with *M. tuberculosis* bacilli and mycobacterial surface glycoconjugates to induce secretion of critical host defense inflammatory cytokines, including tumor necrosis factor (TNF). SIGNR3 signaling was dependent on an intracellular tyrosine-based motif and the tyrosine kinase Syk. Thus, the mouse DC-SIGN homologue SIGNR3 makes a unique contribution to protection of the host against a pulmonary bacterial pathogen.

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Abbreviations used: AraLAM, *Mycobacterium chelonae*–derived LAM; BMM, BM-derived macrophage; Ct, threshold cycle; DC-SIGN, DC-specific intercellular adhesion molecule-3 grabbing nonintegrin; ES, embryonic stem; ITAM, immunoreceptor tyrosine–based activation motif; LM, lipomannan; ManLAM, mannosylated lipoarabinomannan; PRR, pattern recognition receptor; TB, tuberculosis; TLR, Toll-like receptor.

Innate immune defense against pathogens involves interactions between conserved microbial molecular motifs and pattern recognition receptors (PRRs; Janeway and Medzhitov, 2002), such as the Toll-like receptors (TLRs; Ishii et al., 2008) and C-type lectins (Gordon, 2002; Geijtenbeek et al., 2004; Robinson et al., 2006), expressed on host phagocytic cells. The binding of microbial ligands to PRR results in various immune effector functions, including phagocytosis, oxidative burst (Saijo et al., 2007; Taylor et al., 2007), secretion of antimicrobial peptides (Liu et al., 2006), autophagy (Delgado et al., 2008), and the production of inflammatory cytokines, which help the body to resist pathogens either directly or by shaping the adaptive immune response (Medzhitov, 2007). Conversely, inappropriate innate responses, caused by mutations in PRR-encoding genes, for example, may lead to pathogen persistence and profound effects on host susceptibility to infection (Quintana-Murci et al., 2007).

C-type lectins constitute an extensive family of receptors involved in the recognition of endogenous and microbial carbohydrates in a

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calcium-dependent manner (Weis et al., 1998). DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) is a prototypic member of the C-type lectin family (Geijtenbeek et al., 2000). This molecule facilitates broad immune surveillance by DCs and some subsets of macrophages, through interactions with fucose- and mannose-containing glycans on many viruses, bacteria, and parasites (Robinson et al., 2006). In particular, DC-SIGN is an important receptor for *M. tuberculosis*, the pulmonary bacterial pathogen responsible for tuberculosis (TB; Geijtenbeek et al., 2003; Tailleux et al., 2003, 2005; Torrelles et al., 2008). DC-SIGN recognizes mannose residues in glycoproteins and lipoglycans, such as mannosylated lipoarabinomannan (ManLAM), in the mycobacterial cell envelope (Geijtenbeek et al., 2003; Maeda et al., 2003; Tailleux et al., 2003; Pitarque et al., 2005). A transgenic knockin model of "humanized" mice expressing DC-SIGN (Schaefer et al., 2008) has suggested that this lectin may be involved in protection against TB pathogenesis. Other in vitro studies have suggested that DC-SIGN may be exploited by *M. tuberculosis* as a means of evading immune surveillance and persisting in the host (Geijtenbeek et al., 2003). Genetic association studies in TB patients and healthy contacts have led to conflicting results on whether DC-SIGN promotes protection or increases susceptibility to TB (Barreiro et al., 2006; Vannberg et al., 2008); nevertheless, both studies clearly suggested a role for DC-SIGN in the course of the disease. This role remains unclear, particularly because of the lack of an appropriate animal model.

The mouse *DC-SIGN* locus (Park et al., 2001; Powlesland et al., 2006) encompasses seven genes, *Signr1-5* (also called *Cd209b*, *Cd209c*, *Cd209d*, *Cd209e*, and *Cd209a*) and *Signr7-8* (also called *Cd209g* and *Cd209f*), and one pseudogene, *Signr6*, which is also called *Cd209h* (Fig. S1). SIGNR5 (also termed mDC-SIGN or CIRE) was initially described as the mouse orthologue of human DC-SIGN (Park et al., 2001). However, further analyses clearly showed that SIGNR5 differs functionally from human DC-SIGN (Caminschi et al., 2006; Gramberg et al., 2006; Powlesland et al., 2006). Amino acid sequence comparison and reconstruction of the phylogeny of these lectins revealed that mouse SIGNR1, SIGNR3, and SIGNR4 were the homologues most closely related to human DC-SIGN (Fig. S1). In human DC-SIGN, Val^{351} is instrumental in sugar recognition, particularly in binding fucose residues (Guo et al., 2004). The only mouse homologues containing this residue are SIGNR3 and SIGNR4. However, SIGNR4 is unable to bind sugars in vitro, probably because the critical calcium-binding amino acids, Glu^{347} and Glu^{354}, which are present in all other related molecules, have been replaced by Gln residues (Powlesland et al., 2006). SIGNR1 does not contain Val^{351}, and is thus different from human DC-SIGN, as confirmed by the sugar recognition profiles of the two lectins. Mouse SIGNR1, like human DC-SIGN, recognizes mannose residues (Geijtenbeek et al., 2002), but it recognizes these residues as terminal monosaccharides rather than branched glycans, a property common to other C-type lectins outside the DC-SIGN family, such as the mannose-binding lectin (Powlesland et al., 2006). SIGNR1 binds terminal mannose motifs in mycobacterial ManLAM (Koppel et al., 2004), but SIGNR1-deficient mice have no phenotype upon *M. tuberculosis* infection (Wieland et al., 2007). SIGNR3 is the only one of the seven mouse homologues that, like human DC-SIGN, shows dual specificity for both glycans with high mannose content and fucose-containing oligosaccharides, including the $Lewis^X$ antigen (Powlesland et al., 2006). Furthermore, like human DC-SIGN, SIGNR3 forms tetramers, mediates endocytosis, and releases bound ligands in acidic conditions (Powlesland et al., 2006). Collectively, these data suggest that SIGNR3 is the best candidate for a functional orthologue of human DC-SIGN.

We investigated the role of members of the DC-SIGN family in TB in vivo in more detail by generating mice lacking three homologues of human DC-SIGN, namely SIGNR1 and SIGNR3, the homologues that are biochemically most similar to human DC-SIGN, and the more divergent SIGNR5. KO animals were generated directly in a C57BL/6 genetic background so that backcrosses were not required for background purification. We assessed the susceptibility of these mice to *M. tuberculosis* and compared the results obtained with those for WT animals. We report that only SIGNR3-deficient mice have impaired pulmonary defenses against the bacillus, whereas SIGNR1- and SIGNR5-deficient animals show no particular phenotype upon infection. Consistent with this finding, we report that, like human DC-SIGN in patients with TB (Tailleux et al., 2005), SIGNR3 is expressed in lung (myeloid) cells during the course of *M. tuberculosis* infection, whereas it is not expressed in naive animals. Mycobacterial recognition by SIGNR3 induces a signaling cascade requiring the tyrosine kinase Syk and involving a YxxI motif in the cytoplasmic tail of SIGNR3. This motif resembles the immunoreceptor tyrosine–based activation motif (ITAM)-like YxxI/L found in other C-type lectins, such as dectin-1 (Brown, 2006). SIGNR3 signaling activates macrophages, inducing the production of inflammatory cytokines, including TNF, in an NF-κB– and Raf1–ERK-dependent pathway, with these cytokines being key components of protec tion against TB (Flynn et al., 1995; Ladel et al., 1997; Saunders et al., 2000).

RESULTS

SIGNR1 and SIGNR3 recognize *M. tuberculosis* and mycobacterial surface glycoconjugates

We evaluated the capacity of the murine DC-SIGN homologues to recognize mycobacterial ligands, by carrying out binding experiments with ^{125}I-labeled recombinant SIGNR proteins incubated with various mycobacterial lipoglycans, including *M. tuberculosis*–derived ManLAM and lipomannan (LM), *Mycobacterium chelonae*–derived LAM (AraLAM; Fig. 1 a), and the *M. tuberculosis*–derived 19-, 38-, and 45-kD glycoproteins. These molecules were used to coat plastic plates. Like human DC-SIGN, SIGNR3 recognized ManLAM and LM, but did not bind to AraLAM, which has no mannose-capping residues. However, to a lesser extent, SIGNR3, like human DC-SIGN, also recognized mycobacterial glycoproteins,

including the 19-kD antigen (Fig. 1 b). In contrast, recombinant SIGNR5 bound only weakly to these ligands, and this lectin was unable to recognize ManLAM in particular (Fig. 1 b). As previously reported (Koppel et al., 2004), SIGNR1 also bound to ManLAM and bound poorly to AraLAM. We also found that SIGNR1 recognized several other mycobacterial glycoproteins (Fig. 1 b). Thus, SIGNR3 and SIGNR1, but not SIGNR5, can interact with mannose-containing mycobacterial surface molecules. In addition to binding, the expression of SIGNR3 and SIGNR1, but not of SIGNR5, in Rat6 fibroblasts conferred on these cells an ability to mediate endocytosis and to take up ManLAM (Fig. 1 c). SIGNR1 has been reported to interact directly with *M. tuberculosis* (Taylor et al., 2004). We wished to evaluate whether this was also true for SIGNR3, therefore we infected SIGNR3-expressing and control macrophages of the RAW 264.7 line. Cells expressing SIGNR3 displayed markedly higher levels of mycobacterial recognition than control cells (Fig. 2 a). Furthermore, macrophages expressing low levels of SIGNR3 bound fewer bacteria than macrophages expressing higher levels of this lectin in a mixed cell infection assay (Fig. 2 b). Our results provide evidence for the recognition by the DC-SIGN homologue SIGNR3 of *M. tuberculosis* and mycobacterial surface glycoconjugates.

SIGNR1–, SIGNR3–, and SIGNR5–deficient mice develop normally

With the aim of evaluating the importance of the DC-SIGN homologues SIGNR1, SIGNR3, and SIGNR5 for the control of *M. tuberculosis* infection in vivo, we generated three

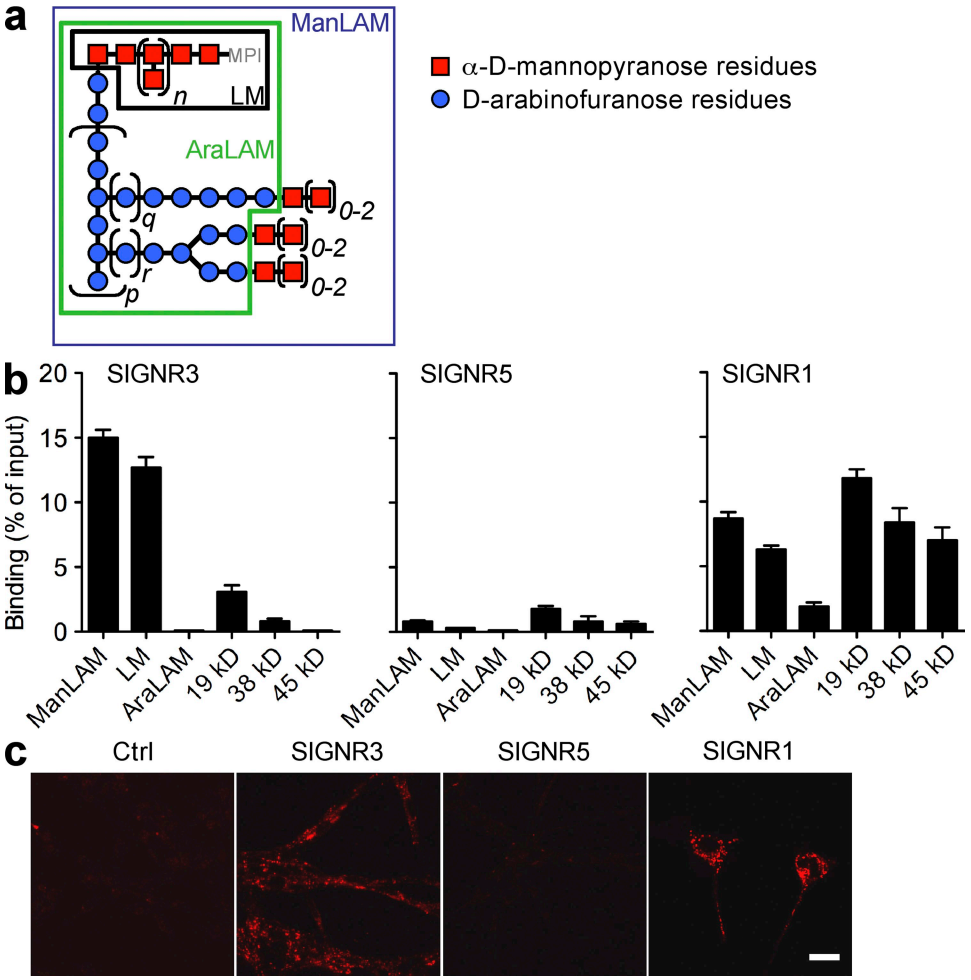

Figure 1. SIGNR1 and SIGNR3 bind mycobacterial ligands, whereas SIGNR5 does not. (a) Schematic representation of the mycobacterial lipoglycans studied. ManLAM, nonmannosylated lipoarabinomannan (AraLAM), and LM, which were used in the study. MPI, myo-phosphatidylinositol. n, p, q, and r indicate variable numbers of motif repeats. (b) ^{125}I-labeled recombinant SIGNR3, SIGNR5, and SIGNR1 were incubated with plastic-associated mycobacterial lipoglycans (ManLAM, AraLAM, and LM) or glycoproteins (19, 38, and 45-kD) for 2 h at room temperature. The plastic plates were then thoroughly washed and the radioactivity was counted. For comparisons, the fractions of the input radioactivity bound to the plates were calculated from the linear portions of the binding curves. Data are mean (± SD) of triplicate samples and were reproduced in two independent experiments. (c) Rat6 fibroblasts stably transfected with vectors encoding SIGNR3, SIGNR5, or SIGNR1. Cells were incubated with biotinylated ManLAM for 1 h at 37°C, washed, and permeabilized, and ManLAM was detected with Texas red–conjugated streptavidin. Data are representative of three independent experiments. Bar, 10 µm.

recombinant KO mouse lines carrying deletions of exons 1–4 of *Signr1* (Fig. S2 a), and of exons 1–5 of *Signr3* (Fig. S3 a) and *Signr5* (Fig. S4 a) in the C57BL/6 background, to overcome the need for backcrosses for the purification of genetic background. Genotyping by PCR and Southern blotting (not depicted) together with RT-PCR analysis confirmed that the *Signr1* (Fig. S2 b), *Signr3* (Fig. S3 b), and *Signr5* (Fig. S4 b) transcripts were not produced in animals with the corresponding inactivations. The expected Mendelian ratio of KO mice was obtained, and these animals were fertile. Detailed histological, hematological, immunological, and metabolic analyses were performed on KO animals and are continuing. Data are available at the Consortium for Functional Glycomics Gateway (http://www.functionalglycomics.org/glycomics/publicdata/phenotyping.jsp).

