Partial Redundancy of the Pattern Recognition Receptors, Scavenger Receptors, and C-Type Lectins for the Long-Term Control of \textit{Mycobacterium tuberculosis} Infection

Nathalie Court, Virginie Vasseur, Rachel Vacher, Cécile Frémond, Yury Shebzukhov, Vladimir V. Yeremeev, Isabelle Maillet, Sergei A. Nedospasov, Siamon Gordon, Padraic G. Fallon, Hiroshi Suzuki, Bernhard Ryffel and Valérie F. J. Quesniaux

\textit{J Immunol} 2010; 184:7057-7070; Prepublished online 19 May 2010; doi: 10.4049/jimmunol.1000164

http://www.jimmunol.org/content/184/12/7057

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2010/05/19/jimmunol.1000164.DC1

**References**

This article cites 63 articles, 35 of which you can access for free at: http://www.jimmunol.org/content/184/12/7057.full#ref-list-1

**Subscription**

Information about subscribing to \textit{The Journal of Immunology} is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Partial Redundancy of the Pattern Recognition Receptors, Scavenger Receptors, and C-Type Lectins for the Long-Term Control of Mycobacterium tuberculosis Infection

Nathalie Court,* Virginie Vasseur,* Rachel Vacher,* Cécile Frémont,* Yury Shebzukhov,† Vladimir V. Yeremeev,*,†,‡ Isabelle Maillet,* Sergei A. Nedospasov,†,‡ Siamon Gordon,§ Padraic G. Fallon,§ Hiroshi Suzuki,§ Bernhard Ryffel,* and Valérie F. J. Quesniaux*

Mycobacterium tuberculosis is recognized by multiple pattern recognition receptors involved in innate immune defense, but their direct role in tuberculosis pathogenesis remains unknown. Beyond TLRs, scavenger receptors (SRs) and C-type lectins may play a crucial role in the sensing and signaling of pathogen motifs, as well as contribute to M. tuberculosis immune evasion. In this study, we addressed the relative role and potential redundancy of these receptors in the host response and resistance to M. tuberculosis infection using mice deficient for representative SR, C-type lectin receptor, or seven transmembrane receptor families. We show that a single deficiency in the class A SR, macrophage receptor with collagenous structure, CD36, mannose receptor, specific ICAM-3 grabbing nonintegrin-related, or F4/80 did not impair the host resistance to acute or chronic M. tuberculosis infection in terms of survival, control of bacterial clearance, lung inflammation, granuloma formation, and cytokine and chemokine expression. Double deficiency for the SRs class A SR types I and II plus CD36 or for the C-type lectins mannose receptor plus specific ICAM-3 grabbing nonintegrin-related had a limited effect on macrophage uptake of mycobacteria and TNF response and on the long-term control of M. tuberculosis infection. By contrast, mice deficient in the TNF, IL-1, or IFN-γ pathway were unable to control acute M. tuberculosis infection. In conclusion, we document a functional redundancy in the pattern recognition receptors, which might cooperate in a coordinated response to sustain the full immune control of M. tuberculosis infection, in sharp contrast with the nonredundant, essential role of the TNF, IL-1, or IFN-γ pathway for host resistance to M. tuberculosis. The Journal of Immunology, 2010, 184: 7057–7070.

The lung, the entry point for Mycobacterium tuberculosis and various other pathogens or particles, relies on the expression of multiple pattern recognition receptors (PRRs) for innate immune defense. The heterogeneity of macrophage receptors and their role in immune recognition has been extensively reviewed (1). In addition to TLRs and nod-like receptors, several types of PRRs, including scavenger receptors (SRs) and C-type lectin receptors, may play a crucial role in the recognition of mycobacteria and downstream signaling (2, 3). Here, we studied in vivo the role in the host response to acute and chronic M. tuberculosis infection of a selection of receptors representative of SRs, C-type lectin receptors, or seven transmembrane receptor families using genetically deficient mice.

The first contact of M. tuberculosis with host cells, such as alveolar macrophages, triggers a robust innate immune response leading to a specific adaptive immune response. In humans and experimental animals, protective immunity to M. tuberculosis infection is regulated by T cells; however, macrophages, dendritic cells (DCs), and several inflammatory cytokines are essential and nonredundant for control of the infection, including IFN-γ, IL-1 and -12, and TNF (4–9). Although receptors, such as TLR2, TLR4, and TLR9, have been implicated in the sensing and innate response to M. tuberculosis (10–13), their role seems limited (14), and other innate receptors may contribute to mounting the adaptive host response. Indeed, we and other investigators showed that although mice deficient for MyD88, the adaptor common to all TLRs but TLR3, are exquisitely sensitive to acute M. tuberculosis infection, they are able to mount an adaptive antimycobacterial response and are partially protected by vaccination with bacillus Calmette-Guérin (BCG) (14, 15).

In addition, M. tuberculosis has developed several strategies to ensure its survival in the host, and several types of receptors contribute to the immunomodulation of the host response by mycobacteria. Among them is inhibition of phagolysosome maturation...
by *M. tuberculosis* lipoarabinomannan through engagement of the human mannose receptor (16, 17). The human C-type lectins mannose receptor and DC-specific ICAM-3-grabbing nonintegrin (DCSIGN) were also implicated in the negative regulation of TLR-induced responses by mannose-capped lipoarabinomannan (18, 19) through Raf-1 kinase-dependent activation and IL-10 secretion, leading to inhibition of Th1-polarized responses (20, 21).

The SR family includes two members in the A subclass that are expressed on lung macrophages and DCs: macrophage receptor with collagenous structure (MARCO) and class A SR types I and II (referred to herein as SR-A) (22–24). MARCO and SR-A have a collagenous structure, bind acetylated low-density lipoprotein and bacteria, are expressed on alveolar macrophages, and promote the uptake and clearance of inhaled particles and bacteria (25–28). MARCO expression is induced on alveolar macrophages after BCG infection (29). Moreover, MARCO was shown recently to tether *M. tuberculosis* trehalose 6,6′-dimycolate (cord factor) to macrophages and activate the TLR2/CD14 signaling pathway (30). The contribution of SR-A to this response was more limited (30); it was reported to downmodulate alveolar proinflammatory cytokine responses to cord factor (31). The roles of SR-A and MARCO in the in vivo response to mycobacteria have not been reported.

