Toll-Like Receptor 4 Expression Is Required to Control Chronic Mycobacterium tuberculosis Infection in Mice

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Endotoxin from Gram-negative bacteria bound to CD14 signals through Toll-like receptor (TLR) 4, while components of Gram-positive bacteria, fungi, and Mycobacterium tuberculosis (M.tb.) preferentially use TLR2 signaling. We asked whether TLR4 plays any role in host resistance to M.tb. infection in vivo. Therefore, we infected the TLR4 mutant C3H/HeJ mice and their controls, C3H/HeN mice, with M.tb. by aerosol. TLR4 mutant mice had a reduced capacity to eliminate mycobacteria from the lungs, spread the infection to spleen and liver, with 10–100 times higher CFU organ levels than the wild-type mice and succumbed within 5–7 mo, whereas most of the wild-type mice controlled infection and survived the duration of the experiment. The lungs of TLR4 mutant mice showed chronic pneumonia with increased neutrophil infiltration, reduced macrophages recruitment, and abundant acid-fast bacilli. Furthermore, the pulmonary expression of TNF-α, IL-12p40, and monocyte chemoattractant protein 1 was significantly lower in C3H/HeJ mice when compared with the wild-type controls. C3H/HeJ-derived macrophages infected in vitro with M.tb. produced lower levels of TNF-α. Finally, the purified mycobacterial glycolipid, phosphatidylinositol mannosides, induced signaling in both a TLR2- and TLR4-dependent manner, thus suggesting that recognition of phosphatidylinositol mannosides in vivo may influence the development of protective immunity. In summary, macrophage recruitment and the proinflammatory response to M.tb. are impaired in TLR4 mutant mice, resulting in chronic infection with impaired elimination of mycobacteria. Therefore, TLR4 signaling is required to mount a protective response during chronic M.tb. infection. The Journal of Immunology, 2002, 169: 3155–3162.

One-third of the world’s population is infected with Mycobacterium tuberculosis (M.tb.), the pathogen of pulmonary tuberculosis. The rational design of improved tuberculosis therapy and vaccine depends on the knowledge of the primary immune response that serves to protect most humans exposed to the pathogen, and an understanding of the factors resulting in the failure to mount protective immunity in humans who are susceptible to tuberculosis. In the first phase, mycobacteria encounter APCs such as macrophages, dendritic cells, and epithelial cells, resulting in their activation. This innate response is followed by the presentation of mycobacterial-derived peptides to Ag-specific, IFN-γ-secreting CD4 T cells and full activation of the adaptive cell-mediated immunity (1). Most of the understanding on the role of T cells and Th1-type cytokines is derived from studies in mice using genetic models or Ab neutralization studies (2). IFN-γ derived from T and NK cells has been shown to be essential, as mice with a disruption of the IFN-γ system are unable to restrict the growth of M.tb. and succumb to the infection (3–7). Additional cytokines, which are critical to control infection and mount protective immunity, include TNF-α, IL-12, and IL-18 (8–13).

The prompt recognition of microorganisms via their signature molecules by APC expressing pattern recognition receptors is an exciting and rapidly developing area (14). Receptors involved in sensing M.tb. patterns include the mannose receptor, complement receptors (CR) CR1, CR3, and CR4 (15), and Toll-like receptor (TLR)2 and TLR4. The mycobacterial envelope contains a wide array of chemically diverse lipids and glycolipids that likely mediate specific interactions with host ligands or membranes and have potent biological activity on eukaryotic cells in vitro systems (10). Mycobacterial lipoprotein-induced mechanisms including cellular activation, mycobacterial killing, and apoptosis have been demonstrated to be mediated by TLR2 (9, 16–20). Both TLR2 and TLR4 can mediate cellular activation by M.tb., specifically, soluble heat-stable and protease-resistant factors via the TLR2 pathway and heat-sensitive membrane-associated factors via both TLR2 and TLR4 (21, 22). Mutations of the gene lps selectively impair endotoxin signal transduction in C3H/HeJ mice, rendering them resistant to endotoxin, yet highly susceptible to Gram-negative infection. C3H/HeJ mice were shown to have a missense mutation in the third exon of TLR4 (23, 24). In addition, TLR4-deficient mice generated by homologous recombination confirm a critical role for TLR4 in endotoxin signaling (25). During M.tb. infection, early macrophage activation may occur through various microbial-receptor engagements. We hypothesized that lack of TLR4 triggering might impair macrophage activation and the development of a subsequent protective response. To assess the role of TLR4 in vivo, we infected the TLR4 mutants, C3H/HeJ and their wild-type controls, C3H/HeN mice with M.tb. aerogenically. We report that M.tb.-infected C3H/HeJ mice have significantly

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reduced recruitment of macrophages to the lungs, lower proinflammatory cytokine production, and succumb to chronic infection.