SIGNR3 is involved in early host resistance to *M. tuberculosis*

We evaluated the impact of an absence of DC-SIGN homologues on resistance to mycobacteria, by infecting KO mice and their WT C57BL/6 counterparts intranasally with 1,000 *M. tuberculosis* CFUs. We recovered lungs and spleens from animals on days 1, 21, and 42 after infection and determined their CFU content. Mice lacking SIGNR1 (Fig. 3 a) and SIGNR5 (Fig. 3 b) displayed no particular lung or spleen phenotype after infection, as shown by comparison with the WT. In contrast, SIGNR3-deficient animals had 5–31 times more bacteria in their lungs 21 d after infection ($14.1 \pm 1.9 \times 10^7$ vs. $2.4 \pm 0.6 \times 10^7$; $n = 7$; $P = 0.0007$), and 3–12 times more bacteria in their lungs 42 d after infection (0.5 ± 0.10^7 vs. $0.1 \pm 0.03 \times 10^7$, $n = 6$, $P = 0.0095$) than did the WT controls (Fig. 3 c). No differences were observed in bacterial colonization of the spleen, with SIGNR3-deficient animals displaying similar levels of colonization to controls ($2.4 \pm 0.4 \times 10^5$ for both genotypes at day 21; $P = 0.9$; 0.4 ± 0.10^5 vs. $0.3 \pm 0.03 \times 10^5$ on day 42; $P = 0.4$; Fig. 3 c). These results were reproduced in two independent experiments and indicated a role for SIGNR3 in early pulmonary resistance to *M. tuberculosis*, but not in mycobacterial dissemination or the extrapulmonary control of infection in secondary lymphoid organs. Although the difference in lung CFUs between WT and SIGNR3-deficient animals persisted in the longer term (after 21 d), infection was controlled in KO mice, as attested by the observed decrease in pulmonary bacterial load. These results suggest that SIGNR3 is involved in the early control of *M. tuberculosis* infection but does not play a major role in mounting an efficient antimycobacterial adaptive immune response. Consistent with this hypothesis, we observed no difference in the formation of granulomatous lesions in SIGNR3-deficient and SIGNR3-containing mice, in

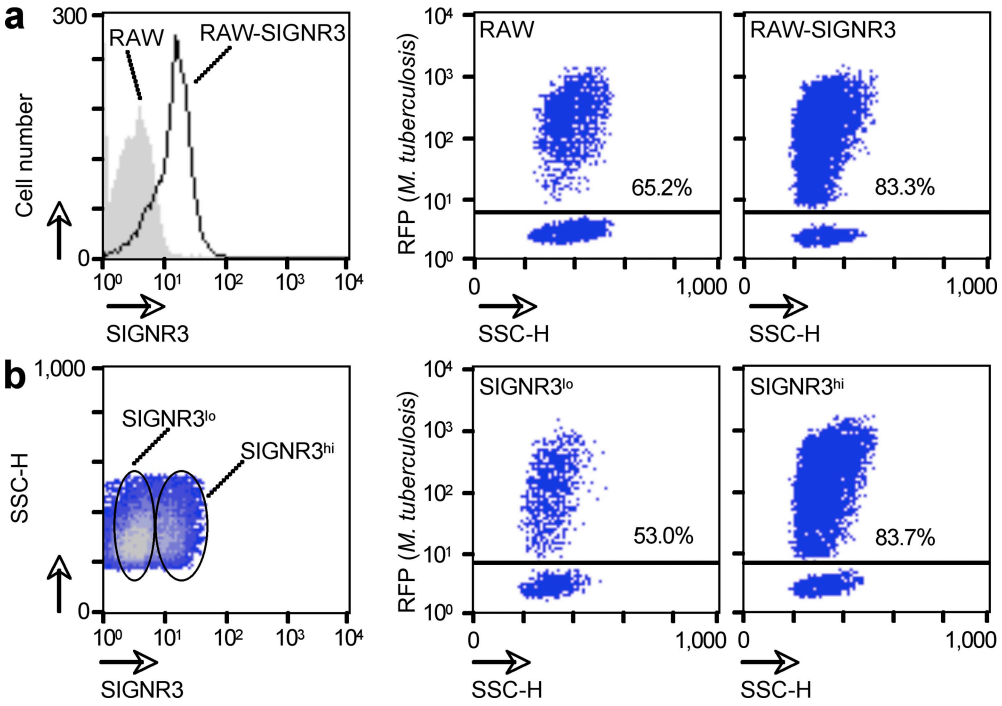

Figure 2. SIGNR3 recognizes *M. tuberculosis* bacilli. (a) RAW and RAW-SIGNR3 cells were analyzed by flow cytometry for SIGNR3 expression (left). Both cell types were infected with *M. tuberculosis* H37Rv expressing RFP at a multiplicity of infection of 5 bacteria per cell, for 4 h at 37°C; cells were then washed, fixed, and analyzed for *M. tuberculosis* content by flow cytometry. (b) A heterogeneous SIGNR3-expressing RAW cell line was analyzed by flow cytometry for SIGNR3 expression. SIGNR3^{lo} and SIGNR3^{+} cells were observed. The cells were infected as in a and further stained with an anti-SIGNR3 antibody that was subsequently detected with an Alexa Fluor 647–conjugated secondary antibody. Mycobacterial content was analyzed by flow cytometry after gating on SIGNR3^{lo} and SIGNR3^{hi} cells. In a and b, the percentages indicate *M. tuberculosis*–positive cells. Experiments were repeated twice and representative panels are shown.

high- (Fig. 4 a) and low-dose infection models (not depicted). Furthermore, SIGNR3-KO animals mounted specific antimycobacterial Th1, Th2, Tc1, and Th17 responses similar in strength and kinetics to those of WT controls (Fig. 4 b). Finally, SIGNR3-KO mice did not die any earlier than WT controls in two independent experiments with the laboratory strain H37Rv and a more virulent strain of the W-Beijing lineage, over infection periods of 170 and 190 d (unpublished data).

SIGNR3 is expressed in the lungs of *M. tuberculosis*–infected mice

In humans infected with *M. tuberculosis*, DC-SIGN is expressed in lung phagocytes, including alveolar macrophages (Tailleux et al., 2005). We investigated whether SIGNR3 displays a similar pattern of expression in mice. SIGNR3 was immunodetected in lung tissues of *M. tuberculosis*-infected mice, but not in those of naive animals or of *M. tuberculosis*–infected SIGNR3-deficient mice (Fig. 5 a). Interestingly,

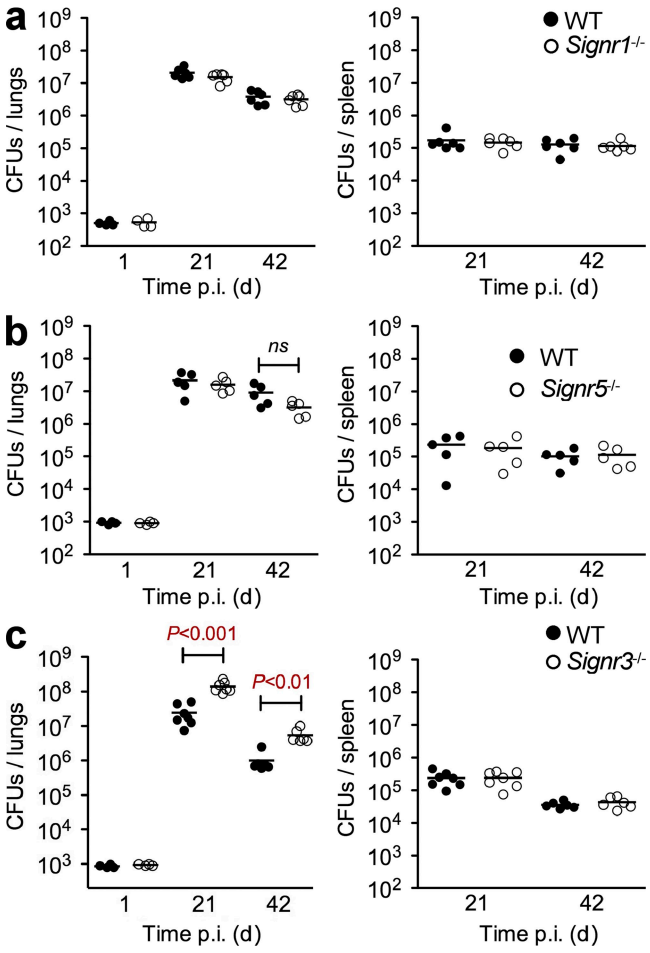

Figure 3. SIGNR3-deficient mice are less resistant to *M. tuberculosis* infection than WT mice. (a–c) SIGNR1- (a), SIGNR5- (b), and SIGNR3-deficient (c) mice (open circles) and their WT counterparts (closed circles) were infected intranasally with 1,000 CFUs of *M. tuberculosis* H37Rv. On days 1, 21, and 42 after infection lungs (left) and spleens (right) were collected from the animals, homogenized, and plated on agar medium for CFU counting. Each circle represents one animal. Data are representative of at least two independent experiments. When not indicated, $P > 0.05$. p.i., postinfection; ns, not significant.

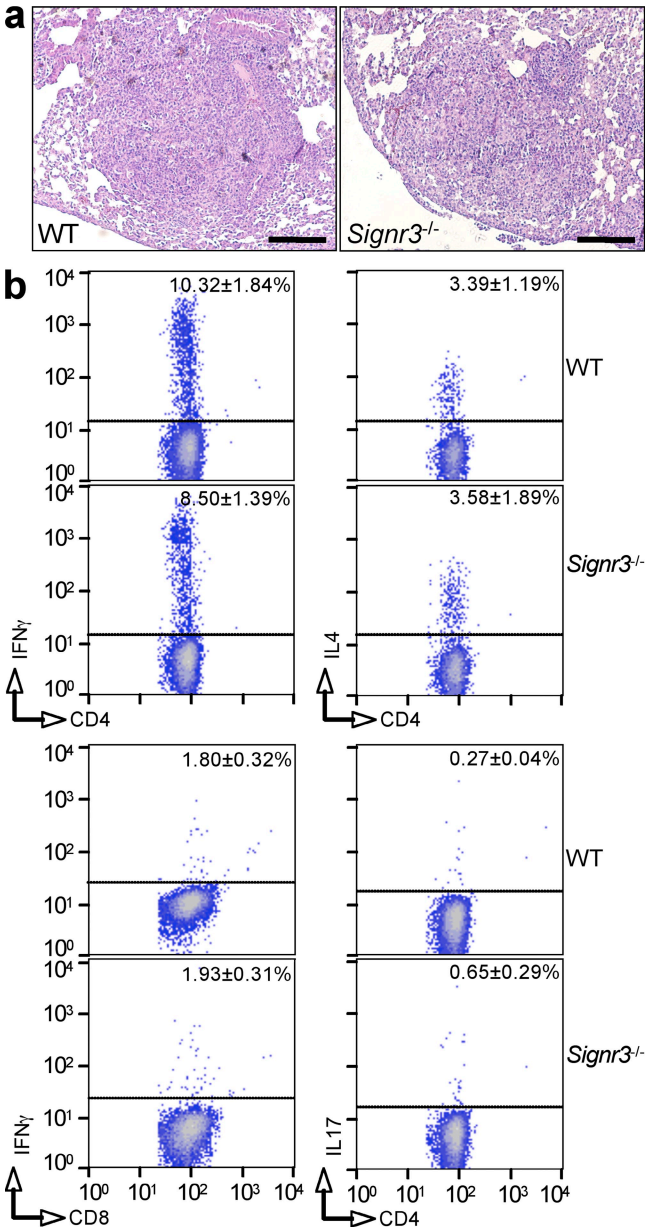

Figure 4. The antimycobacterial adaptive immune response is intact in SIGNR3-deficient mice. (a) Typical pulmonary granulomas observed 21 d after intranasal infection with 1,000 CFU *M. tuberculosis* H37Rv/mouse, from C57BL/6 (left) and SIGNR3-deficient (right) mice. Sections were stained with hematoxylin and eosin. Bars, 150 µm. (b) WT and SIGNR3-KO mice were infected as in a. Lungs were recovered 21 d after infection, and total lung cells were restimulated with purified protein derivative for 6 h. Intracellular IFN-γ, IL-4, and IL-17 and surface CD4 and CD8 staining made it possible to identify pulmonary Th1, Th2, Tc1, and Th17 cells. The data shown are the means (± SD) of quadruplicate samples/mice, reproduced in two independent experiments. As on day 21, no differences between SIGNR3-KO mice and WT controls were observed 15 and 42 d after infection (not depicted).