The class B SR CD36 was shown to be required for the uptake of mycobacteria in *Drosophila* macrophage-like cells (32). CD36 cooperates with TLR2 in sensing bacteria and bacterial ligands, acting as a coreceptor for the induction of proinflammatory cytokines; CD36-deficient mice were unable to control *Staphylococcus aureus* infection (33, 34). Because TLR2 is one of the TLRs most involved in mycobacterial motives recognition, it was of interest to see whether CD36 might also contribute to the TLR2-mycobacterial response.

C-type lectins involved in the recognition of mycobacteria include the mannose receptor and human DC-SIGN family (35, 36). Seven DC-SIGN homolog genes and a pseudogene have been described in mice (37, 38). In this study, we concentrated on addressing the role of murine mannose receptor and specific ICAM-3 grabbing non-integrin related (SIGNR)1, because SIGNR1 was reported to associate with TLR4/myeloid-differentiation factor 2 and modulate downstream signaling under specific conditions (39).

The seven transmembrane receptor, epidermal growth factor module-containing mucin-like hormone receptor 1 (EMR1) murine ortholog F4/80 was also studied. Indeed, F4/80 is upregulated in differentiated macrophages, and it may be implicated in macrophage adhesion and migration (40). F4/80 macrophage expression was shown to be reduced after *Mycobacterium* bovis BCG infection (41). F4/80 was implicated in macrophage-dependent modulation of IFN-γ release by NK cells in response to *Listeria* (42), and it was shown to be required for the induction of Ag-specific effenter regulatory T cells in peripheral tolerance (43).

Little is known about the relative role and potential redundancy of these receptors in the host immune response to acute *M. tuberculosis* infection. In this study, we addressed this question using genetically deficient mice for a selection of receptors representative of SR, C-type lectin receptor, or seven transmembrane receptor families. We show that single deficiency in MARCO, SR-A, CD36, mannose receptor, SIGNR1, or F4/80 or double deficiency for the SRs SR-A plus CD36 or for the C-type lectins mannose receptor plus SIGNR1 did not impair the control of acute or chronic *M. tuberculosis* infection. Mice deficient for proinflammatory cytokine pathways, such as TNF, IL-1R1, the adaptor MyD88, or IFN-γ, were tested in parallel as internal controls for the drastic phenotypes obtained when essential signaling pathways are not functional. The data point to a high redundancy in the PRRs tested for the control of *M. tuberculosis* infection, whereas there is no such redundancy in the proinflammatory TNF, IL-1, or IFN-γ cytokine pathways.

**Materials and Methods**

**Mice**

Mice deficient for mannose receptor (44), MARCO (26), SR-A (45), CD36 (46), SR-A plus CD36 (47), F4/80 (43), SIGNR1 (48), TNF (49), IL-1R1 (50), or MyD88 (51) were bred in our animal facility at the Transgenose Institute (National Center for Scientific Research, Orleans, France). All mice were backcrossed ≥7–10 times on the C57BL/6 genetic background. For experiments, 8–15-wk-old animals were kept in isolators in a biohazard animal unit. The infected mice were monitored regularly for clinical status and weighed weekly. All animal experimental protocols were approved by the Regional Ethics Committee for Animal Experimentation, section “Centre-Limousin” (No. CL2008-011).

**Infection**

*M. tuberculosis* H37Rv (Pasteur Institute, Paris, France) aliquots kept frozen at −80°C were thawed, briefly vortexed, and diluted in sterile saline containing 0.05% Tween 20; clumping was disrupted by 20 repeated aspirations through a 29-gauge needle (Omnican, Braun, Germany). Pulmonary infection with *M. tuberculosis* H37Rv was performed by delivering ~200 bacteria into the nasal cavities (20 μl each) under xylazine-ketamine anesthesia; the inoculum size was verified 24 h postinfection by determining bacterial load in the lungs.

**Bacterial load in tissues**

Bacterial loads in the lung of infected mice were evaluated at different time points postinfection with *M. tuberculosis* H37Rv, as described (52). Organs were weighed, and defined aliquots were homogenized in 0.05% Tween 20 NaCl. Ten-fold serial dilutions of organ homogenates were plated in duplicate onto Middlebrook 7H11 agar plates containing 10% oleic acid/albumin/dextrose/catalase and incubated at 37°C. Colonies were enumerated at 3 wk, and results were expressed as log10 CFU per organ.

**Pulmonary cytokine concentrations**

Cytokine and chemokine concentrations in the lung of infected mice were evaluated in lung homogenates after passage through a 0.20-μm filter using Endogen Search Light Protein array by Perbio Thermo Fisher Scientific (Woburn, MA).

**Histopathological analysis**

For histological analysis, lungs were removed at different time points of infection, fixed in 10% phosphate-buffered formalin, and embedded in paraffin. Two- to 3-μm sections were stained with H&E and a modified Ziehl-Neelsen (ZN) method. The latter involved staining in a prewarmed (60°C) carbol-fuchsin solution for 10 min, followed by destaining in 20% sulfuric acid and 40% ethanol, before counterstaining with methylene blue. Free airway space, lung cellular infiltration, edema, basillci burden, and necrosis were quantified, using a semiquantitative score with increasing severity of changes (0–5), by two independent observers, including a trained pathologist (B.R.).