Materials and Methods

Animal experiments

Female C3H/HeJ (TLR4 mutant), and C3H/HeOuJ and C3H/HeN (both TLR4 wild-type strains) mice used for the experiments were 8–12 wk old (Charles River Breeding Laboratories, Kisslegg, Germany). Mice were kept under specific-pathogen-free conditions in the animal unit at the University of Cape Town (Cape Town, South Africa). Aerosol M. tb infections were performed in a biosafety level 3 area and infected animals were maintained in filter-top cages. The University of Cape Town Animal Ethics Committee approved all protocols used in this study.

Mycobacteria

M. tb. H37Rv and H37Ra (obtained from Prof. G. Kaplan, Rockefeller University, New York, NY, and Prof. G. Marchal, Pasteur, Paris, France, respectively) were grown to mid-log phase in Middlebrook 7H9 medium (Difco, Detroit, MI), supplemented with 10% oleic acid/albumin/dextrose catalase (State Vaccine Institute, Pinelands, South Africa) and 1% glycerol (Merck, Munich, Germany) in 5% CO2 at 37 °C. Aliquots were prepared and frozen at −80 °C. Before use, an aliquot was thawed, briefly vortexed, diluted in sterile saline containing 0.04% Tween 80 (Merck), and clumping was disrupted by aspirating through a 29-gauge needle (Omnican; Braun, Kronberg, Germany) 20 times.

Infection with H37Rv

Mice were infected aerogenically with 50–100 viable CFU using an inhalation exposure system (Glas-Col, Terre Haute, IN). Animals were exposed for 30 min to an aerosol produced by nebulizing 5 ml of a bacterial suspension in 0.9% NaCl solution at a concentration of 2 × 106 bacilli/ml.

Quantitation of viable mycobacteria in organs

The initial infective dose was verified by sacrificing mice 24 h after aerosol exposure. Lungs were aseptically removed, weighed, and homogenized in 0.9% saline containing 0.04% Tween 80, and 10-fold serial dilutions were plated in duplicate onto 7H10 agar (Difco) supplemented with 10% oleic acid/albumin/dextrose catalase and 0.5% glycerol. Plates were incubated at 37 °C and CFU were enumerated 14–18 days later. Thereafter, at specific time points, mice were sacrificed and their lungs, livers, and spleens were aseptically removed and weighed. Two-thirds of each organ was homogenized in 0.9% NaCl solution containing 0.04% Tween 80 for CFU enumeration as described above. Data are presented as log10 CFU per organ (n = 3–4 mice per group).

Histopathology

Lungs, livers, and spleens of mice were fixed for 24 h in 10% buffered Formalin (BDH; Laboratory Supplies, Poole, U.K.) and then embedded in paraffin. Sections were stained with H&E or Ziehl-Neelsen for evaluation of pathologic changes and bacillary load, respectively (26).

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were deparaffinized and rehydrated through decreasing concentrations of alcohol. Sections were stained with a rabbit anti-mouse Ab specific for inducible NO synthase (iNOS; 1/2000; obtained from J. Pfeilschifter, University of Frankfurt, Frankfurt, Germany). Sections were then washed in PBS and incubated for 30 min at room temperature with rat anti-rabbit serum avidin-biotin complex system (Vector Laboratories, Burlingame, CA) followed by dianminobenzidine substrate. Finally, sections were mounted in Immunomount (Thermo Shandon, Pittsburgh, PA) (26).

Bronchoalveolar lavage (BAL)

Control and M. tb.-infected mice were administered a lethal dosage of ketamine. A plastic 20-gauge cannula was inserted into the trachea and the lungs were lavaged with 0.5 ml of sterile PBS. The BAL supernatant was frozen at −80 °C for later detection of cytokines and chemokines by ELISA. The cell pellets were resuspended in 1% BSA/PBS and counted. Cytospins were prepared and stained with Giemsa for differential counts (300 cells/cytospin counted).