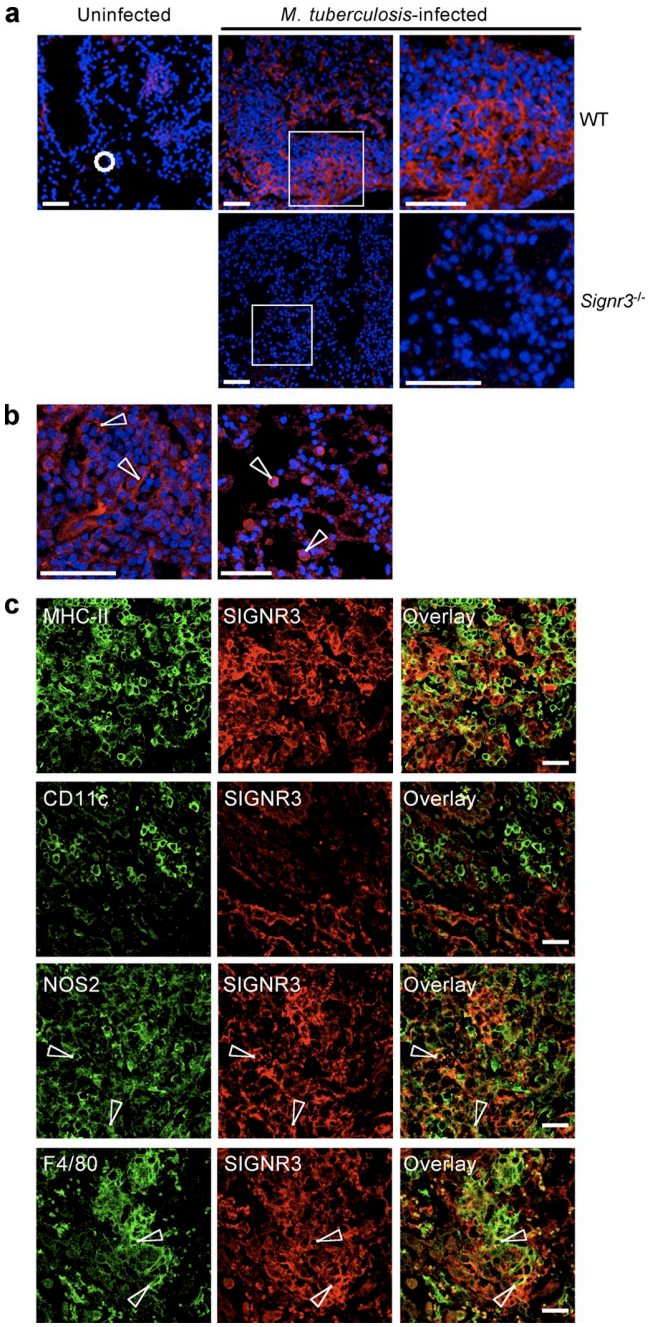

Figure 5. SIGNR3 is expressed in the lungs during *M. tuberculosis* infection. (a) Lung sections from uninfected (left) and *M. tuberculosis*–infected (right) WT (top right) and SIGNR3-KO mice (bottom right) were stained with the nuclear staining agent TO-PRO-3 (blue) and with a rabbit polyclonal anti-SIGNR3 IgG fraction that was subsequently detected with a Cy3-coupled anti–rabbit antibody (red). The images in the right columns are magnified from the white boxes in the middle column. Faint red signals in all panels (illustrated in the circle in the top left image) correspond to autofluorescent erythrocytes (el-Rahman et al., 1995). (b) Lung sections from *M. tuberculosis*–infected WT mice were stained for SIGNR3 as in a. Arrowheads in left image indicate multinucleated giantlike cells; arrowheads in right image indicate alveolar macrophage-like cells in the alveoli. (c) Lung sections from *M. tuberculosis*–infected WT mice were stained for SIGNR3 as in a, and then counterstained (green; Alexa Fluor 488) for MHC-II, CD11c, NOS2, and F4/80, as indicated. Arrowheads indicate colocalization. Pictures are representative of at least two independent experiments using samples collected from different animals. Bars, 50 µm.

within the cellular infiltrates, part of SIGNR3-expressing cells displayed typical morphological features of multinucleated giant cells (Fig. 5 b, top); other SIGNR3-expressing cells were located in the alveoli and resembled alveolar macrophages (Fig. 5 b, bottom). Double immunostaining experiments were used to characterize SIGNR3-expressing cells in the cellular infiltrate; SIGNR3-positive cells expressed no or low levels of MHC-II and CD11c, and some of them expressed NOS2/iNOS and the macrophage marker F4/80 (Fig. 5 c). These observations parallel the reported pattern of DC-SIGN expression in the lungs of patients with TB, with SIGNR3$^+$ cells in the lungs of infected animals being of myeloid origin (Tailleux et al., 2005).

SIGNR3 binding to ManLAM and *M. tuberculosis* induces the secretion of IL-6 and TNF

Lung inflammatory cytokines play a crucial role in host resistance to *M. tuberculosis* (Flynn and Chan, 2001). We investigated whether SIGNR3 stimulation induced cytokine secretion, by stably transfecting RAW 264.7 macrophages with a SIGNR3-encoding vector, and stimulating them with ManLAM for various periods of time. We then quantified cytokine levels in culture supernatants by ELISA. ManLAM stimulation of SIGNR3-expressing cells resulted in the strong induction of IL-6 secretion, as shown by comparison with control cells (Fig. 6 a). Larger amounts of TNF were also produced by SIGNR3-expressing macrophages than by control cells (Fig. 6 b). IL-1β and IL-12p40 were not detected in any conditions in stimulated RAW and RAW-SIGNR3 cells (unpublished data). Nitric oxide, an important mediator of resistance to *M. tuberculosis* in mice and possibly also in humans (Flynn and Chan, 2001), was not produced in larger amounts in SIGNR3-expressing macrophages than in control cells after stimulation with ManLAM (Fig. S5 a). IL-10 was barely induced in ManLAM-treated macrophages, regardless of SIGNR3 expression, and ManLAM did not potentiate IL-10 secretion in LPS-treated SIGNR3-expressing cells (Fig. S5 b). Infection with live *M. tuberculosis* also resulted in higher levels of production of IL-6 (Fig. 6 c) and TNF (Fig. 6 d) in SIGNR3-expressing cells than in control cells. We wanted to use BM-derived phagocytic cells from SIGNR3-deficient animals to confirm these results in a primary context. However, flow cytometry analysis of the macrophages and DCs derived from BM progenitors of WT mice using various classical cytokines, such as M-CSF, GM-CSF, and Flt3-ligand, resulted in no detection of SIGNR3 mRNA or protein in these cells (unpublished data). The treatment of these cells with cytokines, such as IL-4 and IL-13, which are known to induce DC-SIGN expression in human phagocytes (Soilleux et al., 2002; Puig-Kröger et al., 2004), also failed to increase SIGNR3 expression (unpublished data). We could not purify SIGNR3-expressing cells from the lungs of *M. tuberculosis*–infected animals in a sufficiently pure state and in

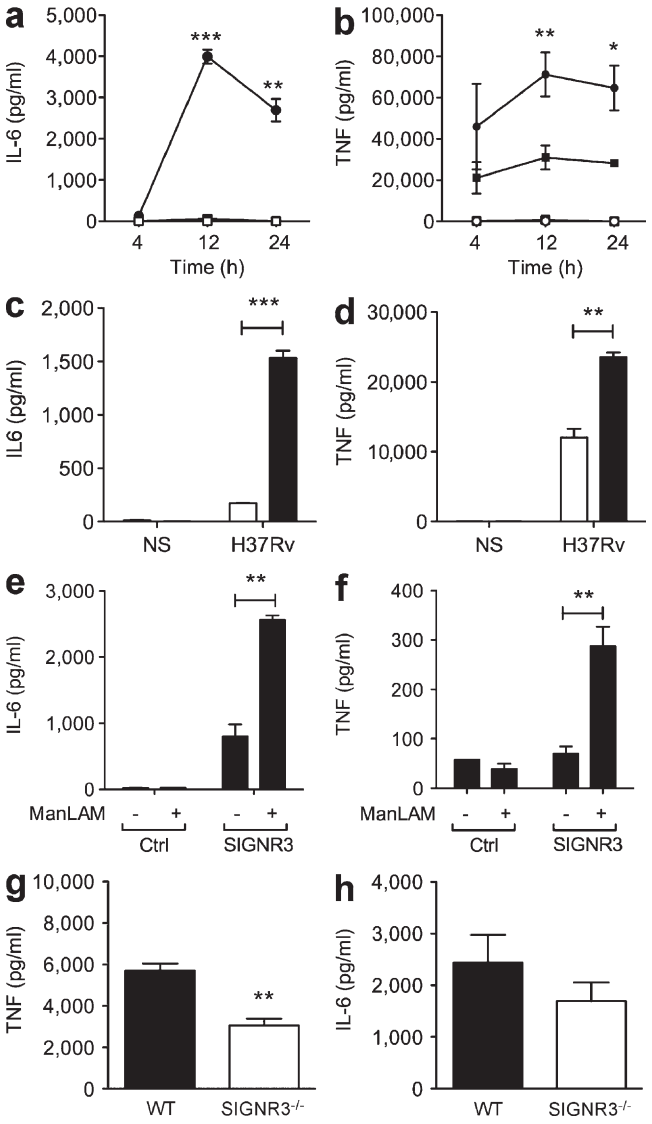

Figure 6. SIGNR3 binding to ManLAM and *M. tuberculosis* induces the secretion of IL-6 and TNF. (a) RAW 264.7–derived macrophages stably transfected with a SIGNR3-encoding (circles) or an empty control (squares) vector were left unstimulated (open symbols) or were stimulated (closed symbols) with 10 µg/ml ManLAM, and IL-6 secretion was measured by ELISA after 4, 12, and 24 h of incubation. (b) TNF secretion by RAW-derived macrophages. The same protocol described for a was followed. (c and d) RAW (open bars) and RAW-SIGNR3 (filled bars) cells were infected with *M. tuberculosis* H37Rv (10 bacteria/cell) or left untreated (NS, nonstimulated) for 4 h; cells were washed and further incubated in bacterium-free medium for 14 h, and IL-6 (c) and TNF (d) were measured by ELISA. (e and f) BMMs from C57BL/6 mice were nucleofected with a control vector (Ctrl) or with a SIGNR3-encoding vector and were stimulated with 10 µg/ml ManLAM (+) or left untreated (−); IL-6 (e) and TNF (f) levels were determined by ELISA after 18 h of incubation. (g and h) WT (filled bars) and SIGNR3-KO mice (open bars) were infected intranasally with 1,000 CFUs *M. tuberculosis* H37Rv, and lungs were analyzed for TNF (g) and IL-6 (h) after 21 d of infection. In a–f, the data shown are the means (± SD) of triplicate samples, reproduced in at least two independent experiments. In g and h, the data shown are the means (± SD) of quadruplicate samples/mouse, reproduced in two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

large enough quantities. We therefore decided to validate the functionality of SIGNR3 signaling in primary cells by nucleofecting BM-derived macrophages (BMMs) with a SIGNR3-encoding vector. SIGNR3 expression in BMMs was validated by RT-PCR (unpublished data). The treatment of these cells with ManLAM again resulted in much higher levels of IL-6 (Fig. 6 e) and TNF (Fig. 6 f) secretion than observed in BMMs transformed with a control vector. IL-6 and TNF play key roles in antimycobacterial immunity, and IL-6– and TNF-deficient mice are particularly susceptible to mycobacteria (Flynn et al., 1995; Ladel et al., 1997; Saunders et al., 2000). We quantified IL-6 and TNF production in the lungs of WT and SIGNR3-deficient animals. On day 21 after infection, the level of TNF was significantly lower in KO than in WT mice (Fig. 6 g). The level of IL-6 was also lower in the lungs of KO animals, although the difference with that in the lungs of WT controls did not reach statistical significance (Fig. 6 h).