**Primary macrophage cultures**

Nonelicited peritoneal macrophages were collected after 0.34 M sucrose lavage, washed, and adhered. Bone marrow cells were isolated from femurs and differentiated into macrophages after culturing at 106 cells/ml for 7 d in DMEM (Sigma-Aldrich, St Louis, MO) supplemented with 20% horse serum and 30% L929 cell-conditioned medium as a source of M-CSF (53). Three days after washing and reculturing in fresh medium, the cell preparation contained a homogeneous population of macrophages. Macrophages were plated in 96-well microculture plates (at 104 cells/well in DMEM supplemented with 10 mM L-glutamine, 25 mM HEPS, 100 U/ml penicillin, and 100 μg/ml streptomycin, without or with 10% FCS for peritoneal macrophages) and stimulated with LPS (*Escherichia coli*, serotype O111:B4, Sigma-Aldrich, at 100 ng/ml), synthetic bacterial lipopeptide ([S-(2,3-bis-(N-palmitoyl-(R)-Cys-(S)-Ser-Lys4-OH)], N-palmitoyl-(R)-Cys-(S)-Ser-LYS-OH), trihydrochloride [Pam3CSK4]; EMC microcollections, Tuebingen, Germany), or MyD88 (Alexis Biochemicals, Lausanne, Switzerland; 30 ng/ml) BCG (Pasteur Institute; multiplicity of infection [MOI] of two bacteria/cell), lipopolysaccharide BCG (kind gift of Prof. G. Marchal, Pasteur Institute; 10 μg/ml), heat-killed *M. bovis* BCG (Pasteur Institute; MOI of two bacteria/cell), or
**Confocal microscopy of mycobacteria internalization**

Macrophage monolayers were established by plating 10^5 cells in 0.2 ml DMEM, as described above, onto sterile glass coverslips and incubating them overnight at 37°C in humidified air containing 5% CO2.

BCG internalization was studied using fluorescent M. bovis BCG expressing GFP (kind gift from V. Snewin, London, U.K.). BCG-GFP stored at −80°C was rapidly thawed, passed through a 25-gauge needle 30 times and then through a 30-gauge needle 10 times, sonicated six times for 15 s, and immediately added to the cultures at an MOI of 1. After 2 h at 37°C under a humidified atmosphere containing 5% CO2, the medium was removed and fixed with paraformaldehyde 4% in PBS for 20 min at 37°C or overnight at 4°C. After fixation, macrophages on coverslips were washed once in warm PBS for 10 min. Cells were permeabilized for 3 min with 0.1% Triton X-100 in PBS, washed 10 min with PBS, quenched with 50 mM NH4Cl for 30 min, washed 10 min with PBS, preincubated for 30 min with 1% BSA in PBS, and washed again in PBS. To stain F-actin, macrophages were incubated for 20 min with β-phallloid conjugated to rhodamine in 5 U/ml (Molecular Probes, Eugene, OR), followed by two 5-min washes in PBS. Coverslips were mounted using DAKO mounting medium with DAPI. BCG-GFP internalization was assessed using a fluorescence Leica DM IRBE microscope (Leica, Reuil Malmaison, France; ×100 oil immersion objective) by counting the macrophages containing one or two isolated intracellular bacteria and the noninfected macrophages in 10 fields of view per slide (three or four slides per group).

### Analysis of PRR gene expression

Total RNA was isolated from lungs by TRizol reagent (Sigma-Aldrich) and purified using an RNasey Mini Kit (Qiagen, Valencia, CA) at the indicated times after *M. tuberculosis* infection. For quantitative RT-PCR, 1 μg total RNA was pretreated by DNaseI and converted to cDNA by ImProm-II Reverse Transcriptase (Promega, Madison, WI) using random nanomer primers. Quantitative PCR reactions were performed using the Brilliant III SYBR QPCR kit and Stratagene Mx3005P thermocycler (Agilent, Palo Alto, CA). The following program was used: 95°C for 15 min and 40 cycles at 95°C for 10 s and 60°C for 30 s.

Primers used for quantitative PCR included β-actin forward primer: 5′-CCCTGAgCGCgAGTCCTTG-3′; β-actin reverse primer: 5′-TAAAC-CgCgCgCTCcTAACgCgTCC-3′; SR-A1/Msr1 forward primer: 5′-TgAAC-AgAgACgAgCTgC-3′ and reverse primer: 5′-ggAggggCCCAT-TTTTAgCgTc-3′; CD63 forward primer: 5′-TCgAGCaAgAcACgCgTA-3′; and reverse primer: 5′-TTgTgAAgCgAgCgAgTA-3′. M. tuberculosis infected with *H.37Rv* (heat-killed for 40 min at 80°C; two bacteria/cell).

### Results

#### Controlled acute *M. tuberculosis* infection in the absence of the C-type lectins mannose receptor or SIGN1

Because both C-type lectins (mannose receptor and DC-SIGN) are involved in the human response to mycobacteria and may contribute to the immunomodulation of the host response by mycobacteria, we next addressed the role of the murine receptors in the response to acute *M. tuberculosis* infection. Mice deficient for SIGN1 or mannose receptor infected with *M. tuberculosis* suffered the loss of body weight similar to WT mice during the first 3 mo postinfection (Fig. 2A), in contrast to mice deficient for TNF or IL-1R1, which succumbed within 3–4 wk of infection. Although mice deficient for IL-1R1 or TNF exhibited pulmonary bacterial loads 1–3 log_{10} greater than WT controls 4 wk postinfection, SIGN1- or mannose receptor-deficient mice controlled the infection as well as WT mice for the first 8–12 wk of the experiment (Fig. 2B, 2C). They showed lung weights similar to WT mice at 2–3 mo of infection, whereas IL-1R1- or TNF-deficient mice had increased lung weight, indicative of marked lung inflammation at 4 wk postinfection (Fig. 2D, 2E). Macroscopically, the lungs of SIGN1- or mannose receptor-deficient mice were essentially similar to those of WT mice, whereas large and numerous confluent nodules occurred in IL-1R1- or TNF-deficient mice at 4 wk of infection (Fig. 2F, 2G). Although SIGN1-deficient mice showed similar lung weight to WT controls, histologically they presented increased lung cell infiltration and edema at 4 wk, a time point at which extensive necrotic lesions had developed in susceptible IL-1R1-deficient mice (Fig. 2H, 2I). In addition, lungs of SIGN1-deficient mice displayed some accumulation of mycobacteria, albeit not as marked as the uncontrolled bacilli growth seen in IL-1R1-deficient mice, although solely scattered isolated bacilli were found in WT lungs compared with wild-type (WT) controls 3–7 wk postinfection (Fig. 1B, 1C). SR-A- and MARCO-deficient mice had bacterial loads similar to WT mice in the lungs up to 13–16 wk postinfection (Fig. 1B, 1C). Increased lung weight, a sign of marked inflammation, is usually seen in IL-1R1- or IFN-γR-deficient mice; SR-A- or MARCO-deficient mice had a lung weight similar to the WT controls up to 13–16 wk postinfection (Fig. 1D, 1E), indicating no obvious organ inflammation.