Purification and characterization of phosphatidyl-myo-inositol tetra- and hexamannosides (PIM4–6)

The preparation of PIM 4–6 was a generous gift from Dr. G. Pazo (Institut de Pharmacologie et de Biologie Structurale, Toulouse, France). The PIM-containing lipidic extract was obtained by phenolic purification from M. tb strain H37Rv as described elsewhere (27). Briefly, PIMs were located in the acetone-insoluble fraction of the chloroform/methanol (1:1, v/v) mycobacterial extracts. The contaminating neutral compounds were eliminated by QMA anion exchange chromatography, irrigated with neutral eluents. Then phospholipids were eluted with ammonium acetate-containing organic solvents. The PIM composition was analyzed by electrospray mass spectrometry (EMI-MS) in negative mode. The tri- and tetra-acetylated forms of PIMs appeared to be the most abundant acyl forms. The level of contaminating LPS was determined using a quantitative Limulus lyase assay (BioWhittaker, Walkersville, MD) and were <1 pg of endotoxin/μg of material; this amount of LPS does not elicit the degree of activation induced by PIMs.

U373 and Ba-F3 cell cultures and stimulation

The human astrocyte cell line U373 (American Type Culture Collection, Manassas, VA) was cultured in RPMI 1640 medium supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) supplemented with 10% FCS. Ba-F3-transfected cells were cultured in RPMI 1640 with antibiotics, 10% FCS, and IL-3 (28).

U373 cells were cultured in AIM V medium supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) in Terasaki plates or 96-well round-bottom microtiter plates (Nunc, Roskilde, Denmark) in the presence of increasing concentrations of PIM4–6 complexes. Supernatants were harvested 16 h later and stored at −20 °C until IL-6 assays by ELISA were performed.

The Ba-F3 cell line was stably expressing TRLR4, MD-2, and p55IgLuc, an NF-κB-dependent luciferase reporter construct (28). For the luciferase assay, transfornants were washed and cultured in fresh medium containing heat-inactivated serum. Individual wells containing 2 × 104 cells were left untreated or were stimulated with TNF-α or PIM4–6, as indicated. Cells were then incubated for an additional 6 h before harvesting and lysis in 50 μl in lysis buffer (Promega, Madison, WI). Luciferase assays were performed using the Luciferase Assay System (Promega) according to the manufacturer’s instructions.

Primary macrophage cultures and infection

Bone marrow cells were isolated from femurs and cultivated (104/ml) for 7 days in DMEM supplemented with 20% horse serum and 30% L929 cell-conditioned medium (as source of M-CSF, as described in Ref. 29). Three days after washing and reculturing in the same medium, the cell preparation contained a homogenous population of macrophages. The bone marrow-derived macrophages were stimulated with LPS (Escherichia coli, serotype O111:B4, 10 ng/ml; Sigma-Aldrich, St. Louis, MO) for 4 h (29). Alternatively the macrophages were infected with H37Ra at a ratio of 1:1 for 4 h and the supernatants were analyzed for TNF-α by ELISA.

ELISA for cytokines and chemokines

Mice were sacrificed as described above, entire lungs removed, and a piece of equal mass placed in 1 ml of 4°C PBS solution. The contents were transferred into sterile 10-ml perspex mortars and homogenized, centrifuged at 14,000 rpm, supernatants aliquoted, and stored at −80°C until further use. Before use in ELISA, aliquots were thawed and centrifuged at 14,000 rpm, and supernatants were used for cytokine/chemokine quantification by ELISA.

ELISA for TNF-α, IFN-γ, IL-12p40, and monocyte chemotactic protein 1 (MCP-1) were performed on BAL supernatants or lung homogenates (sensitivity <15 pg/ml; R&D Systems, Abingdon, U.K., and BD Pharmingen, San Diego, CA). Quantification of human IL-6 in culture supernatants was performed using a sandwich ELISA (BD Pharmingen) with a modified protocol using streptavidin-peroxidase (Amersham, Arlington Heights, IL). Absorbance was measured on a Cytoflour ELISA reader (PerSeptive Biosystems). Data are expressed as mean ± SD.
Statistical analysis

Statistical evaluation of differences between the experimental groups was determined with the Mann-Whitney U test and Student’s t test (level of significance of $p < 0.05$).