SIGNR3 signaling is dependent on Tyr^{27} and does not require TLR2

We next investigated the molecular bases of SIGNR3-mediated signaling and IL-6 and TNF production. Although ManLAM is a poor ligand for TLR2 (Nigou et al., 2008), we investigated the possible involvement of TLR2 in SIGNR3-dependent IL-6 and TNF secretion after ManLAM recognition, and the possibility that SIGNR3 signals on its own. Neutralizing anti-TLR2 antibodies did not inhibit the cytokine secretion induced by SIGNR3 stimulation with ManLAM, although the antibody was clearly active in cells stimulated with the TLR2 ligand lipopeptide $Pam_3Cys\text{-}SK_4$ (Fig. 7 a). The cytoplasmic tail of SIGNR3 contains a tyrosine residue (Y^{27}) in the vicinity of an ITAM-like motif (Fig. 7 b). As in other C-type lectins, such as dectin-1/CLEC-7A and CLEC-2, this residue may be involved in signal transduction. We thus transfected macrophages with a vector encoding a $Y^{27}S$ mutant form of SIGNR3. Like WT SIGNR3, the mutated form of the receptor was correctly immunodetected in the cytoplasm and at the plasma membrane of transfected cells (Fig. 7 c), and the two forms were expressed at similar levels (Fig. 7 d). The $Y^{27}S$ mutation strongly impaired SIGNR3 signal transduction and the induction of IL-6 and TNF (Fig. 7 e) secretion after stimulation with ManLAM. The secretion of IL-6 and TNF was also weaker in *M. tuberculosis*–infected macrophages expressing the mutated form of SIGNR3 than in cells expressing the WT lectin (Fig. 7 f). As a control, macrophages expressing the WT or the mutated form of SIGNR3 responded similarly to non-SIGNR3 ligands, such as FSL-1 (TLR2/6 ligand) and Pam_3CSK_4 (TLR1/2 ligand), as shown by IL-6 and TNF production (Fig. 7 g).

SIGNR3 signaling depends on the tyrosine kinase Syk and the Raf1–ERK and NF-κB pathways

We investigated the impact of SIGNR3 on the secretion of IL-6 and TNF using chemical inhibitors to target various steps in the regulation of intracellular transduction pathways. We showed that SIGNR3 signaling was dependent on both the NF-κB and the Raf1–ERK pathways (Fig. 8, a and b).

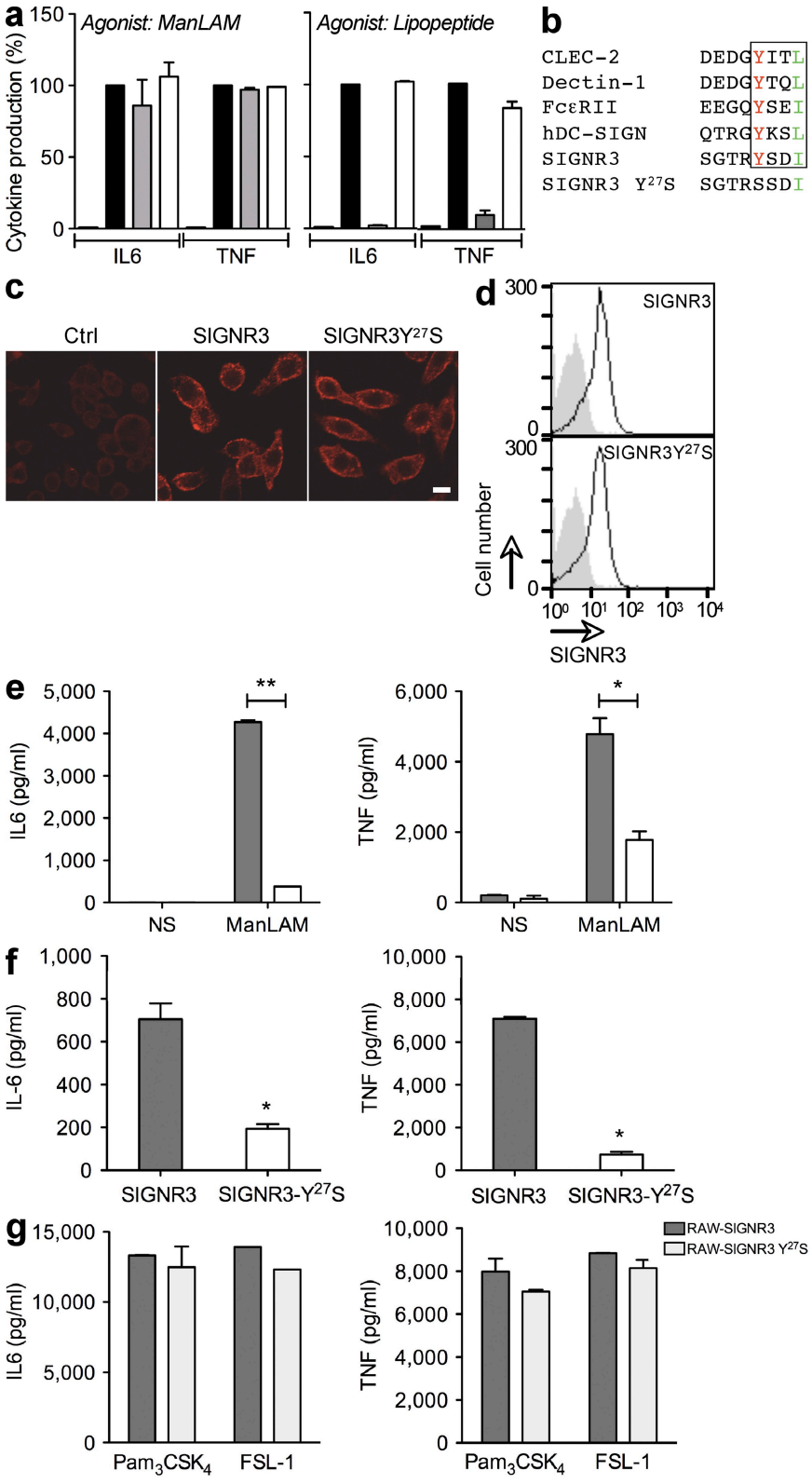

Figure 7. SIGNR3 signaling is TLR2–independent and involves intracellular Tyr^{27}. (a) SIGNR3-expressing RAW 264.7 macrophages were left unstimulated (dashed bars) or were stimulated with 10 µg/ml ManLAM or 0.5 µg/ml lipopeptide Pam_3-$CysSK_4$ in the absence (black bars) or presence of anti-TLR2 antibody (gray bars) or the corresponding isotype control (white bars). After 18 h of incubation, the secretion of IL-6 and TNF (as indicated) was measured by ELISA. Data represent cytokine production as a mean percentage (± SD) of production by cells stimulated in the absence of antibody (black

ERK activation after signal transduction by a C-type lectin was reminiscent of recent findings for the Syk-coupled C-type lectin dectin-1 (Rogers et al., 2005). Specific inhibition of the tyrosine kinase Syk by piceatannol abolished SIGNR3 signaling and cytokine secretion in a dose-dependent manner (Fig. 8 c). In contrast, this Syk inhibitor had no effect on TLR2-induced cytokine production (Fig. 8 c). The more specific Syk inhibitor R406 gave similar results (Fig. 8 d). Finally, SIGNR3 trans-expression in BMMs from Syk-deficient mice (Turner et al., 1995) resulted in much lower levels of IL-6 production upon ManLAM treatment than were observed for BMMs with active Syk (Fig. 8 e). Thus, the recognition of mycobacterial sugar motifs by the C-type lectin SIGNR3 induces TLR2-independent, Syk-dependent secretion of the proinflammatory cytokines IL-6 and TNF.

SIGNR3 collaborates with TLR2 to induce inflammatory cytokines

SIGNR3 signaling after ManLAM recognition was TLR2-independent. However, mycobacteria express TLR2 ligands, some of which, including the 19-kD antigen (Brightbill et al., 1999), are also recognized by C-type lectins (Pitarque et al., 2005). We therefore decided to evaluate the effect of SIGNR3 in the context of macrophage stimulation by mycobacterial TLR2 ligands such as the 19-kD antigen. The 19-kD protein is recognized by SIGNR3 (Fig. 1 b), and SIGNR3 allows nonphagocytic cells to take up the antigen by endocytosis (Fig. 9 a). SIGNR3 expression in RAW macrophages clearly increased the secretion of IL-6 and TNF (Fig. 9 b) after stimulation with the 19-kD protein, as shown by comparison with control cells. The blocking of TLR2 with an antibody reduced cytokine secretion in SIGNR3$^+$ cells after 18 h of stimulation (Fig. 9 c), confirming that TLR2 was required for 19-kD protein–induced signaling. Finally, IL-6 secretion was partially impaired in Syk-deficient BMMs trans-expressing SIGNR3 and stimulated with the 19-kD antigen (Fig. 9 d). The LAM and LM contents of the 19-kD lipoprotein fraction were assessed by carbohydrate quantification based on capillary electrophoresis (Nigou et al., 2000), after total acid hydrolysis, and were found to be <3% (unpublished data). Thus, in addition to signaling on its own, SIGNR3 can "collaborate" with TLR2 to induce proinflammatory cytokine secretion after the recognition of mycobacterial agonists.

DISCUSSION

This study provides evidence that the functional DC-SIGN homologue SIGNR3 contributes to early host resistance to *M. tuberculosis* in experimentally infected mice, and that Syk-coupled SIGNR3 signaling triggers secretion of the protective inflammatory cytokines IL-6 and TNF in a NF-κB and Raf1–ERK–dependent manner. SIGNR3 signaling is triggered by ManLAM, which is only a weak TLR2 ligand (Means et al., 1999; Nigou et al., 2008), as well as by LM, a strong TLR2 ligand (Nigou et al., 2008). SIGNR3 activation is entirely dependent on an ITAM-like motif in the cytoplasmic tail of the lectin, and is not altered by anti-TLR2 neutralizing antibodies. In addition to signaling on its own, SIGNR3 may act with TLR2, in an additive manner, to increase cytokine secretion after recognition of dual ligands such as the 19-kD protein.

The mouse *DC-SIGN* locus differs from its human homologue in several ways. In particular, it has been intensely remodeled through gene duplication, generating seven paralogous genes and one pseudogene, whereas the human *DC-SIGN* gene is flanked by only one paralogue, *L-SIGN*. Sequence analysis and phylogeny reconstruction alone could not identify the true mouse orthologue of human DC-SIGN (Powlesland et al., 2006). However, biochemical studies clearly demonstrated that only SIGNR3 displayed dual specificity for mannose- and fucose-containing oligosaccharides and glycans, like human DC-SIGN. It is therefore not surprising that, like DC-SIGN, SIGNR3 binds to and internalizes *M. tuberculosis* ManLAM through its mannose capping residues. We also showed that SIGNR3 bound other mycobacterial lipoglycans, such as LM, and glycoproteins, such as the 19-kD antigen, and whole *M. tuberculosis* bacilli. SIGNR1 also recognizes *M. tuberculosis* ManLAM, as previously reported (Koppel et al., 2004), in addition to mycobacterial glycoproteins. However, the properties of SIGNR1 are not consistent with strong functional similarities to human DC-SIGN. Indeed, SIGNR1 recognizes terminal monosaccharides rather than mannose-branched oligosaccharides (Powlesland et al., 2006). Moreover, SIGNR1 is expressed in

bars, 100%). 100% of IL-6 secretion after stimulation with ManLAM and lipopeptide corresponded to 9.5 ± 1.8 and 5.4 ± 0.5 ng/ml, respectively. 100% of TNF secretion after stimulation with ManLAM and lipopeptide corresponded to 8.4 ± 0.2 and 9.4 ± 0.2 ng/ml, respectively. (b) Amino acid sequence alignment of the ITAM-like signaling motif (YXXL/I) in CLEC-2, dectin-1/CLEC-7A, FcεRII, hDC-SIGN, and the corresponding sequence in SIGNR3 and in a Y27S-mutated form of SIGNR3. The critical tyrosine residue is shown in red; the second typical residue is shown in green, the ITAM-like motif is indicated by a boxed area. (c and d) SIGNR3- and SIGNR3(Y^{27}S)-expressing RAW 264.7 macrophages were stained with a rat anti-SIGNR3 antibody, which was subsequently detected with an Alexa Fluor 594– (c) or an Alexa Fluor 647–conjugated (d) anti–rat monoclonal antibody, and were analyzed by confocal microscopy (c) or by flow cytometry (d). Data are representative of two independent experiments. Bar: (c) 10 µm. (e) RAW 264.7 macrophages expressing SIGNR3 (gray bars) and SIGNR3(Y^{27}S) (white bars) were left unstimulated (NS) or were stimulated with 10 µg/ml ManLAM. After 18 h of incubation, the secretion of IL-6 (left) and TNF (right) was assessed by ELISA. (f) RAW 264.7 macrophages expressing SIGNR3 (gray bars) and SIGNR3(Y^{27}S) (open bars) were infected with *M. tuberculosis* H37Rv (10 bacteria/cell). Cells were treated as in Fig. 6 (c and d), and IL-6 (left) and TNF (right) levels were determined by ELISA. (g) RAW 264.7 macrophages expressing SIGNR3 (dark gray bars) and SIGNR3(Y^{27}S) (light gray bars) were stimulated with 0.5 µg/ml Pam_3CSK_4 or 0.1 µg/ml FSL-1 (Pam_2CGDPKHPKSF). After 18 h of incubation, the secretion of IL-6 (left) and TNF (right) was assessed by ELISA. In a and e–g, the data shown are the means (± SD) of triplicate samples, reproduced in at least two independent experiments. *, $P < 0.05$; **, $P < 0.01$.

