Pulmonary immune response during (myco)bacterial infection
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CHAPTER 8

Toll-Like Receptor 4 Plays a Limited Protective Role in Pulmonary Tuberculosis in Mice

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Peter Speelman, and Tom van der Poll
Toll-like receptors (TLRs) play an essential role in the innate recognition of microorganisms by the host. To determine the role of TLR4 in host defense against lung tuberculosis, TLR4 mutant (C3H/HeJ) and wild type (Wt, C3H/HeN) mice were intranasally infected with live *Mycobacterium tuberculosis*. Overall the differences between TLR4 mutant and Wt mice were modest. Yet, TLR4 mutant mice were more susceptible to pulmonary tuberculosis, as indicated by a reduced survival and an enhanced mycobacterial outgrowth. Lung infiltrates were more profound in TLR4 mutant mice and contained more (activated) T cells. Splenocytes of infected TLR4 mutant mice demonstrated a reduced capacity to produce the protective type 1 cytokine interferon-γ upon antigen-specific stimulation, indicating that TLR4 may be involved in the generation of acquired T cell-mediated immunity. These data suggest that TLR4 plays a limited protective role in host defense against lung infection by *M. tuberculosis*. 

ABSTRACT
INTRODUCTION

Tuberculosis represents a serious threat to global health. An estimated one third of the world’s population is infected with *Mycobacterium tuberculosis*, resulting in approximately eight million new cases of tuberculosis and two million deaths each year (1). An adequate cell-mediated immune response plays a key role in resistance against tuberculosis (2). In murine models of mycobacterial infection, type 1 cytokines, in particular interferon-γ (IFN-γ), are essential for protective immunity (3, 4). The development of a protective T helper 1 response during tuberculosis is driven by interleukin (IL)-12, which is produced by macrophages upon phagocytosis of *M. tuberculosis* (5), and like IFN-γ plays a pivotal role in the control of mycobacterial infection (6).

Innate recognition of mycobacterial products is the first step in a chain of events that results in an effective host defense against infection with *M. tuberculosis*. The innate immune system serves to mount a rapid protective response in the early phase of an infection, and also instructs the (later) adaptive T cell mediated immune response (7). Until recently it was unclear how *M. tuberculosis* activates macrophages to initiate an innate immune response. The discovery of the Toll-like receptor (TLR) family has provided important insight into the recognition of various microbial pathogens by the host (8, 9). Two members of the TLR family have been demonstrated to mediate *M. tuberculosis* induced intracellular signaling *in vitro*, i.e. TLR2 and TLR4. Indeed, experiments with Chinese hamster ovary (CHO) cells and murine macrophages transfected with human TLR2 and/or TLR4 have established that viable *M. tuberculosis* bacilli activate these cells via both TLR2 and TLR4 (10, 11). In addition, thioglycollate-elicited peritoneal macrophages from TLR4 deficient mice produced less tumor necrosis factor-α (TNF) upon stimulation with whole cell lysates from *M. tuberculosis* than normal wild type macrophages, also indicating that this microorganism activates cells via TLR4 and another receptor, presumably TLR2 (12). TLR2 has emerged as the signaling receptor for purified mycobacterial products, including arabinose-capped lipoarabinomannan (AraLAM), culture supernatant soluble tuberculosis factor, mycolylarabinogalactan-peptidoglycan complex, the 19 kDa antigen and total lipids (10, 11, 13-18).

Knowledge of the role of TLRs in host defense against mycobacterial infection *in vivo* is highly limited. The only investigation reported to date involved a study in which
intraperitoneal administration of *M. bovis* bacillus Calmette-Guérin (BCG) was found to result in an increased mortality of C3H/HeJ mice, which have a nonfunctional TLR4, relative to mice with a normal TLR4 gene, as measured three days after the infection (11). The impact of TLR4 deficiency on mycobacterial outgrowth and host responses was not reported. Since tuberculosis almost exclusively is acquired via the respiratory route and typically follows a chronic course, we were interested in the consequences of TLR4 deficiency on pulmonary infection with virulent *M. tuberculosis*. Therefore, we induced lung tuberculosis in wild type C3H/HeN and TLR4 deficient C3H/HeJ mice by intranasal inoculation with *M. tuberculosis* (H37Rv), and monitored the course of the infection and host responses.

**MATERIAL & METHODS**

*Mice*
Pathogen-free 8-9 week old female C3H/HeJ (TLR4 mutant) and C3H/HeN (Wt) mice were purchased from Charles River (Someren, the Netherlands) and were maintained in biosafety level 3 facilities. C3H/HeJ mice have been demonstrated to have a missense mutation in the third exon of TLR4 predicted to result in a Pro712→His substitution, yielding a nonfunctional TLR4 (19, 20). All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands).