Results

Reduced TNF-α production by C3H/HeJ mice in response to endotoxin and mycobacteria

As previously established, the C3H/HeJ mice with a mutant allele of the TLR4 gene are protected from endotoxic shock and do not manifest any of the symptoms which afflict the C3H/HeN mice. C3H/HeJ mice injected i.p. with 100 μg LPS produce insignificant levels of TNF-α relative to C3H/HeN mice (Fig. 1A). This hypersensitivity to LPS is confirmed ex vivo by the diminished production of TNF-α by C3H/HeJ-derived whole blood cells in response to LPS stimulation (Fig. 1B).

Bone marrow-derived macrophages were stimulated with LPS (100 ng/ml) or infected with M.tb. H37Ra (1:1), and TNF-α levels were quantified in the supernatant 4 h later. The TNF-α production in macrophages from C3H/HeJ mice was significantly reduced when compared with that from C3H/HeOuJ control mice (Fig. 1C). The residual TNF-α from C3H/HeJ macrophages is likely mediated via TLR2 triggering, as macrophages deficient in TLR2 released significantly reduced TNF-α after infection with M.tb. H37Ra (data not shown).

Reduced survival of C3H/HeJ mice infected with mycobacteria

To assess the susceptibility of the TLR4 mutants to an M.tb. infection, C3H/HeN and C3H/HeJ mice were infected by aerosol (100 CFU/lung). The mice were weighed routinely and mortality was recorded. Although one of five C3H/HeN mice succumbed to the infection during the 250 days, all C3H/HeJ mice displayed a decrease in body weight from 4 mo onward (data not shown) and died between 160 and 200 days postinfection (Fig. 2). The experiment was repeated twice, confirming the reduced resistance of the TLR4 mutant mice. The present data suggest that TLR4 signaling confers protection against M.tb. infection, which occurs in the late chronic stage.

Reduced clearance of mycobacteria from the lung and extrapulmonary sites in C3H/HeJ mice

In view of the increased susceptibility of C3H/HeJ mice with a late lethal outcome, the distribution, growth and elimination of mycobacteria was investigated. The infective dose in the lungs was determined to be ~100 CFU/lung at day 1. CFU were not detectable in the liver and spleen at this time point. C3H/HeN mice appear to control the M.tb. infection as noted by a slight, but not significant decline of viable bacilli in the lungs 2 mo postinfection (Fig. 3A) and insignificant growth at extrapulmonary sites (Fig. 3, B and C). In contrast, C3H/HeJ mice have a reduced ability to control M.tb. infection as noted by a significant increase in lung CFU 2 mo postinfection, increasing further at 4 mo to 100-fold higher levels when compared with C3H/HeN mice (Fig. 3A). Dissemination from the lungs occurred at the same rate initially (at 1 mo) as in the C3H/HeN mice, but the infection progressed more rapidly in C3H/HeJ mice, reaching 100-fold higher CFU levels in liver and spleen, respectively (Fig. 3, B and C).

Therefore, TLR4 signaling appears to be required to control the local growth and dissemination of M.tb. infection from the lung.

Chronic inflammatory responses in C3H/HeJ mice infected with mycobacteria

Postmortem analysis revealed nodular changes in the lung, which were more prominent in C3H/HeJ mice (data not shown), and the
lung weights showed a slight increase in relative weights, which did not reach significance (data not shown). The relative organ weights are an indirect indication of generalized inflammatory cell recruitment and were significantly increased in the spleen of C3H/HeJ mice (data not shown). The microscopy of the lungs revealed an early inflammatory response with small granulomas at 28 days postinfection in the lungs of C3H/HeN mice (Fig. 4A) and C3H/HeJ mice (Fig. 4B); the C3H/HeJ mice, however, had more prominent neutrophil infiltration in the lung that can also be seen in the spleen. At 140 days postinfection, massive chronic inflammation with abundant mononuclear cell infiltrates and neutrophils prevailed in the lungs of the C3H/HeJ mice (Fig. 4D), resulting in the obliteration of most of the airspace, which was less apparent in C3H/HeN lungs (Fig. 4C). In contrast, the extent of the granulomatous response was slightly more prevalent in the lung of control C3H/HeN mice.