Granuloma formation, the result of a structured cell-mediated immune response, is crucial for controlling mycobacterial growth. We next examined lung morphology and asked whether the SRs are essential for granuloma formation upon *M. tuberculosis* infection. Macroscopically, the lungs of SR-A- or MARCO-deficient mice displayed no obvious difference from WT controls, in sharp contrast to the lungs of IL-1R1- or IFN-γR-deficient mice, which displayed large subpleural and confluent nodules at 3–7 wk postinfection (Fig. 1F, 1G). Microscopic investigation of the lungs of SR-A- or MARCO-deficient mice revealed granuloma formation, characterized by the accumulation of macrophages accompanied by lymphocytic perivascular and peribronchiolar cuffing, similar to WT mice (Fig. 1H–K). In contrast, IL-1R1- or IFN-γR-deficient mice had severe inflammation, with significant reduction of ventilated alveolar spaces and massive mononuclear and neutrophil infiltration with extensive confluent necrosis in the absence of proper granuloma formation (Fig. 1A). Scattered intracellular mycobacteria were present in the lung of SR-A- and MARCO-deficient mice, similar to WT mice, in contrast to the abundant mycobacteria observed extracellularly in the lung of IL-1R1– and IFN-γR–deficient mice (Fig. 1L–O). Thus, the absence of SR-A or MARCO did not affect the host response to acute *M. tuberculosis* infection; the animals survived, controlled the infection, and displayed appropriate granulomatous responses, with no excessive inflammation of the lungs.

**Controlled acute *M. tuberculosis* infection in the absence of the **C-type lectins mannose receptor or SIGN1

Because both C-type lectins (mannose receptor and DC-SIGN) are involved in the human response to mycobacteria and may contribute to the immunomodulation of the host response by mycobacteria, we next addressed the role of the murine receptors in the response to acute *M. tuberculosis* infection. Mice deficient for SIGN1 or mannose receptor infected with *M. tuberculosis* survived the loss of body weight similar to WT mice during the first 3 mo postinfection (Fig. 2A), in contrast to mice deficient for TNF or IL-1R1, which succumbed within 3–4 wk of infection. Although mice deficient for IL-1R1 or TNF exhibited pulmonary bacterial loads 1–3 log_{10} greater than WT controls 4 wk postinfection, SIGN1- or mannose receptor-deficient mice controlled the infection as well as WT mice for the first 8–12 wk of the experiment (Fig. 2B, 2C). They showed lung weights similar to WT mice at 2–3 mo of infection, whereas IL-1R1- or TNF-deficient mice had increased lung weight, indicative of marked lung inflammation at 4 wk postinfection (Fig. 2D, 2E). Macroscopically, the lungs of SIGN1- or mannose receptor-deficient mice were essentially similar to those of WT mice, whereas large and numerous confluent nodules occurred in IL-1R1– or TNF-deficient mice at 4 wk of infection (Fig. 2F, 2G). Although SIGN1-deficient mice showed similar lung weight to WT controls, histologically they presented increased lung cell infiltration and edema at 4 wk, a time point at which extensive necrotic lesions had developed in susceptible IL-1R1-deficient mice (Fig. 2H, 2I). In addition, lungs of SIGN1-deficient mice displayed some accumulation of mycobacteria, albeit not as marked as the uncontrolled bacilli growth seen in IL-1R1-deficient mice, although solely scattered isolated bacilli were found in WT lungs.
FIGURE 1. Controlled acute *M. tuberculosis* infection in the absence of the SRs MARCO or SR-A. A, Mice deficient for MARCO, SR-A, IFN-γR, or IL-1R1 and WT mice were exposed to aerogenic *M. tuberculosis* H37Rv and monitored for body weight. Mean values of *n* = 8–15 mice per group from two or three experiments with *n* = 3–4 cytokine pathway-deficient control mice. Significant body weight change was found after week 3 for IL-1R1 knockout (KO) and week 4 for IFN-γR KO mice, which rapidly lost weight and succumbed, and after weeks 3 and 9 for SR-A KO and MARCO KO mice, respectively.
controls (Fig. 2H, 2M). However, the discrete phenotype of SIGNR1-deficient mice was transient, and little difference was seen at a later time point during infection (Fig. 2J, 2N). Granuloma formation was normal in the lungs of mannose receptor-deficient mice (Fig. 2K, 2L), whereas TNF-deficient mice had severe inflammation, with marked reduction of ventilated alveolar space and extensive confluent necrosis in the absence of granuloma formation (Fig. 2K). Scattered mycobacteria were visible in the lung of mannose receptor-deficient mice, similar to WT controls, whereas uncontrolled and extensive bacterial growth occurred in the absence of TNF (Fig. 2O, 2P). Thus, SIGNR1 or mannose receptor deficiency did not affect the host response to acute M. tuberculosis infection; the animals survived, controlled the infection, and displayed granulomatous responses, with no excessive inflammation of the lungs, suggesting a functional redundancy of these receptors. This was in sharp contrast to the cytokine TNF or IL-1R1 pathway, which were both essential for the control of infection.