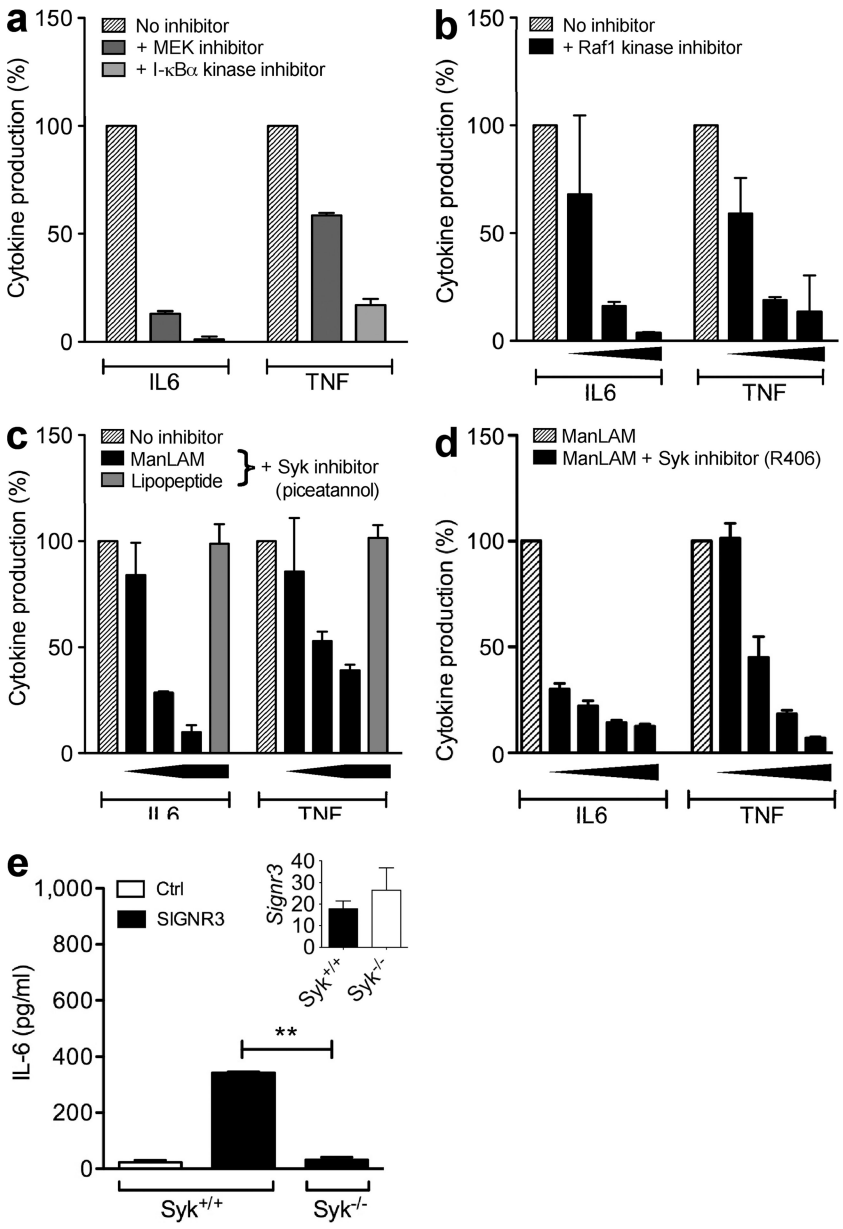

Figure 8. SIGNR3 signaling involves Syk and Raf1. (a) SIGNR3-expressing RAW 264.7 macrophages were stimulated with 10 µg/ml ManLAM in the absence (dashed bars) or presence of the 10 µM ERK1/2 kinase inhibitor U0126 (dark gray bars) or 10 µM I-κBα kinase inhibitor BAY117082 (light gray bars). After 18 h of incubation, the secretion of IL-6 and TNF (as indicated) was measured by ELISA. (b) SIGNR3-expressing RAW 264.7 macrophages were stimulated with 10 µg/ml ManLAM in the absence (dashed bars) or presence (black bars) of various concentrations of the Raf1 kinase inhibitor GW5074 (0.1, 1, 10 µM). After 18 h of incubation, the secretion of IL-6 and TNF (as indicated) was measured by ELISA. (c) SIGNR3-expressing RAW 264.7 macrophages were stimulated with 10 µg/ml ManLAM or 0.5 µg/ml Pam_3-Cys-SK_4 lipopeptide in the absence (dashed bars) or presence (black bars for ManLAM, gray bars for lipopeptide) of various concentrations of the Syk inhibitor piceatannol (0.1, 1, and 10 µM for ManLAM, and 10 µM for lipopeptide). After 18 h of incubation, the secretion of IL-6 and TNF (as indicated) was measured by ELISA. (d) SIGNR3-expressing RAW 264.7 macrophages were stimulated with 10 µg/ml ManLAM in the absence (dashed bars) or presence (black bars) of increasing concentrations of the Syk inhibitor R406 (0.5, 1, 2, and 5 µM). After 18 h of incubation, the secretion of IL-6 and TNF (as indicated) was measured by ELISA. (e) BMMs from C57BL/6 ($Syk^{+/+}$) or from $Syk^{-/-}$ animals were nucleofected with a control vector (Ctrl) or with a SIGNR3-expressing vector (SIGNR3). Cells were stimulated with 10 µg/ml ManLAM, and IL-6 levels were determined by ELISA on cell supernatants after 18 h of incubation. For technical reasons, TNF secretion could not be assessed in these settings. In a–d, data are shown as in Fig. 5 a, and in a–e, the data shown are the means (± SD) of triplicate samples, reproduced in three (a–d) and two (e) independent experiments.

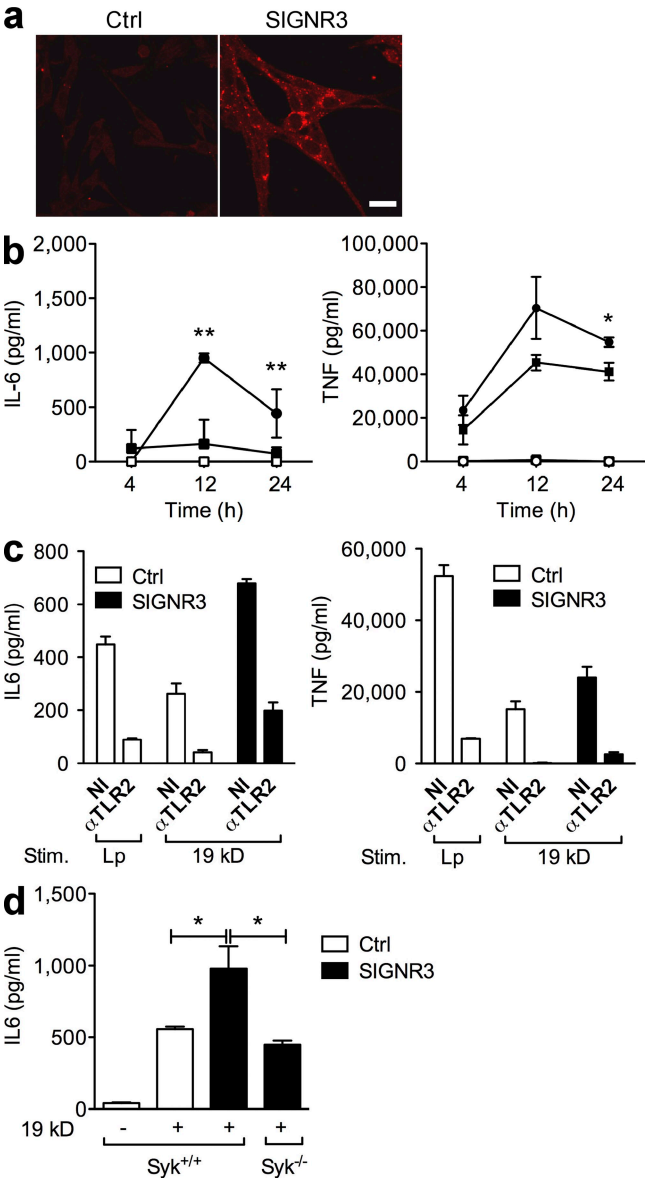

Figure 9. SIGNR3 collaborates with TLR2 to induce cytokine secretion. (a) Rat6 fibroblasts were stably transfected with a control (Ctrl) or a SIGNR3-encoding (SIGNR3) vector. Cells were incubated with 19-kD protein for 1 h at 37°C, washed, and permeabilized, and the 19-kD antigen was immunodetected with a Texas red–conjugated secondary antibody. Data are representative of three independent experiments. Bar, 10 µm. (b) RAW 264.7–derived macrophages stably transfected with a SIGNR3-encoding (circles) or an empty control (squares) vector were left unstimulated (open symbols) or were stimulated (closed symbols) with 0.3 µg/ml of 19-kD protein, and the secretion of IL-6 (left) and TNF (right) was measured by ELISA after 4, 12, and 24 h of incubation. *, $P < 0.05$; **, $P < 0.01$. (c) RAW 264.7–derived macrophages stably transfected with a SIGNR3-encoding (black bars) or an empty control (open bars) vector were incubated with an anti-TLR2 blocking antibody or left with no inhibitor (NI) for 1 h, after which cells were stimulated (Stim.) with 0.3 µg/ml of 19-kD protein or 0.5 µg/ml Pam_3CSK_4 lipopetide (Lp). We measured the secretion of IL-6 (left) and TNF (right) by ELISA after 18 h of incubation. (d) BMMs from C57BL/6 ($Syk^{+/+}$) or from $Syk^{-/-}$ animals were nucleofected with a control vector (Ctrl) or with a SIGNR3-expressing vector (SIGNR3). Cells were stimulated with 0.3 µg/ml 19-kD protein, and IL-6 levels were determined by ELISA in the cell supernatants after 18 h of incubation. Data shown as in Fig. 8 e. For technical reasons, TNF secretion could not be assessed in these settings. In b–d, the data shown are the means (± SD) of triplicate (b and c) and duplicate (d) samples, reproduced in at least two independent experiments.

liver endothelial cells (Geijtenbeek et al., 2002), with an expression pattern similar to that of L-SIGN in humans. The other mouse SIGNR lectins recognize different sugar moieties, such as N-acetyl glucosamine for SIGNR2, or have no sugar-binding activity, as is the case for SIGNR4 (Powlesland et al., 2006). Thus, our findings support the hypothesis that human DC-SIGN and mouse SIGNR3 are functional orthologues that are duplicated differently within and between species during the course of evolution to fulfill different functions (Bashirova et al., 2003).

We found that the early pulmonary resistance of SIGNR3-deficient mice to *M. tuberculosis* was weaker than that of WT animals, although the infection was controlled in the longer term in KO animals. In contrast, SIGNR5- and SIGNR1-KO animals were as resistant to the bacillus as WT control mice. The greater permissiveness to infection of SIGNR3-deficient mice was consistent with the ability of the lectin to signal after binding its mycobacterial ligands, and to induce secretion of the inflammatory cytokines IL-6 and TNF in vitro in macrophages, and in vivo in the lungs of experimentally infected animals, at least regarding TNF. These cytokines are essential for the control of mycobacterial infections (Flynn et al., 1995; Ladel et al., 1997; Saunders et al., 2000). Although we could not evince a dramatic decrease in IL-6 secretion in the lungs of SIGNR3-KO animals 21 d after infection (Fig. 6 g), the level of IL-6 transcript in these mice was reduced significantly as compared with WT controls (unpublished data). Moreover our results obtained in vitro undoubtedly show that SIGNR3 signaling contributes to IL-6 secretion. Although we can only speculate at this stage about the potential involvement of IL-6 in SIGNR3-mediated protection against *M. tuberculosis*, our results for SIGNR3-KO mice are reminiscent of those reported for IL-6 KO mice (Saunders et al., 2000). Indeed, in both mouse lines, early control of mycobacterial infection is impaired in the lungs, but not in other organs, such as the spleen, and the adaptive antimycobacterial immune response, longer term control of the infection, and survival of the infected animals were normal. The possible roles of IL-6 in the early control of *M. tuberculosis* infection have been discussed in detail by Saunders et al. (2000), and further work will be needed to firmly establish whether IL-6 plays a part in SIGNR3-mediated protection against the TB bacillus in vivo.