*Experimental infection*
Pulmonary tuberculosis was induced as described previously (21-23). In brief, a virulent laboratory strain of *M. tuberculosis* H37Rv was grown in liquid Dubos medium containing 0.01% Tween 80 for 4 days. A replicate culture was incubated at 37°C, harvested at mid-logarithmic phase, and stored in aliquots at -80°C. For each experiment, a vial was thawed and washed two times with sterile 0.9% NaCl. Mice were lightly anesthetized by inhalation with isoflurane (Upjohn, Ede, the Netherlands) and intranasally inoculated with 50 µl of mycobacterial suspension. The intranasal route of infection has been described previously by us and others, and results in a reproducible infection of the lung with subsequent dissemination to liver and spleen (21, 22, 24, 25). Bacterial counts recovered from lungs one day postinfection were shown previously to be similar to the number of bacteria in the inoculum (22). Exact inoculum strength was determined by
plating tenfold dilutions of the suspension on Middlebrook 7H11 agar plates immediately after inoculation. Mice were inoculated with $10^5$ Colony Forming Units (CFUs) *M. tuberculosis*. After 2 and 5 weeks, groups of 12-13 mice per time point were anesthetized by FFM (fentanyl citrate 0.079 mg/ml, fluanisone 2.5 mg/ml, midazolam 1.25 mg/ml in H$_2$O) and sacrificed by bleeding out the vena cava inferior. Lungs and one lobus of the liver were removed aseptically and homogenized with a tissue homogenizer (Biospec Products, Bartlesville, OK) in 5 volumes of sterile 0.9% NaCl, and 10-fold serial dilutions were plated on Middlebrook 7H11 agar plates to determine bacterial loads. Colonies were counted after 21 days of incubation at 37°C. CFUs are provided as total number in the lungs or as total per gram liver tissue. For cytokine measurements, lung homogenates were diluted 1:1 in lysis buffer (150 mM NaCl, 15 mM Tris, 1mM MgCl$_2$H$_2$O, 1 mM CaCl$_2$, 1% Triton X-100, 100 µg/ml Pepstatin A, Leupeptin and Aprotinin, pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g for 15 min after which the supernatants were sterilized using a 0.22 µm filter (Corning, Corning, NY) and stored at -20°C until further use.

**Histologic examination**

Lungs for histologic examination were harvested at 2 and 5 weeks after infection and fixed in 4% formaldehyde and then embedded in paraffin. Four µm thick sections were stained with haematoxylin and eosin, and analyzed for the total area of inflammation and granuloma formation by a pathologist who was blinded for groups.

**Flow cytometry**

Pulmonary cell suspensions were obtained using an automated disaggregation device (Medimachine System; Dako, Glostrup, Denmark) and processed as described previously (21, 22). Total leukocytes in pulmonary cell suspensions were counted by using a hemacytometer and TÜRK's solution (Merck, Darmstadt, Germany). The number of macrophages, granulocytes and lymphocytes were calculated from these totals, using cytopsin preparations stained with modified Giemsa stain (Diff-Quick, Baxter, McGraw Perk, IL). For FACS analysis, cells were brought to a concentration of $4 \times 10^6$ cells/ml FACS buffer (PBS supplement with 0.5% BSA, 0.01% NaN$_3$, and 100 mM EDTA). Immunostaining for cell surface molecules was performed for 30 min at 4°C using directly labeled Abs against CD3 (anti-CD3 PE), CD4 (anti-CD4 CyChrome), CD8 (anti-CD8 FITC, anti-CD8 PerCP), CD25 (anti-CD25 FITC), and CD69 (anti-CD69 FITC).
ROLE OF TLR4 IN TB

All Abs were used in concentrations recommended by the manufacturer (PharMingen, San Diego, CA). To correct for specific staining, an appropriate control Ab (rat IgG2; PharMingen) was used. The number of positive cells was obtained by setting a quadrant marker for nonspecific staining. FACS analysis was performed using Calibrite (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Splenocyte stimulation

Single cell suspensions were obtained by crushing spleens through a 40 μm cell strainer (Becton Dickinson, Franklin Lakes, NJ). Erythrocytes were lysed with ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 100 mM EDTA, pH 7.4) and the remaining cells were washed twice. Splenocytes were resuspended in RPMI medium (Bio Whittaker) containing 10% FCS and 1% antibiotic-antimycotic (Life Technologies), seeded in 96-well round bottom culture plates at a cell density of 1 x 10⁶ cells in triplicate, and stimulated with 10 μg/ml tuberculin-purified protein derivative (PPD, Statens Seruminstitut, Copenhagen, Denmark). In a separate experiment, round bottom plates were coated overnight with anti-CD3 Ab (clone nr. 145.2c11) and washed twice with sterile PBS. 1 x 10⁶ cells (in triplicate) were added to each well and diluted with RPMI containing anti-CD28 Ab (1 μg/ml, PharMingen, San Diego, CA). Supernatants of both experiments were harvested after a 48 h incubation period at 37°C in 5% CO₂. Cytokine levels were analyzed by ELISA.