The bacterial burden assessed by the Ziehl-Neelsen stain revealed at 28 days postinfection similar numbers of acid-fast bacilli in the two groups of mice (Fig. 5, A and B), but at 140 days a significant difference was observed. Although bacilli were noticed primarily in foamy macrophages of the C3H/HeJ lungs (Fig. 5D), they were virtually undetectable in the C3H/HeN lungs (Fig. 5C). The expression of iNOS as assessed by immunostaining was comparable in the lung of C3H/HeJ mice when compared with C3H/HeN mice (Fig. 5, F and E).

The dissemination of the infection and inflammatory process was assessed in liver and spleen sections. Hepatic granulomas at 28 days postinfection were small in both C3H/HeN (Fig. 6A) and C3H/HeJ mice (Fig. 6B). At 140 days postinfection, the hepatic granulomas were increased in size and numbers in C3H/HeJ mice (Fig. 6D) when compared with C3H/HeN mice (Fig. 6C). Therefore, the inflammatory response in the liver was more prominent in C3H/HeJ mice coincident with the increased bacterial load and extrapulmonary dissemination.

**Increased neutrophil recruitment into the bronchi of C3H/HeJ mice**

To quantify the inflammatory cell recruitment, BAL was performed on C3H/HeN and C3H/HeJ mice 4 wk after aerogenic M.tb. infection, and BAL cells retrieved from the lavages were counted and differentiated to determine the type of cells recruited into the bronchoalveolar space. Although the total cell counts were comparable for the two groups, the C3H/HeN mice had a significantly larger proportion of macrophages than the C3H/HeJ mice 4 wk postinfection (Fig. 7A), but the lymphocyte population appeared to be comparable (Fig. 7B). Interestingly, there were a significant number of neutrophils being recruited into the alveoli of the C3H/HeJ mice at this time point, which was not apparent in the C3H/HeN lungs (Fig. 7C). These data concur with the histological finding of increased lung parenchymal infiltration by neutrophils in the C3H/HeJ mice (data not shown). Therefore, pulmonary recruitment of macrophages was reduced, while recruitment of neutrophils was significantly increased in the TLR4 mutants relative to the C3H/HeN control mice.

**Decreased cytokine and chemokine production in the lungs of C3H/HeJ mice**

To compare the level of proinflammatory cytokines and chemokines produced in response to aerosol M.tb. infection, BAL fluid and lung homogenates were taken at 4 wk postinfection and assessed by ELISA. The production of IL-12p40 was significantly reduced in the BAL fluid of the C3H/HeJ mice (Fig. 8A), while no significant differences were observed for TNF-α and IFN-γ (Fig. 8, B and C). To further assess the cytokine and chemokine production

![Figure 4](https://example.com/figure4.png)

**FIGURE 4.** Representative microscopic alterations in lungs of C3H/HeN and C3H/HeJ mice at 28 days (A and B) and 140 days (C and D) after aerosol M.tb. infection. The early pneumonitis with small granulomas at 28 days was similar in C3H/HeN and C3H/HeJ mice (A and B), while at later stages (140 days) chronic pneumonia with abundant macrophages and neutrophils was more prominent in the TLR4 mutant C3H/HeJ (D) than in C3H/HeN control mice (C). H&E stain.
in the lung environment, infected lungs were homogenized and supernatants were quantified. The cytokines IL-12p40 and TNF-α were significantly reduced in the lung homogenates of C3H/HeJ mice when compared with the C3H/HeN mice (Fig. 9, A and C). The level of IFN-γ in the C3H/HeJ lung homogenates was slightly lower than in C3H/HeN homogenates (Fig. 9B). The monocyte and T lymphocyte chemokine MCP-1 was significantly lower in C3H/HeJ mice than in C3H/HeN mice (Fig. 9D). Therefore, increased

**FIGURE 5.** Abundant acid-fast bacilli in TLR4 mutant strain, and evidence of macrophage activation as assessed by the expression of iNOS2. Ziehl-Neelsen stain at 28 days (A and B) and at 140 days (C and D) and iNOS2 immunostaining (E and F) 56 days after aerosol *M. tb.* infection in lungs of C3H/HeN (A, C, and E) and C3H/HeJ (B, D, and F) mice. Four mice per group were examined and representative sections were selected.