**Controlled acute M. tuberculosis infection in the absence of EMR1 receptor F4/80**

Because F4/80, the murine ortholog of the seven transmembrane receptor EMR1, was implicated in macrophage-dependent modulation of IFN-γ release by NK cells in response to Listeria (42), we next asked whether this receptor was essential for the control of M. tuberculosis infection. Mice deficient for F4/80 infected with M. tuberculosis survived and gained body weight like WT mice during the first 3 mo postinfection (Fig. 3A), whereas mice deficient for MyD88 succumbed within 3 wk of infection. F4/80-deficient mice controlled bacterial growth similar to WT mice up to 13 wk, whereas MyD88-deficient mice exhibited 2 log10 greater bacterial load within 3 wk of infection (Fig. 3B). F4/80-deficient mice showed no overt lung inflammation, with lung weight similar to WT mice for the 13 wk of the experiment (Fig. 3C). Granuloma formation was normal in F4/80-deficient mice, macroscopically (Fig. 3D) and microscopically at 3 and 13 wk postinfection, with some giant cells found at 13 wk in F4/80-deficient mice (Fig. 3E, 3F), whereas large confluent nodules with necrosis and defective granuloma formation occurred in MyD88-deficient mice. Scattered mycobacteria were present in the lung tissue of F4/80-deficient and WT mice (Fig. 3G, 3H), in contrast to the abundant mycobacteria observed in the extracellular space in the lung of MyD88-deficient mice 3 wk postinfection. Thus, acute M. tuberculosis infection could be controlled in the absence of F4/80.

**Long-term M. tuberculosis infection is controlled in the absence of a single PRR**

Because none of the receptors tested seemed to be essential for the control of acute infection, we next asked whether the individual PRRs might contribute to the control of chronic M. tuberculosis infection. Mice deficient for MARCO, SIGNR1, or F4/80 were infected with M. tuberculosis and followed for 9 mo. MARCO- and SIGNR1-deficient mice gained body weight during the first 3 mo postinfection, body weight stabilized thereafter, and they survived the 9 mo of the experiment. F4/80-deficient mice also gained weight and survived the first months of infection, but 3 of 12 mice died at later time points (days 174, 188, and 258) (Fig. 4A). Lung bacterial load in all groups was essentially similar to that in WT mice at 6 or 9 mo postinfection (Fig. 4B). None of the groups showed signs of exacerbated lung inflammation, as documented by increased lung weight over WT controls (Fig. 4C). Macroscopically, the lungs of all groups, including WT mice, showed nodules 6 and 9 mo postinfection (Fig. 4D, 4E). Histologically, granulomata were well formed in all groups, with overall similar cell infiltration and prominent lymphocyte infiltration in SIGNR1-deficient mice (Fig. 4F, 4G, 4J). Although lungs of MARCO–, SIGNR1–, or F4/80-deficient mice showed reduced ventilated alveolar spaces compared with WT controls at 6 mo (Fig. 4F, 4J), all groups, including WT controls, had similarly reduced free air space, with no apparent necrosis, by 9 mo of infection (Fig. 4G, 4K). Lipid crystals were observed in the lungs in all groups at these late time points of infection. Despite no significant differences in bacterial load, lung sections of MARCO-deficient mice presented more mycobacteria aggregates than WT and other deficient mice (Fig. 4H–K).

**Control of M. tuberculosis infection in the absence of two scavenger or C-type lectin receptors**

We then addressed the potential redundancy between PRRs of the same scavenger family with regard to the response to M. tuberculosis infection. We tested the resistance to infection of mice doubly deficient for SR-A plus the class B SR CD36 or doubly deficient for the C-type lectins mannose receptor plus SIGNR1. Mice doubly deficient for SR-A plus CD36 (Fig. 6A) and WT control mice were infected with M. tuberculosis and followed up to 9 mo postinfection. Most mice survived for the duration of the experiment with no body weight decrease, except for three of eight SR-A plus CD36-deficient mice that lost weight and had to be killed on days 165, 216, and 265; TNF-deficient mice succumbed within the first 3–4 wk of infection. Double deletion of SR-A plus CD36 resulted in a slight increase in lung bacterial load at 4 wk postinfection (Fig. 6B), which also was seen microscopically on lung sections, with larger foci of mycobacteria compared with the few scattered bacilli seen in WT mice (Fig. 6H). However, the bacterial burden was not as great as that seen in TNF-deficient mice at this time point (Fig. 6B, 6H); thereafter, the SR-A plus CD36-deficient mice controlled the bacterial load at 2, 6, and 9 mo postinfection (Fig. 6B, 6I). There was no sign of exacerbated lung inflammation in SR-A plus CD36-deficient mice up to 9 mo postinfection (Fig. 6C), and the pulmonary cytokine compared with WT control, three groups that survived and gained body weight during the first 3 mo postinfection. The number of viable bacteria present in the lungs (B, C) and the lung relative weight (D, E) were measured at 3–16 wk postinfection. Mean ± SD of n = 4 representative of one of two independent experiments. ***p ≤ 0.01; ****p ≤ 0.001. Macroscopic examination of the postinfection (F) and of MARCO- and IFN-γR–deficient mice and WT extensive inflammation and necrosis in infected IL-1R1–deficient lungs (G) and followed for 9 mo. MARCO- and SIGNR1-deficient mice gained body weight during the first 3 mo postinfection, body weight stabilized thereafter, and they
FIGURE 2. Controlled acute *M. tuberculosis* infection in the absence of the C-type lectins SIGNR1 and mannose receptor. A, Mice deficient for SIGNR1, mannose receptor, TNF, or IL-1R1 and WT mice were exposed to *M. tuberculosis* H37Rv and monitored for body weight. Mean values of *n* = 8–15 mice per
and chemokine concentrations were essentially normal in these mice (Fig. 5). Macroscopically, lungs of SR-A plus CD36-deficient mice presented large nodules (Fig. 6D), and histological sections revealed reduced free air space, with more cells infiltrating the lungs and edema, compared with WT controls at 1 mo postinfection. However, both groups presented similar lung morphology 9 mo postinfection (Fig. 6G), and no necrotic area could be detected as in the TNF-deficient mice.

In parallel, we infected mice doubly deleted for mannose receptor plus SIGNR1 with *M. tuberculosis* and monitored their body weight up to 5 mo. Mice lacking both lectins were able to control the infection with no loss in body weight, except for 2 of 13 mice who lost weight and had to be killed on days 69 and 98 (Fig. 7A). Doubly deficient mice showed no impairment of bacterial growth control, as seen at 1, 2, and 5 mo postinfection (Fig. 7B), and no obvious inflammation of the lungs (Fig. 7C), whereas mice lacking TNF or IL-1R1 succumbed (Fig. 7D).