Cytokine measurements

IFN-γ and IL-4 were measured in lung homogenates and spleen cell supernatants by specific ELISA’s according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

Statistical analysis

All data are expressed as mean ± SEM. Differences between groups were analyzed by Mann-Whitney U test. For comparison of survival curves Kaplan-Meier analysis with a log rank test was used. P < 0.05 was considered to represent a statistically significant difference.
RESULTS

Survival
To investigate the role of TLR4 in the outcome of tuberculosis, mice were intranasally inoculated with mycobacteria and were monitored in 2 separate, consecutive experiments. In the first study, mice were observed for 15 weeks. As shown in Fig.1A, no mortality was seen in the Wt group. In contrast, 7/12 (58%) of the TLR4 mutant mice died ($P = 0.002$). The second experiment showed a trend toward enhanced mortality of TLR4 mutant mice: mortality after 23 weeks in the Wt group was 10/13 (77%) while all of the TLR4 mutant mice had died (non-significant; Figure 1B).

![Figure 1](image)

**Figure 1** Survival of TLR4 mutant and Wt mice after intranasal infection with *M. tuberculosis*. The results of two independent experiments are shown. N = 12 – 13 mice per group in each experiment. $P$ value $<0.05$ indicates statistical difference between groups. NS = non significant.

Mycobacterial outgrowth
Because of the differences in survival between TLR4 mutant and Wt mice, we determined whether differences in mycobacterial load existed in earlier phases of the infection. At 2 and 5 weeks post-infection, outgrowth of *M. tuberculosis* in lungs of both mouse strains was compared. The numbers of CFUs recovered from lungs were not different between TLR4 mutant and Wt mice 2 weeks post-infection (data not shown). However, as shown in Figure 2A, at 5 weeks post-infection, TLR4 mutant lungs contained 2.8 times more viable mycobacteria compared to Wt lungs ($P = 0.003$). Since *M. tuberculosis* is known to disseminate in mice, mycobacterial numbers were also determined in a distant organ - the liver. The mycobacterial load in liver tissue was similar in TLR4 mutant and Wt mice 2 weeks after *M. tuberculosis* inoculation (data not shown). At 5 weeks post-infection, however, the number of CFUs in liver tissue of TLR4 mutant
mice was 3.2-fold higher compared to the bacterial load in liver tissue of Wt mice \( (P = 0.05; \text{Figure 2B}) \). In a subsequent separate experiment we tried to determine mycobacterial outgrowth at a time point later than 5 weeks. However, this experiment was confounded due to the fact that in the 6th week post-infection, 5/13 (38%) of the TLR4 mutant mice died while none of the Wt mice died \( (P = 0.02) \), again suggesting that TLR4 mutant are more susceptible to \textit{M. tuberculosis} infection.

![Figure 2](image)

Figure 2 Enhanced growth of \textit{M. tuberculosis} in (A) lungs and (B) liver of TLR4 mutant mice compared to Wt mice at 5 wk post-infection. Data are mean and SEM CFUs from 12–13 mice per group. *\( P < 0.05 \) vs Wt mice.

**Histology**

Histopathological analysis of the lungs two weeks after infection showed a slight interstitial infiltrate consisting of macrophages, lymphocytes and occasional neutrophils adjacent to small airways and vessels in Wt mice (Fig. 3A). At the same time, the lungs of TLR4 mutant mice showed larger areas of inflammation (Fig. 3B). At 5 weeks post-infection, the inflammatory infiltrates became more diffuse and intense, and involved between 50 and 60% of the analyzed lung surface in Wt mice (Fig. 3C). At this time point, the inflammation was also more pronounced in TLR4 mutant mice with an involvement of 70 to 80% of the lung surface (Fig. 3D).

**Cellular composition of lung infiltrates**

To obtain more insight into the cellular composition of the pulmonary infiltrates, we determined whole lung leukocyte counts and differentials, and further analyzed lymphocyte subsets and activation status by flow cytometry (Table I). In accordance with the histopathology, the lungs of TLR4 mutant mice contained more leukocytes than
those of Wt mice. This was caused by increased absolute numbers of macrophages, lymphocytes and granulocytes. Subtyping of lymphocytes showed that the percentage of CD4+ and CD8+ cells was similar in both groups. The percentages of activated (CD25+ or CD69+) T cells were higher in lungs of TLR4 mutant mice, especially at 5 weeks postinfection in the CD8+ population.