We showed that SIGNR3-mediated cytokine secretion required an intact tyrosine residue within the YxxL/I ITAM-like motif in the cytoplasmic tail of the lectin, and that this process was associated with Syk and dependent on both the NF-κB and Raf1–ERK pathways. These findings resemble

those for another myeloid C-type lectin, dectin-1 (Brown, 2006), which mediates protection against fungal infections through the secretion of inflammatory cytokines and an oxidative burst in a Syk- and CARD9/Bcl10-dependent manner, via its cytoplasmic YxxL motif. Like SIGNR3, dectin can signal on its own, or in collaboration with TLR2 to modulate inflammatory responses. Several C-type lectins carry intracellular ITAM-like motifs, and Syk-mediated signaling may be a common feature of this PRR family.

Our results raise intriguing questions about the potential similarity of DC-SIGN and SIGNR3 functions in humans and mice, and about the overall beneficial or detrimental nature of the DC-SIGN–mediated immune response to *M. tuberculosis* in vivo for the host. Interestingly, SIGNR3 in infected mice and DC-SIGN in patients with TB have similar patterns of expression, with both lectins induced during the infection process in cells of myeloid origin (Fig. 5; Tailleux et al., 2005). In vitro, DC-SIGN may signal in a TLR-dependent (Geijtenbeek et al., 2003; Caparrós et al., 2006; Gringhuis et al., 2007) or -independent (Hodges et al., 2007) manner. In human DCs, DC-SIGN binding to ManLAM (Geijtenbeek et al., 2003) or agonist antibodies (Caparrós et al., 2006) has been reported to increase secretion of the antiinflammatory cytokine IL-10 if it coincides with TLR4 stimulation by bacterial LPS. The intracellular Tyr_{31} residue in the cytoplasmic tail of DC-SIGN does not appear to be required for signaling in this case (Gringhuis et al., 2007). We observed no such increase in IL-10 secretion in SIGNR3-expressing cells stimulated with both ManLAM and LPS, but it is likely that macrophages and DCs differ in this respect. However, the TLR-independent stimulation of DC-SIGN requires Tyr_{31} and leads to the induction of *TNF* (Hodges et al., 2007), consistent with our findings in mouse SIGNR3.

M. tuberculosis and *Mycobacterium*-derived compounds can interact with TLRs in vitro, leading to cell activation and cytokine production (Jo, 2008). In vivo, TLR2 plays a key role in the production of TNF and IL-12, and TLR2-deficient mice have been reported to die earlier than WT controls after *M. tuberculosis* infection (Reiling et al., 2002), although these results were not confirmed in a more recent study of TLR2-4-9 triple KO mice (Hölscher et al., 2008). In contrast, the role of TLR4 in TB remains unclear. *M. tuberculosis*-derived compounds can stimulate TLR4 (Means et al., 1999; Takeuchi et al., 2000) in vitro, but TLR4 does not appear to be involved in antimycobacterial immunity in vivo, at least in mice (Reiling et al., 2002; Hölscher et al., 2008). Further investigations are required to determine whether DC-SIGN acts together with TLR4 to potentiate IL-10 production during the natural course of TB in vivo in humans. Such a mechanism might result in immune evasion by the bacillus (Geijtenbeek et al., 2003). It might also make it possible to control inflammation and to limit host tissue damage, thereby contributing to host protection (Neyrolles et al., 2006). These results are compatible with a model in which, depending on the cell context, DC-SIGN may signal either as a single PRR along a pathway requiring the Tyr^{31} residue, or in association with TLR4, independently of Tyr^{31}. The net effect of these multiple types of PRR stimulation on the immune response to *M. tuberculosis* remains unclear. The results obtained in this study with mice and the 19-kD protein, a mycobacterial TLR2 ligand also recognized by SIGNR3, suggest that SIGNR3 signaling synergizes with TLR2 for the production of protective inflammatory cytokines.

In summary, our results reveal, for the first time, the role of a C-type lectin, the DC-SIGN homologue SIGNR3, in innate resistance to *M. tuberculosis* in vivo. In addition to mycobacteria, DC-SIGN recognizes a plethora of viruses, bacteria, and parasites. The identification of SIGNR3 as the most likely functional orthologue of DC-SIGN and the availability of a mouse line in which this receptor has been inactivated should facilitate further investigations of the role of this lectin in several infectious diseases.

MATERIALS AND METHODS

Binding experiments. Mycobacterial ManLAM, LM, and AraLAM were purified as previously described (Pitarque et al., 2005). The 19-kD lipoprotein was produced as previously described (Turner et al., 2004). The 38- and 45-kD proteins were obtained from the Mycobacteria Research Laboratories at Colorado State University (grant NIH HHSN266200400091c). Aliquots of mycobacterial antigens (50 ml) diluted to 2 ng/ml in a 1:1 ethanol/water mixture were dried overnight in 96-well Immulon 4 plates (Thermo Fisher Scientific). Wells were blocked by incubation with 5% bovine serum albumin in Tris-buffered saline for 2 h at 37°C and rinsed twice with binding buffer (150 mM NaCl, 25 mM Tris-Cl, pH 7.8, and 2 mM $CaCl_2$). Tetrameric streptavidin complexes of biotin-tagged carbohydrate recognition domains from the mouse SIGNs were prepared as previously described (Powlesland et al., 2006), transferred to buffer containing sodium bicine, pH 8.5, in place of Tris buffer, reacted with radioiodinated Bolton-Hunter reagent (GE Healthcare), and repurified by affinity chromatography. Binding experiments were performed by incubation for 2 h at room temperature, with the labeled SIGNs diluted in binding buffer containing 0.5% bovine serum albumin. Wells were washed three times with binding buffer, and radioactivity was counted in a Wallac Wizard γ counter. At the maximum concentrations tested, 0.4 μg/ml for SIGNR1, 12 μg/ml for SIGNR3, and 6 μg/ml for SIGNR5, only SIGNR3 began to show saturation. For comparisons, we calculated the fraction of the input radioactivity that bound from the linear portions of the binding curves.

Cell lines. Rat6 fibroblasts producing SIGNR1, SIGNR3, and SIGNR5 were cultured as previously described (Powlesland et al., 2006). RAW 264.7 macrophages (TIB71; American Type Culture Collection) were cultured in RPMI 1640 GlutaMAX (Invitrogen) supplemented with 10% FCS (Sigma-Aldrich), 50 μM β-mercaptoethanol (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 100 U/ml penicillin/streptomycin (Invitrogen).

Genetic constructs and transfection. SIGNR3 cDNA was amplified from magnetic cell sorting–enriched preparations of $CD11c^+$ lung cells with the following oligonucleotides: SIGNR3Fd, 5′-CCACATGAGTGACTCCATGGAATCAAAG-3′; SIGNR3Rv, 5′-CTATTTGGTGGTGCATGATGAGGTTG-3′ by PCR (*Pfu*; Stratagene). The cDNA was inserted into the pUNO expression vector (Invivogen) to generate pUNO-SIGNR3. The Y27S mutant was generated from pUNO-SIGNR3 by PCR-mediated mutagenesis, with the Phusion Site-Directed Mutagenesis kit (Finnzymes), used according to the manufacturer's protocol, and the following oligonucleotides: S3-Y/S-27-Fd, 5′-GAGTGGCACCAGGTCTTCTGATATCAGCTC-3′; S3-Y/S-27-Rv, 5′-ATCAAACACTCTTCATCCTCTGGAATGACC-3′, generating pUNO-SIGNR3YS. RAW 264.7 cells were stably transfected with pUNO, pUNO-SIGNR3, and pUNO-SIGNR3YS by

nucleofection (Amaxa kit V), according to the manufacturer's protocol (Cell Line Nucleofector kit V for RAW 264.7 cells). Stable transfectants were selected on medium containing 40 μg/ml blasticidin (Invivogen), and were subsequently cultured in the presence of 10 μg/ml blasticidin. Transgene expression was assessed by qRT-PCR and immunostaining.

BMMs and nucleofection. BM cells from $Syk^{-/-}$ mice (Turner et al., 1995; provided by E. Schweighoffer and V. Tybulewicz, National Institute for Medical Research, London, England, UK) were prepapred by O. Joffre and C. Reis e Sousa. BM cells were flushed from the femurs and tibias of 6–8-wk-old female C57BL/6 or $Syk^{-/-}$ (Turner et al., 1995) mice, and resuspended in complete RPMI supplemented with 20 ng/ml recombinant M-CSF (Miltenyi Biotec). Macrophages were allowed to differentiate for 7 d. BMM nucleofection was performed as described in the Mouse Macrophage Nucleofector kit (Lonza). In brief, after the differentiation of fresh or frozen BM-derived progenitors, cells were harvested in cell dissociation solution (Sigma-Aldrich) and washed twice in PBS. We then resuspended 1 million cells in 100 μl Nucleofector Solution, to which we added 2 μg DNA (pUNO or pUNO::SIGNR3). Cell/DNA suspensions were then transfered into certified cuvettes, and the Y-001 nucleofection program was run. Nucleofected cells were then resuspended in 500 μl of preequilibrated medium (RPMI 1640, 20% FCS, 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM GlutaMAX), gently transfered into culture plates, and incubated in a humidified incubator at 37°C, in an atmosphere containing 5% CO_2. After overnight incubation, the medium was changed and cells were used for analyses 24 h after nucleofection.

Mycobacteria. *M. tuberculosis* was cultured at 37°C in Middlebrook 7H9 broth (Difco) supplemented with 10% ADC (5% bovine serum albumin fraction V, 2% dextrose, and 0.003% beef catalase; Difco) and 0.05% Tween 80 (Sigma-Aldrich), or in agar Middlebrook 7H11 broth (Difco) supplemented with OADC (0.05% oleic acid, ADC; Difco). DsRed-encoding *M. tuberculosis* was previously constructed for DsRed-BCG (Abadie et al., 2005).

Immunofluorescence and confocal microscopy. Cells were cultured on glass coverslips in 24-well plates (5×10^5 cells per well, in a volume of 1 ml). Biotinylated ManLAM and LM were prepared as previously described (Pitarque et al., 2005). Rat6 cells were pulsed with biotinylated ManLAM (20 μg/ml), biotinylated LM (20 μg/ml), and the 19-kD protein (5 μg/ml) for 2 h at 37°C, and washed three times with saline. Cells were fixed in 4% paraformaldehyde in saline and incubated for 20 min in 1% BSA (Sigma-Aldrich), 0.1% FCS (Sigma-Aldrich), and 0.05% saponin (Sigma-Aldrich) in saline, to block nonspecific binding and to permeabilize the cells. Streptavidin-Alexa Fluor 594 (Invitrogen) was used to detect Biot-LAM and Biot-LM. Primary rabbit serum against 19-kD (a gift from D. Young, National Institute for Medical Research, London, England, UK) and secondary goat anti–rabbit–Cy5 (GE Healthcare) were used to detect the 19-kD antigen. After labeling, coverslips were set in Fluoromount G (SouthernBiotech) on microscope slides. RAW 264.7 cells were fixed by incubation with Cytofix/Cytoperm buffer (BD) for 20 min at room temperature, blocked by incubation for 20 min at room temperature in Perm/Wash buffer (BD), and incubated for an additional 20 min at room temperature with ImageiT FX signal enhancer (Invitrogen). The cells were then incubated for 1 h at room temperature with a primary rat IgG2a antibody directed against SIGNR3 (DSR3-1.1; Wethmar et al., 2006). Primary antibodies were detected with a goat anti–rat IgG-conjugated with Alexa Fluor 594 (Invitrogen). Slides were observed by confocal microscopy, using an LSM510 apparatus (Carl Zeiss, Inc.). For flow cytometry analysis, the anti-SIGNR monoclonal antibody was detected with an Alexa Fluor 647–conjugated goat anti–rat IgG antibody.

Cell stimulation and ELISA. Cells were stimulated at 37°C for various periods of time with 10 μg/ml ManLAM, 1 μg/ml 19-kD protein, or 0.5 μg/ml PamCys3 lipopeptide (Invivogen) or *M. tuberculosis* at a multiplicity of infection of 5 bacteria/cell. For *M. tuberculosis*–infected cells, infection rate was quantified by flow cytometry using a DsRed-expressing bacterium. In some experiments, a blocking anti-TLR2 antibody (Clone T2.5; eBioscience) at a concentration of 50 μg/ml or the appropriate control isotype (mouse IgG1a; eBioscience) was used. LPS was used at a concentration of 100 ng/ml (Invivogen). In some experiments, cell signaling inhibitors were used at the concentrations indicated in the figure legends: U0126 (ERK1/2 kinase), BAY117082 (NF-κB), GW5074 (Raf1 kinase), piceatannol (Syk), or R406 (Syk), which were all purchased from Calbiochem, except for R406 (provided by J. Schmitz, Rigel Pharmaceuticals, Inc., San Francisco, CA), and diluted in dimethylsulfoxide (Sigma-Aldrich). After stimulation, cell culture, supernatants were harvested and TNF, IL-6, and IL-10 were detected by ELISA (OptEIA kits; BD) according to the kit manufacturer's instructions. Nitric oxide production was measured with Griess reagent (Invitrogen).