**FIGURE 3.** Controlled acute *M. tuberculosis* infection in the absence of F4/80. A, Mice deficient for F4/80 or MyD88 and WT mice were exposed to *M. tuberculosis* H37Rv and monitored for body weight (mean values of *n* = 6–7 mice per group from two experiments with *n* = 5 MyD88-deficient control mice). A significant change in body weight was found at week 3 for MyD88 knockout (KO) mice, which succumbed. The change in body weight was nonsignificant for F4/80 KO mice compared with WT controls; both groups survived and gained body weight during the first 3 mo postinfection. The number of viable bacteria present in the lungs (B) and the lung relative weight (C) were measured at 3 and 13 wk postinfection (mean ± SD of *n* = 3–5 lungs from one experiment). **B**, CFU/lung (log10). **C**, Lung weight (% BW). **D**, Macroscopic view of the lungs of F4/80- and MyD88-deficient mice and WT controls at week 3 showed large and confluent nodules in MyD88-deficient mice, whereas F4/80-deficient mice were more similar to WT mice. Microscopic examination showing extensive inflammation and necrosis in infected MyD88-deficient lungs (E) at week 3 with abundant mycobacteria in the extracellular space (G), whereas lungs of F4/80-deficient mice exhibited well-defined granuloma with few mycobacteria, comparable to WT lungs at 3 wk (E, G) or 13 wk (F, H) postinfection. E and F, H&E, original magnification ×100. G and H, ZN staining, original magnification ×1000. Arrows point to ZN positive bacilli.
FIGURE 4. Chronic *M. tuberculosis* infection is controlled in SIGNR1-, MARCO-, or F4/80-deficient mice. A, Mice deficient for SIGNR1, MARCO, or F4/80 and WT mice were exposed to aerogenic *M. tuberculosis* H37Rv and monitored for body weight (mean values of *n* = 6–12 mice per group). No
TNF-deficient mice had $\geq 2 \log_{10}$ greater bacterial loads and sharply increased lung weight 1 mo postinfection, when they had to be terminated. Macroscopic (Fig. 7D) and microscopic (Fig. 7F, 7H) observations showed no clear difference in nodule formation, cell infiltration, granuloma formation, or mycobacteria spreading in the lungs of WT mice and mice lacking mannose receptor plus SIGNR1, in contrast to TNF-deficient mice, which exhibited large necrotic areas and uncontrolled bacterial growth at 1 mo of infection (Fig. 7F, 7H). At 5 mo of infection, there was no difference in bacterial load or lung weight between WT controls and mice deficient in mannose receptor plus SIGNR1 (Fig. 7B, 7C). However, mice lacking both receptors exhibited a slight increase in pulmonary cell infiltration compared with WT controls, as well as visible mycobacteria (Fig. 7G, 7H), whereas bacilli were barely detectable in WT controls (data not shown).

Therefore, the double deficiency in SRs (SR-A plus CD36) or in both C-type lectins (mannose receptor plus SIGNR1) did not result in a systematic impairment of the long-term control of $M. tuberculosis$ infection.

**Competence of PRR-deficient macrophages for TNF release in response to mycobacteria**

TNF plays an important role in the control of local immune response to intracellular pathogens, such as $M. tuberculosis$. Therefore, we investigated the ability of PRR-deficient resident peritoneal macrophages or bone marrow-derived macrophages to secrete TNF in response to mycobacteria in vitro (Fig. 8). Peritoneal macrophages deficient for MARCO or SR-A produced levels of TNF comparable to WT macrophages after stimulation with $M. bovis$ BCG or $M. tuberculosis$ H37Rv (Fig. 8A). In contrast, macrophages lacking SR-A plus CD36 exhibited a slight decrease in TNF production in response to mycobacteria, which was also seen, to a lower level, with CD36 deficiency (Fig. 8A). As expected, macrophages deficient for CD36 did not respond to MALP2, that interacts and signals through TLR2 and CD36, whereas they responded normally to Pam3CSK4, an agonist of TLR2/TLR1 heterodimers (Fig. 8B).

Similarly, resident peritoneal macrophages deficient in SIGNR1 or doubly deficient for mannose receptor plus SIGNR1 produced TNF in response to mycobacteria, albeit at slightly reduced levels in the latter cells (Fig. 8C). Deficiency in F4/80 had no effect on the TNF response of bone marrow-derived macrophages to mycobacteria (Fig. 8D). Comparable results were obtained for the production of NO, an important mycobactericidal mediator, in all macrophages tested (data not shown). Therefore, none of the PRRs tested was essential for the in vitro TNF response of macrophages stimulated with mycobacteria, but they might cooperate to result in a full inflammatory response.

**Internalization of BCG into macrophages from PRR-deficient mice**

Several PRRs, including C-type lectins and complement receptors, have been implicated in the recognition and binding of $M. tuberculosis$ in human cells (35, 36). Therefore, we asked whether murine homologs are important for the internalization of $Mycobacterium bovis$ BCG. We showed previously that the absence of TLR2 plus TLR4 or of MyD88 led to a reduction in BCG-GFP internalization by one third or one half, respectively (54). Macrophages lacking SIGNR1, mannose receptor, mannose receptor plus SIGNR1, MARCO, SR-A, CD36, or SR-A plus CD36 were incubated with BCG-GFP for 2 h, and the proportion of macrophages infected with one or two mycobacteria was analyzed using confocal microscopy. Macrophages from MARCO-, SR-A-, or CD36-deficient mice internalized mycobacteria at a similar rate to WT controls, whereas in the absence of SR-A and CD36, there was a decrease in BCG internalization by resident peritoneal (Fig. 8E) and bone marrow-derived macrophages (Fig. 8F), similar to what was seen in the absence of MyD88 (46% reduction; data not shown).
shown). In contrast, deficiency in SIGNR1 or mannose receptor plus SIGNR1 led to a slight increase in BCG uptake by peritoneal macrophages (Fig. 8G), whereas bone marrow-derived macrophages from mannose receptor-deficient mice presented a similar internalization rate to WT cells (Fig. 8H). Therefore, the absence of any one of the receptors tested did not compromise BCG internalization, whereas the absence of SR-A plus CD36 resulted in a reduced internalization.