**FIGURE 6.** Increased numbers and size of hepatic granulomas of TLR4 mutant C3H/HeJ mice 140 days after aerosol infection. Hepatic granulomas from C3H/HeN (B and C) and C3H/HeJ (C and D) mice examined at 28 days (A and B) were comparable, but at 140 days (C and D) after aerosol *M. tb.* infection the granulomas were more prominent in the TLR4 mutant mice. H&E staining.
susceptibility to \textit{M. tb} infection appears to be associated with a reduced induction of proinflammatory cytokines.

\textbf{Mycobacterial PIM induces a TLR4-dependent signaling pathway}

To define structures that may be associated with the TLR4-dependent immunity, we have tested the biological activities of a purified mycobacterial glycolipidic anchor. The main cell wall glycolipid from \textit{Mycobacterium} is a lipoarabinomannan (LAM). Heterogeneity in biological responses to LAM has been linked to the terminal mannose residues and to mycobacteria strains (30, 31). In contrast, reversed-phase purified PIM is a biosynthetic precursor of the larger glycolipid LAM and represents a homogeneous preparation with a phosphatidylinositol moiety with acyl groups consisting of palmitic and tuberculostearic acids which has been characterized by mass spectrometry (31). Although LAM and PIM have been shown to activate via a TLR2-dependent pathway, we examined the biological activities related to the CD14- and TLR4-dependent pathways induced by a defined preparation of PIM\textsubscript{4-6} (27). Using the astrocytic U373 cell line, no IL-6 was produced after stimulation with PIM in the absence of serum, whereas IL-6 production was increased in a dose-dependent manner on the presence of recombinant CD14 (Fig. 10A). Since U373 are TLR2-negative and TLR4-positive cells, the phospholipidic anchor PIM may induce inflammatory cytokine in a CD14-dependent manner through TLR4. The level of LPS in the PIM\textsubscript{4-6} preparation was found be \(<1\) pg of endotoxin/\(\mu\)g of material by the \textit{Limulus} assay, thus excluding LPS contamination. To further characterize the induction of TLR4 by this lipidic structure, stable TLR4 and MD-2 Ba-F3 transformants were stimulated with increasing concentrations of PIM\textsubscript{4-6}. Whereas no induction was observed in control cells, PIM\textsubscript{4-6} induced NF-\beta transactivation in a dose-dependent manner in MD-2- and TLR4-positive cells (Fig. 10B). As demonstrated previously, TLR2 transfectants responded to our PIM preparation (data not shown). These results identify mycobacterial PIM as one of the simplest defined structures known to have TLR4 agonist activity. Taken together, PIM structures induce responses...
through CD14 and TLR4 and behave in a fashion similar to the amphiphilic molecule LPS derived from Gram-negative bacteria.

Discussion

We demonstrate here for the first time that innate immune recognition by TLR4 is required and may be involved in protective mechanisms during chronic M. tuberculosis infection. The TLR4 mutant, C3H/HeJ mice, has an increased susceptibility to M. tuberculosis infection associated with reduced macrophage recruitment and diminished proinflammatory cytokine response, which might be the key factors for reduced immune response. Whereas C3H/HeJ mice were apparently highly effective in controlling the primary infection, host immune protection to M. tuberculosis was insufficient during long-term persistence in vivo.

The recognition of mycobacterial components may include several pattern recognition receptors including the mannose receptors, CRs CR1, CR3, and CR4 (15), and TLR-2 and TLR-4. The 19-kDa lipoprotein binds and signals through TLR2, resulting in IL-12 secretion; furthermore, mycobacterial killing and apoptosis are primarily TLR2 dependent (9, 16–19). Whereas it has been suggested that both TLR2 and TLR4 can mediate cellular activation by M. tuberculosis-derived soluble heat-stable and heat-sensitive cell-associated mycobacterial factors distinct from mycobacterial cell wall glycolipid LAM (22), several reports have described a TLR2-dependent pathway induced by LAM and PIM (21, 32), suggesting that the main signaling pathway appears to be TLR2. These results do not differ significantly from other groups as our preparation of PIM4-induced signaling via TLR2 (data not shown) as demonstrated by Jones et al. (32), but additionally signaled via TLR4. We show that PIM samples obtained following a purification protocol for glycolipidic molecules and characterized by mass spectrometry induce, indeed, a TLR4-dependent activation (31). This work demonstrates a potential mechanism for the recognition of M. tuberculosis via PIM in a TLR4-dependent manner, which might be partly responsible for the phenotype observed for the TLR4 mutants.