KO mice. The C57BL/6J mouse BAC clone RP23-12K14 (BACPAC Resources Center at Children's Hospital Oakland Research Institute, Oakland, CA), based on a sequence from the Ensembl database, spans the *CD209a (SIGNR5)*, *CD209b (SIGNR1)*, and *CD209d (SIGNR3)* genes. Using the BAC DNA as a template, we amplified homologous fragments by PCR. Two homologous fragments of the *CD209a* gene were inserted into the replacement KO vector *pKO*. Three homologous fragments of *CD209b* and *CD209d* were inserted separately into the conditional KO vector *pKO-3loxp*. The three resulting targeted vectors were used to transfect Bruce 4 embryonic stem (ES) cells, a C57BL/6 ES cell line. The homologous recombinant ES cell clones for each targeted gene were identified by Southern blotting. The selected ES clones carrying the homologous recombinant *CD209a* allele were injected into Albino B6 (The Jackson Laboratory) blastocysts to generate *CD209a* chimeric mice. Homologous recombinant ES clones for *CD209b* and *CD209d* were further transfected with the *pMC-Cre* vector encoding the Cre recombinase and cultured with 2 μM ganciclovir (Sigma-Aldrich). The ganciclovir-resistant ES cell subclones bearing floxed alleles were stored in liquid nitrogen. ES clones with the delta allele were injected into Albino B6 blastocysts, to generate chimeric mice. Male chimeras were crossed with Albino B6 females. The resulting KO mice were backcrossed with C57BL/6 three times, and then intercrossed. The homozygous KO of *CD209a*, *CD209b*, and *CD209d* was confirmed by PCR, Southern blotting, and RT-PCR using the following primers: SIGNR3Fd, 5′-TTGGTCCTGCAGCTGCTTTC-3′; SIGNR3Rv, 5′-ACCGACATTGTTGGGCTCC-3′; SIGNR5Fd, 5′-AAATGGGGAAGAGGCAGCTTC-3′; SIGNR5Rv, 5′-CAGCCTTCAACTGGGTCAGTTC-3′; β-actinFd, 5′-CCACACTGTGCCCATCTACGAG-3′; β-actinRv, 5′-CAGCACTGTGTTGGCATAGAGG-3′.

Mouse infection. 6–8-week-old female C57BL/6J mice were purchased from CER Janvier. All mice were housed in pathogen-free conditions and treated according to institutional animal care protocol no. 20080318/9, approved by the Regional Ethics Committee of Midi-Pyrénées (France) for Animal Experimentation (authorization no. MP/01/36/06/08). For high-dose infections, mice were anesthetized with a cocktail of ketamine (100 mg/kg; Merial) and xylasine (15 mg/kg; Bayer). Mice were infected intranasally with 10^3 CFUs of *M. tuberculosis* in 20 ml of saline/0.01% Tween 80. Mice were killed by CO_2 asphyxiation after various times. Lungs and spleens were harvested, homogenized, and plated on agar for colony counts. For low-dose infections, stock solutions of *M. tuberculosis* were diluted in sterile distilled water and pulmonary infection was achieved with a pediatric nebulization system. For mice infected with a dose of 100 CFU/lung, animals were exposed for 20 min to an aerosol generated by nebulizing ∼5 ml of a suspension containing 5×10^6 live bacteria/ml. Inoculum size was checked 24 h after infection by determining the bacterial load in the lungs of infected mice.

Intracellular cytokine staining and analysis of lymphocyte populations. For antigen-specific restimulation and flow cytometry analysis, single-cell suspensions were prepared from the lungs of *M. tuberculosis*–infected mice at various time points. Mice were anesthetized and injected intraperitoneally

with 150 U heparin sodium (Sanofi-Aventis). Lungs were perfused through the right ventricle with warm PBS. Lungs were removed once they appeared white. Single-cell preparations were then obtained by mechanical dissociation, using the gentle MACS Dissociator (Miltenyi Biotec). We followed the protocol for the preparation of single-cell suspensions from mouse lung with collagenase D described by the manufacturer. The live lung cells recovered were counted and diluted in complete RPMI (Invitrogen) supplemented with 10% fetal calf serum (Dutscher) and penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively; Invitrogen). We used intracellular cytokine staining kits (BD) for the detection of intracellular IFN-γ, IL-4, and IL-17. In brief, single-cell suspensions were prepared 15, 21, and 42 d after infection. Cells were then plated at a density of 7×10^6 cells/ml in complete RPMI. Cells were incubated with 20 μg/ml mycobacterial purified protein derivative (Aventis-Pasteur) in 24-well plates for 1 h. They were then incubated with Golgi-Stop (brefeldin; BD) for 5 h at 37°C. Nonspecific antibody binding was blocked by incubation with a cocktail containing anti-FcRIII/II mAb (clone 2.4G2). Cells were washed and incubated with optimal concentrations of anti–CD4-FITC or anti–CD8-FITC (both from BD). After staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD), and the IFN-γ and IL-17 accumulating within the cells were stained with PE-labeled anti–IFN-γ and –IL-17 mAb (BD), respectively. The IL-4 accumulating within the cells was stained with biotin-labeled anti–IL-4 mAb (eBiosciences) and PE-labeled streptavidin. Fluorescence intensity was analyzed on a FACSCalibur (BD) machine, with gating on $CD4^+$ or $CD8^+$ lymphocytes identified on the basis of FSC-SSC profile.

Histology. The lungs were removed aseptically from the killed mice. They were fixed in zinc fixative and embedded in paraffin. Serial 4-μm sections were cut and stained with hematoxylin and eosin. For immunohistochemistry, lungs were fixed with zinc for 2 d at 4°C. After fixation, the tissues were dehydrated in graded ethyl alcohol concentrations and embedded in paraffin at 37°C. Immunohistochemistry was performed as described previously (Tailleux et al., 2005). Sections (5 μm thick) were immunostained with the following mAbs: an affinity-purified anti-SIGNR3 rabbit IgG (Wethmar et al., 2006), biotin anti-CD11c antibody (eBioscience), anti-F4/80 (Invitrogen), biotin anti-IA^d (BD), anti-NOS2 (Santa Cruz Biotechnology, Inc.), Cy3-conjugated secondary mAb (GE Healthcare), Alexa Fluor 488–conjugated secondary mAb and Alexa Fluor 488–conjugated streptavidin (Invitrogen). Nuclei were counterstained with To-Pro 3 iodide (Invitrogen). Stainings were analyzed using a confocal microscope (Carl Zeiss, Inc.) and the software LSM 510 v3.2 (Zeiss).

RNA extraction and qRT-PCR. Total RNA was extracted from cells using the RNeasy minikit (QIAGEN) according to the manufacturer's instructions. RNA was treated with RNase-free DNase (Ambion). The concentration and purity of the extracted RNA were assessed with a NanoDrop 1000 apparatus (NanoDrop). A two-step RT-PCR was performed with the SuperScript First-Strand Synthesis System (Invitrogen) and random hexamer primers. Specific primers for quantifying IL-6, TNF, and the house-keeping gene HPRT were used: TNF sense 5′-TCTCATCAGTTCTATGGCCC-3′; TNF antisense 5′-GGGAGTAGACAAGGTACAAC-3′; IL-6 sense 5′-GTTCTCTGGGAAATCGTGGA-3′; IL-6 antisense 5′-TGTACTCCAGGTAGCTATGG-3′; HPRT sense 5′-GATTCAACTTGCGCTCATCTTA-3′; and HPRT antisense 5′-GTTGGATACAGGCCAGACTTTGTTG-3′. All primers were selected to span the predicted splice sites. PCR was performed with a 25-μl reaction mixture, in triplicate, with a sequence detection system (model ABI Prism 7300; Applied Biosystems) and the Power SYBR-Green PCR mastermix (Applied Biosystems). The threshold cycle (Ct; the number of the cycle at which the amount of the amplified gene of interest reached a fixed threshold) was then determined. Relative mRNA levels were calculated by the comparative Ct method. Relative target mRNA levels, normalized with respect to an endogenous control (*HPRT*) and in particular calibrator conditions, were expressed as $2^{-\Delta\Delta Ct}$ (fold), where ΔCt = Ct of the target gene − Ct of the control gene (*HPRT*), and $\Delta\Delta Ct = \Delta Ct$ of the studied set of conditions − ΔCt of the calibrator conditions, as previously described (Livak and Schmittgen, 2001).

Statistical analyses. Data were analyzed using the Student's *t* test, with Welch's correction in cases of unequal variance, as assessed with the *F* test.

Online supplemental material. Fig. S1 depicts comparative genomic protein sequence and phylogeny analysis of human DC-SIGN, its paralogue L-SIGN, and their homologues in the mouse. Fig. S2–S4 illustrate the strategy used to inactivate SIGNR1, SIGNR3, and SIGNR5, respectively, and show RT-PCR analysis of the KO and control animals. In Fig. S5, we show that SIGNR3 expression in macrophages does not influence nitric oxide production and does not enhance IL-10 secretion after TLR4 stimulation by LPS. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090188/DC1.

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REFERENCES

Abadie, V., E. Badell, P. Douillard, D. Ensergueix, P.J. Leenen, M. Tanguy, L. Fiette, S. Saeland, B. Gicquel, and N. Winter. 2005. Neutrophils rapidly migrate via lymphatics after *Mycobacterium bovis* BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. *Blood*. 106:1843–1850. doi:10.1182/blood-2005-03-1281

Barreiro, L.B., O. Neyrolles, C.L. Babb, L. Tailleux, H. Quach, K. McElreavey, P.D. Helden, E.G. Hoal, B. Gicquel, and L. Quintana-Murci. 2006. Promoter variation in the DC-SIGN-encoding gene CD209 is associated with tuberculosis. *PLoS Med*. 3:e20. doi:10.1371/journal.pmed.0030020

Bashirova, A.A., L. Wu, J. Cheng, T.D. Martin, M.P. Martin, R.E. Benveniste, J.D. Lifson, V.N. KewalRamani, A. Hughes, and M. Carrington. 2003. Novel member of the CD209 (DC-SIGN) gene family in primates. *J. Virol*. 77:217–227. doi:10.1128/JVI.77.1.217-227.2003

Brightbill, H.D., D.H. Libraty, S.R. Krutzik, R.B. Yang, J.T. Belisle, J.R. Bleharski, M. Maitland, M.V. Norgard, S.E. Plevy, S.T. Smale, et al. 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science*. 285:732–736. doi:10.1126/science.285.5428.732

Brown, G.D. 2006. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol*. 6:33–43. doi:10.1038/nri1745

Caminschi, I., A.J. Corbett, C. Zahra, M. Lahoud, K.M. Lucas, M. Sofi, D. Vremec, T. Gramberg, S. Pöhlmann, J. Curtis, et al. 2006. Functional comparison of mouse CIRE/mouse DC-SIGN and human DC-SIGN. *Int. Immunol*. 18:741–753. doi:10.1093/intimm/dxl011

Caparrós, E., P. Munoz, E. Sierra-Filardi, D. Serrano-Gómez, A. Puig-Kröger, J.L. Rodríguez-Fernández, M. Mellado, J. Sancho, M. Zubiaur, and A.L. Corbí. 2006. DC-SIGN ligation on dendritic cells results in ERK and PI3K activation and modulates cytokine production. *Blood*. 107:3950–3958. doi:10.1182/blood-2005-03-1252

Delgado, M.A., R.A. Elmaoued, A.S. Davis, G. Kyei, and V. Deretic. 2008. Toll-like receptors control autophagy. *EMBO J.* 27:1110–1121. doi:10.1038/emboj.2008.31

el-Rahman, A., M.A. Hammouda, and A. Fakeir. 1995. Flow cytometric evaluation of erythrocyte response to oxidant stress. *Cytometry*. 20:19–22. doi:10.1002/cyto.990200105

Flynn, J.L., and J. Chan. 2001. Immunology of tuberculosis. *Annu. Rev. Immunol.* 19:93–129. doi:10.1146/annurev.immunol.19.1.93

Flynn, J.L., M.M. Goldstein, J. Chan, K.J. Triebold, K. Pfeffer, C.J. Lowenstein, R. Schreiber, T.W. Mak, and B.R. Bloom. 1995. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity*. 2:561–572. doi:10.1016/1074-7613(95)90001-2

Geijtenbeek, T.B., R. Torensma, S.J. van Vliet, G.C. van Duijnhoven, G.J. Adema, Y. van Kooyk, and C.G. Figdor. 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell*. 100:575–585. doi:10.1016/S0092-8674(00)80693-5

Geijtenbeek, T.B., P.C. Groot, M.A. Nolte, S.J. van Vliet, S.T. Gangaram-Panday, G.C. van Duijnhoven, G. Kraal, A.J. van Oosterhout, and Y. van Kooyk. 2002. Marginal zone macrophages express a murine homologue of DC-SIGN that captures blood-borne antigens in vivo. *Blood*. 100:2908–2916. doi:10.1182/blood-2002-04-1044

Geijtenbeek, T.B., S.J. Van Vliet, E.A. Koppel, M. Sanchez-Hernandez, C.M. Vandenbroucke-Grauls, B. Appelmelk, and Y. Van Kooyk. 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* 197:7–17. doi:10.1084/jem.20021229

Geijtenbeek, T.B., S.J. van Vliet, A. Engering, B.A. 't Hart, and Y. van Kooyk. 2004. Self- and nonself-recognition by C-type lectins on dendritic cells. *Annu. Rev. Immunol.* 22:33–54. doi:10.1146/annurev.immunol.22.012703.104558