Discussion

Several types of PRRs have been identified in sensing, binding, or signaling M. tuberculosis motives, including TLRs, nod-like receptors, complement receptors, SRs, and C-type lectin receptors. An in vitro study indicated that blocking complement and mannose receptors did not completely abrogate binding of M. tuberculosis to human macrophages (2). Further blocking of class A SRs abrogated nearly all binding, indicating that they are important mediators of M. tuberculosis–macrophage interactions (2). In this study, we attempted a comparative study of receptors of the different classes of PRRs to assess their importance in the control of an acute or chronic M. tuberculosis infection.

The role of receptors representative of SR, C-type lectin receptor, or seven transmembrane receptor families in the host response to acute and chronic M. tuberculosis infection was studied using genetically deficient mice. We and other investigators showed previously that although mycobacteria produce agonist molecules able to trigger TLR2, TLR4, and TLR9, the role of these receptors in the control of in vivo M. tuberculosis infection is limited (10, 12, 14). Therefore, it is essential to understand the involvement of
the other PRRs in mounting the host innate and adaptive responses and in controlling *M. tuberculosis* infection.

Expression of MARCO and SR-A is rapidly induced on macrophages after BCG infection, and a role for these SRs in the host antibacterial defense was suggested (29, 55). SR-A expression was increased in the lung of *M. tuberculosis*-infected mice 2–4 wk postinfection, as revealed by microarray analysis (data not shown) and confirmed by quantitative PCR (Supplemental Fig. 1). This is in agreement with the strong SR-A upregulation reported 3–9 wk after *M. tuberculosis* infection in mice (56), as well as in human pulmonary macrophages from tuberculosis patients (57). However, MARCO expression was not affected 1–4 wk after *M. tuberculosis* infection in our model (Supplemental Fig. 1). MARCO was recently shown to be essential for *M. tuberculosis* cord factor recognition and activation of the TLR2/CD14 signaling pathway by macrophages (30), whereas the contribution of SR-A to this response was more limited and associated with TLR2 and TLR4 (30). In this study, although mycobacterial aggregates seemed slightly more prominent in MARCO-deficient mice, we showed no impairment in the control of acute or chronic *M. tuberculosis* infection in the absence of MARCO or SR-A.

Decreased expression of the class B SR CD36 was reported on monocytes isolated from pulmonary, pleural, or miliary tuberculosis patients or after in vitro infection with *M. tuberculosis* (58). In our

---

**FIGURE 7.** Mice doubly deficient for C-type lectins mannose receptor and SIGNR1 control chronic *M. tuberculosis* infection. A, Mice doubly deficient for mannose receptor plus SIGNR1 were exposed to *M. tuberculosis* H37Rv, in parallel with mannose receptor- or TNF-deficient mice and WT mice, and monitored for body weight (mean values of *n* = 4–6 mice per group; no significant body weight changes compared with WT controls). Lung bacterial load (B) and lung relative weight (C) were measured at 1, 2, and 5 mo postinfection (mean ± SD of *n* = 3–6; two-way comparisons between C57BL/6 and mannose receptor plus SIGNR1 double knockout (dKO) mice or between C57BL/6 and mannose receptor KO mice were nonsignificant; two-way comparisons between C57BL/6 and TNF KO. ***p < 0.001. Macroscopic views (D, E) and microscopic examination (F, G) of the lungs at 1 mo (D, F) and 6 mo (E, G) postinfection showed large and confluent nodules in TNF-deficient mice, whereas the lungs of mice deficient for mannose receptor plus SIGNR1 were similar to those of WT mice. At 1 mo postinfection, TNF-deficient mice had uncontrolled bacterial growth (H), whereas WT controls and mannose receptor plus SIGNR1-deficient mice presented few mycobacteria. At 6 mo, mice lacking both lectins presented larger foci of bacilli than mice lacking mannose receptor only (I), whereas WT controls had undetectable bacilli (data not shown). F and G, H&E, original magnification ×100. H and I, ZN staining, original magnification ×1000. Arrows point to ZN positive bacilli.
ROLE OF PRRs IN MYCOBACTERIAL INFECTION

The human C-type lectins mannose receptor and DC-SIGN are implicated in the recognition of mycobacteria, and they were implicated in the negative regulation of TLR-induced response, likely contributing to M. tuberculosis immune-evasion strategies (35, 36). Among the seven murine SIGNR homologs, the absence of SIGNR1, SIGNR3, or SIGNR5 did not hamper survival after M. tuberculosis infection (59, 60). In this study, we concentrated on addressing the role of murine mannose receptor and SIGNR1, because SIGNR1 may associate with TLR4/myeloid-differentiation factor 2 and modulate downstream signaling (39). Mannose receptor expression was decreased in the lung of M. tuberculosis-infected mice 3 wk postinfection in our model, as revealed by microarray analysis (not shown) and confirmed by quantitative PCR (Supplemental Fig. 1), whereas SIGNR1 expression was very low and decreased further on day 21 postinfection. Human mannose receptor was implicated in the phagocytosis of mycobacteria by macrophages (16, 17), but we did not see any effect of the deficiency in the murine mannose receptor on BCG uptake in vitro. However, macrophages lacking SIGNR1 or both C-type lectins (mannose receptor and SIGNR1) showed a slightly increased macrophage uptake of BCG, which was associated with a reduced production of TNF in vitro. In vivo, mice deficient for mannose receptor plus SIGNR1 seemed to have a more marked pathology 5 mo postinfection, with high cell infiltration and mycobacteria foci in the lung; one mouse each had to be killed at 2 and 4 mo of infection. Therefore, there was a slight phenotype in the absence of mannose receptor in combination with SIGNR1; overall, this did not impair the resistance to acute or chronic M. tuberculosis infection, excluding an essential model, CD36 expression in the lung was slightly decreased 2–4 wk after M. tuberculosis infection, as revealed by microarray analysis (data not shown) and confirmed by quantitative PCR (Supplemental Fig. 1). The inverse regulation of SR-A and CD36 expression after M. tuberculosis infection might also contribute to the functional compensation between the SRs. CD36 cooperates with TLR2 in sensing bacteria, acting as a coreceptor for the induction of proinflammatory cytokines (33). Because TLR2 is one of the TLRs most involved in mycobacterial motives recognition, it was of interest to see whether CD36 might also contribute to the TLR2–mycobacterial response. The absence of SR-A plus CD36 partially impaired macrophage internalization of BCG and TNF release in response to mycobacteria. In vivo, the absence of SR-A plus CD36 seemed to slightly compromise the control of M. tuberculosis growth and inflammatory response in the lungs 4 wk postinfection, which normalized thereforer for up to 9 mo of infection. However, the long-term control of M. tuberculosis infection was compromised because three mice had to be terminated 5–9 mo postinfection. These phenotypes were clearly not as marked as those seen in the absence of cytokines, such as TNF or IL-1 pathways, but they suggest that the contribution of several receptor families might be necessary for full, long-term control of the infection.
involvement of either receptor and suggesting a functional redundancy of both receptors for the host response to M. tuberculosis.