TLR4 signaling in response to M. tuberculosis appears to be required for long-term resistance as the TLR4 mutant, C3H/HeJ mice, succumbed to chronic M. tuberculosis infection, while the control C3H/HeN survived. These data concur with those obtained recently with the vaccine strain M. bovis bacillus Calmette-Guérin (BCG)-infected C3H/HeJ mice, demonstrating that TLR4 has a protective role, although the i.p. infection route chosen appears to be rather non-physiologic (33).

These data are in contrast to findings by Chackerian et al. (34), who demonstrated a high susceptibility in the C3H strain of mice, which is TLR4 independent. The reasons for the difference may be related to the route of infection, the high infective dose, and the mycobacterial strain difference. Several studies have shown an enhanced sensitivity of TLR4-deficient C3H/HeJ mice when compared with C3H/HeN mice to a broad range of infectious models (35), including E. coli-induced pyelonephritis (36), Salmonella typhimurium, and Leishmania donovani (37). Subsequently, several groups demonstrated that the dramatic susceptibility of the C3H/HeJ mice to Salmonella was due to a reduced recruitment of macrophages and an inherent activation defect in these cells (38, 39, 40, 41). The increased susceptibility of C3H/HeJ mice to M. tuberculosis infection may be likely due to a decreased TLR4-dependent activation of proinflammatory cytokines and chemokine production induced by mycobacterial structures such as PIM. We indeed demonstrated reduced pulmonary TNF-α, IL-12p40, and MCP-1 production in C3H/HeJ mice. Tsuji et al. (42) reported that BCG-derived cell wall skeleton induced maturation of dendritic cells via TNF-α in a TLR4-dependent manner. In the absence of TLR4, BCG cell wall skeleton induced significantly less TNF-α from macrophages, leading to poor maturation of dendritic cells concomitant with impaired secretion of IL-12p40, IL-6, and surface expression of CD40, CD80, CD83, and CD86 (42). The diminished cytokines/chemokine production might be a consequence of reduced macrophage recruitment and activation, impairing the control of mycobacterial growth and leading to a significant increase in the bacterial load of C3H/HeJ mice. Over time, the clinical pattern of M. tuberculosis-infected TLR4 mutants show an increasing infiltration of inflammatory cells and increasing number of granuloma in infected organs associated with a defective clearance of pathogens leading to a lethal outcome. The more prominent neutrophil infiltration in organs from C3H/HeJ mice suggests that neutrophil migration is TLR4 independent. This is in agreement with a study by Haziot et al. (43), which demonstrated dramatically enhanced neutrophil recruitment in response to either LPS or E. coli in a TLR4-independent manner. Consistent with our findings, Means et al. (33) demonstrate that M. tuberculosis-induced TNF-α production by RAW 264.7 macrophages was blocked by E5531, a TLR4 antagonist. Furthermore, they demonstrate normal production of NO in the presence of the TLR4 antagonist, which suggests a TLR4-independent pathway for NO induction, which is in agreement with our findings by iNOS2 immunostaining. The reduced IL-12p40 production might be a consequence of reduced recruitment of macrophages and activation thereof. A reduction in IL-12 levels was observed in C3H/HeJ mice in endodontic inflammatory lesions in response to a mixed anaerobic infection (44). This was accompanied by a comparable level of IFN-γ, which is analogous to our findings of decreased IL-12p40, but comparable IFN-γ. Furthermore, nucleosome remodeling at the IL-12p40 promoter was reported to be dependent on TLR4 signaling (45).

In conclusion, TLR4 is one of several pattern recognition receptors involved in the recognition of M. tuberculosis including TLR-2, CR1, 3, and 4, and the mannose receptor. Early in vitro studies blocking CR3 indicated that CR3 was the major phagocytic receptor for M. tuberculosis (46), but subsequent experiments with CR3-deficient mice revealed no differences compared with wild-type mice with respect to survival and bacterial burden (47) The absence of TLR4 can be partly compensated for by the other pattern recognition receptors, but does indeed lead to an impaired activation of APCs as demonstrated by decreased IL-12p40 production. This impaired APC activation might affect the extent of CD4 T cell priming and ultimately antituberculosis immunity. Thus, immune responses to mycobacteria are at least in part TLR4 dependent. C3H/HeJ mice succumbed to chronic M. tuberculosis infection, and diminished proinflammatory response, macrophage recruitment, and activation are prime factors for reduced host immunity.

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