Gordon, S. 2002. Pattern recognition receptors: doubling up for the innate immune response. *Cell*. 111:927–930. doi:10.1016/S0092-8674(02)01201-1

Gramberg, T., I. Caminschi, A. Wegele, H. Hofmann, and S. Pöhlmann. 2006. Evidence that multiple defects in murine DC-SIGN inhibit a functional interaction with pathogens. *Virology*. 345:482–491. doi:10.1016/j.virol.2005.10.008

Gringhuis, S.I., J. den Dunnen, M. Litjens, B. van Het Hof, Y. van Kooyk, and T.B. Geijtenbeek. 2007. C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. *Immunity*. 26:605–616. doi:10.1016/j.immuni.2007.03.012

Guo, Y., H. Feinberg, E. Conroy, D.A. Mitchell, R. Alvarez, O. Blixt, M.E. Taylor, W.I. Weis, and K. Drickamer. 2004. Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. *Nat. Struct. Mol. Biol.* 11:591–598. doi:10.1038/nsmb784

Hodges, A., K. Sharrocks, M. Edelmann, D. Baban, A. Moris, O. Schwartz, H. Drakesmith, K. Davies, B. Kessler, A. McMichael, and A. Simmons. 2007. Activation of the lectin DC-SIGN induces an immature dendritic cell phenotype triggering Rho-GTPase activity required for HIV-1 replication. *Nat. Immunol.* 8:569–577. doi:10.1038/ni1470

Hölscher, C., N. Reiling, U.E. Schaible, A. Hölscher, C. Bathmann, D. Korbel, I. Lenz, T. Sonntag, S. Kröger, S. Akira, et al. 2008. Containment of aerogenic *Mycobacterium tuberculosis* infection in mice does not require MyD88 adaptor function for TLR2, -4 and -9. *Eur. J. Immunol.* 38:680–694. doi:10.1002/eji.200736458

Ishii, K.J., S. Koyama, A. Nakagawa, C. Coban, and S. Akira. 2008. Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe*. 3:352–363. doi:10.1016/j.chom.2008.05.003

Janeway, C.A. Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol.* 20:197–216. doi:10.1146/annurev.immunol.20.083001.084359

Jo, E.K. 2008. Mycobacterial interaction with innate receptors: TLRs, C-type lectins, and NLRs. *Curr. Opin. Infect. Dis.* 21:279–286. doi:10.1097/QCO.0b013e3282f88b5d

Koppel, E.A., I.S. Ludwig, M.S. Hernandez, T.L. Lowary, R.R. Gadikota, A.B. Tuzikov, C.M. Vandenbroucke-Grauls, Y. van Kooyk, B.J. Appelmelk, and T.B. Geijtenbeek. 2004. Identification of the mycobacterial carbohydrate structure that binds the C-type lectins DC-SIGN, L-SIGN and SIGNR1. *Immunobiology*. 209:117–127. doi:10.1016/j.imbio.2004.03.003

Ladel, C.H., C. Blum, A. Dreher, K. Reifenberg, M. Kopf, and S.H. Kaufmann. 1997. Lethal tuberculosis in interleukin-6-deficient mutant mice. *Infect. Immun.* 65:4843–4849.

Liu, P.T., S. Stenger, H. Li, L. Wenzel, B.H. Tan, S.R. Krutzik, M.T. Ochoa, J. Schauber, K. Wu, C. Meinken, et al. 2006. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science*. 311:1770–1773. doi:10.1126/science.1123933

Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 25:402–408. doi:10.1006/meth.2001.1262

Maeda, N., J. Nigou, J.L. Herrmann, M. Jackson, A. Amara, P.H. Lagrange, G. Puzo, B. Gicquel, and O. Neyrolles. 2003. The cell surface receptor DC-SIGN discriminates between *Mycobacterium* species through selective recognition of the mannose caps on lipoarabinomannan. *J. Biol. Chem.* 278:5513–5516. doi:10.1074/jbc.C200586200

Means, T.K., S. Wang, E. Lien, A. Yoshimura, D.T. Golenbock, and M.J. Fenton. 1999. Human toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. *J. Immunol.* 163:3920–3927.

Medzhitov, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature*. 449:819–826. doi:10.1038/nature06246

Neyrolles, O., B. Gicquel, and L. Quintana-Murci. 2006. Towards a crucial role for DC-SIGN in tuberculosis and beyond. *Trends Microbiol.* 14:383–387. doi:10.1016/j.tim.2006.07.007

Nigou, J., A. Vercellone, and G. Puzo. 2000. New structural insights into the molecular deciphering of mycobacterial lipoglycan binding to C-type lectins: lipoarabinomannan glycoform characterization and quantification by capillary electrophoresis at the subnanomole level. *J. Mol. Biol.* 299:1353–1362. doi:10.1006/jmbi.2000.3821

Nigou, J., T. Vasselon, A. Ray, P. Constant, M. Gilleron, G.S. Besra, I. Sutcliffe, G. Tiraby, and G. Puzo. 2008. Mannan chain length controls lipoglycans signaling via and binding to TLR2. *J. Immunol.* 180:6696–6702.

Park, C.G., K. Takahara, E. Umemoto, Y. Yashima, K. Matsubara, Y. Matsuda, B.E. Clausen, K. Inaba, and R.M. Steinman. 2001. Five mouse homologues of the human dendritic cell C-type lectin, DC-SIGN. *Int. Immunol.* 13:1283–1290. doi:10.1093/intimm/13.10.1283

Pitarque, S., J.L. Herrmann, J.L. Duteyrat, M. Jackson, G. Stewart, F. Lecointe, B. Payré, O. Schwartz, D. Young, G. Marchal, et al. 2005. Deciphering the molecular bases of *Mycobacterium tuberculosis* binding to the lectin DC-SIGN reveals an underestimated complexity. *Biochem. J.* 392:615–624.

Powlesland, A.S., E.M. Ward, S.K. Sadhu, Y. Guo, M.E. Taylor, and K. Drickamer. 2006. Widely divergent biochemical properties of the complete set of mouse DC-SIGN-related proteins. *J. Biol. Chem.* 281:20440–20449. doi:10.1074/jbc.M601925200

Puig-Kröger, A., D. Serrano-Gómez, E. Caparrós, A. Domínguez-Soto, M. Relloso, M. Colmenares, L. Martínez-Muñoz, N. Longo, N. Sánchez-Sánchez, M. Rincon, et al. 2004. Regulated expression of the pathogen receptor dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin in THP-1 human leukemic cells, monocytes, and macrophages. *J. Biol. Chem.* 279:25680–25688. doi:10.1074/jbc.M311516200

Quintana-Murci, L., A. Alcaïs, L. Abel, and J.L. Casanova. 2007. Immunology *in natura*: clinical, epidemiological and evolutionary genetics of infectious diseases. *Nat. Immunol.* 8:1165–1171. doi:10.1038/ni1535

Reiling, N., C. Hölscher, A. Fehrenbach, S. Kröger, C.J. Kirschning, S. Goyert, and S. Ehlers. 2002. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with *Mycobacterium tuberculosis*. *J. Immunol.* 169:3480–3484.

Robinson, M.J., D. Sancho, E.C. Slack, S. LeibundGut-Landmann, and C. Reis e Sousa. 2006. Myeloid C-type lectins in innate immunity. *Nat. Immunol.* 7:1258–1265. doi:10.1038/ni1417

Rogers, N.C., E.C. Slack, A.D. Edwards, M.A. Nolte, O. Schulz, E. Schweighoffer, D.L. Williams, S. Gordon, V.L. Tybulewicz, G.D. Brown, and C. Reis e Sousa. 2005. Syk-dependent cytokine induction

by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity*. 22:507–517. doi:10.1016/j.immuni.2005.03.004

Saijo, S., N. Fujikado, T. Furuta, S.H. Chung, H. Kotaki, K. Seki, K. Sudo, S. Akira, Y. Adachi, N. Ohno, et al. 2007. Dectin-1 is required for host defense against *Pneumocystis carinii* but not against *Candida albicans*. *Nat. Immunol.* 8:39–46. doi:10.1038/ni1425

Saunders, B.M., A.A. Frank, I.M. Orme, and A.M. Cooper. 2000. Interleukin-6 induces early gamma interferon production in the infected lung but is not required for generation of specific immunity to *Mycobacterium tuberculosis* infection. *Infect. Immun.* 68:3322–3326. doi:10.1128/IAI.68.6.3322-3326.2000

Schaefer, M., N. Reiling, C. Fessler, J. Stephani, I. Taniuchi, F. Hatam, A.O. Yildirim, H. Fehrenbach, K. Walter, J. Ruland, et al. 2008. Decreased pathology and prolonged survival of human DC-SIGN transgenic mice during mycobacterial infection. *J. Immunol.* 180:6836–6845.

Soilleux, E.J., L.S. Morris, G. Leslie, J. Chehimi, Q. Luo, E. Levroney, J. Trowsdale, L.J. Montaner, R.W. Doms, D. Weissman, et al. 2002. Constitutive and induced expression of DC-SIGN on dendritic cell and macrophage subpopulations *in situ* and in vitro. *J. Leukoc. Biol.* 71:445–457.

Tailleux, L., O. Schwartz, J.L. Herrmann, E. Pivert, M. Jackson, A. Amara, L. Legres, D. Dreher, L.P. Nicod, J.C. Gluckman, et al. 2003. DC-SIGN is the major *Mycobacterium tuberculosis* receptor on human dendritic cells. *J. Exp. Med.* 197:121–127. doi:10.1084/jem.20021468

Tailleux, L., N. Pham-Thi, A. Bergeron-Lafaurie, J.L. Herrmann, P. Charles, O. Schwartz, P. Scheinmann, P.H. Lagrange, J. de Blic, A. Tazi, et al. 2005. DC-SIGN induction in alveolar macrophages defines privileged target host cells for mycobacteria in patients with tuberculosis. *PLoS Med.* 2:e381. doi:10.1371/journal.pmed.0020381

Takeuchi, O., K. Takeda, K. Hoshino, O. Adachi, T. Ogawa, and S. Akira. 2000. Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. *Int. Immunol.* 12:113–117. doi:10.1093/intimm/12.1.113

Taylor, P.R., G.D. Brown, J. Herre, D.L. Williams, J.A. Willment, and S. Gordon. 2004. The role of SIGNR1 and the beta-glucan receptor (dectin-1) in the nonopsonic recognition of yeast by specific macrophages. *J. Immunol.* 172:1157–1162.

Taylor, P.R., S.V. Tsoni, J.A. Willment, K.M. Dennehy, M. Rosas, H. Findon, K. Haynes, C. Steele, M. Botto, S. Gordon, and G.D. Brown. 2007. Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* 8:31–38. doi:10.1038/ni1408

Torrelles, J.B., A.K. Azad, L.N. Henning, T.K. Carlson, and L.S. Schlesinger. 2008. Role of C-type lectins in mycobacterial infections. *Curr. Drug Targets*. 9:102–112. doi:10.2174/138945008783502467

Turner, M., P.J. Mee, P.S. Costello, O. Williams, A.A. Price, L.P. Duddy, M.T. Furlong, R.L. Geahlen, and V.L. Tybulewicz. 1995. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature*. 378:298–302. doi:10.1038/378298a0

Turner, J., K.M. Dobos, M.A. Keen, A.A. Frank, S. Ehlers, I.M. Orme, J.T. Belisle, and A.M. Cooper. 2004. A limited antigen-specific cellular response is sufficient for the early control of *Mycobacterium tuberculosis* in the lung but is insufficient for long-term survival. *Infect. Immun.* 72:3759–3768. doi:10.1128/IAI.72.7.3759-3768.2004

Vannberg, F.O., S.J. Chapman, C.C. Khor, K. Tosh, S. Floyd, D. Jackson-Sillah, A. Crampin, L. Sichali, B. Bah, P. Gustafson, et al. 2008. CD209 genetic polymorphism and tuberculosis disease. *PLoS One*. 3:e1388. doi:10.1371/journal.pone.0001388

Weis, W.I., M.E. Taylor, and K. Drickamer. 1998. The C-type lectin superfamily in the immune system. *Immunol. Rev.* 163:19–34. doi:10.1111/j.1600-065X.1998.tb01185.x

Wethmar, K., Y. Helmus, K. Lühn, C. Jones, A. Laskowska, G. Varga, S. Grabbe, R. Lyck, B. Engelhardt, M.G. Bixel, et al. 2006. Migration of immature mouse DC across resting endothelium is mediated by ICAM-2 but independent of beta2-integrins and murine DC-SIGN homologues. *Eur. J. Immunol.* 36:2781–2794. doi:10.1002/eji.200526311

Wieland, C.W., E.A. Koppel, J. den Dunnen, S. Florquin, A.N. McKenzie, Y. van Kooyk, T. van der Poll, and T.B. Geijtenbeek. 2007. Mice lacking SIGNR1 have stronger T helper 1 responses to *Mycobacterium tuberculosis*. *Microbes Infect.* 9:134–141. doi:10.1016/j.micinf.2006.10.018