F4/80 was proposed to be involved in cell adherence and migration because it is expressed at a low level on circulating monocytes, whereas it is upregulated in differentiated macrophage populations (40). F4/80 expression was shown to be downregulated on peritoneal macrophages after local M. bovis BCG infection (41), which may be compatible with an increased migration upon infection. Although no significant change in pulmonary F4/80 expression was noted 1–2 wk after airway M. tuberculosis infection, a trend for higher expression was seen at day 21 by quantitative PCR, and a sharp increase was noted on day 28 (Supplemental Fig. 1) (Y. Shebzukhov and S.A. Nedospasov, unpublished observations).

Although Abs to F4/80 revealed a functional requirement for F4/80 in the production of TNF, IL-12, or IFN-γ after splenocyte exposure to Listeria monocytogenes (42), no phenotype was reported in F4/80-deficient mice (43), with the exception of a role for F4/80 in the generation of efferent CD8 regulatory T cells (43). Little was known of the potential role for F4/80 in the host response to M. tuberculosis. We showed in this study that F4/80 is dispensable for macrophage TNF response to mycobacteria and that the absence of F4/80 does not compromise the control of acute and chronic M. tuberculosis infection.

Several of the receptors studied were reported to modulate the cytokine response mediated by TLR receptors, such as C-type lectins downregulating TLR4-induced IL-12 responses (18, 20), CD36 acting as a TLR2 coreceptor for the induction of proinflammatory cytokines (33), or SR-A and MARCO distinctly regulating IL-12 release (62). TLR2 is essential in mediating the response to killed mycobacteria and to several molecularly identified mycobacterial Ags, although it does not seem indispensable for the in vitro response to live mycobacteria (63), raising the question about whether other receptors might be involved in this response. The release of TNF, a cytokine central to the control of mycobacterial infection, was largely unaffected in macrophages deficient for MARCO, SR-A, CD36, F4/80, SGNR1, or mannose receptor upon mycobacterial stimulation. A partial reduction in TNF release was observed in macrophages doubly deficient for SR-A plus CD36 or for mannose receptor plus SGNR1. Additionally, the pattern of pulmonary expression of IL-12p40, -12p70, and -23, IFN-γ, IL-1α, -1β, and -1ra; TNF-α; and the chemokines CCL2 (JE/MPC-1) and CXCL1 (KC) was not drastically affected after 9 mo of M. tuberculosis infection in mice deficient for SGNR1, MARCO, F4/80, or SR-A plus CD36. Therefore, none of the single PRRs tested was essential for the in vivo TNF response of macrophages stimulated with mycobacteria or for the in vivo lung cytokine and chemokine expression in late infection, but they might cooperate to provide a full inflammatory response.

The essential role of several proinflammatory cytokine pathways, such as TNF, IL-1R1, the adaptor MyD88, or IFN-γ, although already well documented (4–9), was reassessed in the current study. The cytokine pathway-deficient mice served as internal controls in each experiment, illustrating the type of extreme phenotype that can be expected when an essential signaling pathway is not functional, as well as controlled for the reproducibility and virulence of the M. tuberculosis infections in the different experiments. More importantly, we showed that abrogation of several proinflammatory cytokine pathways leads to similarly drastic phenotypes, confirming that none of the cytokines can compensate for the other, whereas the disruption of several PRRs leads only to moderate phenotypes. Thus, the redundancy seems much greater at the level of PRRs than at the level of the downstream cytokine pathways activated.

In this study, we documented a functional redundancy in the different PRRs, in sharp contrast to the proinflammatory cytokine TNF, IL-1-, and IFN-γ pathways, which are each essential and cannot compensate for each other in the control of M. tuberculosis infection. Although most cytokines are recognized by a unique or a limited set of receptors, mycobacteria express multiple molecular motives recognized by multiple receptors and susceptible to triggering the activation of different signaling pathways. Our results suggest that different receptors or receptor families might cooperate in a coordinated response to sustain the full immune control of M. tuberculosis infection.

Acknowledgments
We thank Prof. M.C. Nussenzwieg (The Rockefeller University, New York, NY) for the kind gift of the mannose receptor-deficient mice, Prof. S. Akira (Osaka University, Osaka, Japan) for sharing the MyD88-deficient mice, Prof. A.N. McKenzie (Medical Research Council, Cambridge, U.K.) for giving access to the SGNR1-deficient mice, Dr. M. Febbraio (Cleveland Clinic, Cleveland, OH) for the kind gift of the CD36-deficient mice, and Dr. Josachim Mollenkopf (Max Planck Institute for Infection Biology, Berlin, Germany) for skillful help with the microarray analysis.

Disclosures
The authors have no financial conflicts of interest.

References


**Suppl. Figure 1: PRR gene expression during *M.tuberculosis* infection**

Analysis of PRR gene expression was performed by quantitative RT-PCR on days 0, 14 or 21 after infection and normalized to β-actin message. (n=3-4 mice per point; 2 way comparisons between day 14 or 21 post-infection and day 0 control are indicated; **p<0.01, *p<0.05; no significant change was found for F4/80 and Marco expression**