**Lung IFN-γ and IL-4 levels**

Th1 cells are essential for early host defense in *M. tuberculosis* infection (2). We therefore investigated whether the deteriorated outcome of TLR4 mutant mice was associated with a change in the Th1 and Th2 cytokine profile in the pulmonary compartment. Th1 (IFN-γ) and Th2 (IL-4) cytokines were measured in lung homogenates. As shown in Fig. 4, IFN-γ levels were similar at 2 weeks post-infection. After 5 weeks, IFN-γ was elevated in TLR4 mutant mice compared to Wt mice (*P* = 0.005). This finding paralleled the mycobacterial load in the lungs. In contrast, the Th2 cytokine IL-4 was produced in lower concentrations in TLR4 mutant mouse lungs in comparison with Wt mice.
**Table I** Cell subsets in total lungs in TLR4 mutant and Wt mice during pulmonary tuberculosis

<table>
<thead>
<tr>
<th>Cells</th>
<th>2 weeks post-infection</th>
<th>5 weeks post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TLR4 mutant</td>
<td>Wt</td>
</tr>
<tr>
<td>Leukocytes x10^6</td>
<td>13.1 ± 0.9*</td>
<td>9.5 ± 0.0</td>
</tr>
<tr>
<td>Macrophages x10^6</td>
<td>2.0 ± 0.2</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>(16.5 ± 1.6%)</td>
<td>(19.0 ± 3.5%)</td>
<td>(12.6 ± 1.0%)</td>
</tr>
<tr>
<td>Granulocytes x10^6</td>
<td>5.0 ± 0.5*</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>(39.2 ± 1.5%)</td>
<td>(35.6 ± 2.3%)</td>
<td>(52.5 ± 2.3%)</td>
</tr>
<tr>
<td>Lymphocytes x10^6</td>
<td>5.6 ± 0.5</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>(44.3 ± 1.7%)</td>
<td>(45.5 ± 2.0%)</td>
<td>(34.7 ± 2.1%)</td>
</tr>
<tr>
<td>% CD4+</td>
<td>63.4 ± 0.9</td>
<td>65.0 ± 2.1</td>
</tr>
<tr>
<td>% CD8+</td>
<td>25.5 ± 3.1</td>
<td>21.9 ± 1.7</td>
</tr>
<tr>
<td>% CD4/CD69+</td>
<td>7.7 ± 1.3*</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>% CD4/CD25+</td>
<td>8.2 ± 1.1</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>% CD8/CD69+</td>
<td>2.7 ± 1.5</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>% CD8/CD25+</td>
<td>1.2 ± 0.2</td>
<td>0.7 ± 0.3</td>
</tr>
</tbody>
</table>

Cell subsets in the lungs of TLR4 mutant and Wt mice infected with *M. tuberculosis* 2 and 5 weeks post-infection. Total leukocyte counts per left lung. Differential counts per left lung and, in parenthesis, as percentage of total leukocytes. Flow cytometry results are expressed as the percent of CD4+, CD8+, CD25+ and CD69+ T-cells within the CD3+ population in the left lung. *P < 0.05 vs Wt at the same time point.

at 5 weeks (P = 0.017), while at 2 weeks, no significant difference in IL-4 concentrations was observed (Fig. 4). As a consequence, there was a relative shift towards a Th1 response in the pulmonary compartment of TLR4 mutant mice.

![Figure 4 IFN-γ and IL-4 concentrations in lungs of TLR4 mutant and Wt mice. N = 12-13 mice per group. Data are expressed as mean and SEM. *P < 0.05 vs Wt mice.](image)

**Th1 response of ex vivo stimulated spleen cells**

To obtain more insight in mechanisms contributing to the decreased survival of TLR4 mutant mice and the enhanced mycobacterial outgrowth (at 5 weeks) in TLR4 mutant mouse lungs in comparison with Wt mice, the ability of splenocytes, harvested 5 weeks post-infection from TLR4 mutant and Wt mice, to produce a Th1 response after *M. tuberculosis* infection was tested. IFN-γ production by splenocytes upon *ex vivo* stimulation
with either the T cell stimulator anti-CD3/anti-CD28 or PPD, was reduced 3.3 and 4.4 times respectively in TLR4 mutant mice compared to Wt mice ($P = 0.025$ and $P = 0.004$ resp., Fig. 5). The Th2 cytokine IL-4 was not detectable in supernatants of anti-CD3/anti-CD28 or PPD stimulated splenocytes from either mouse strain (data not shown).

**DISCUSSION**

TLRs play a pivotal role in the induction of an innate immune response to various infectious agents. *In vitro* studies have implicated TLR2 and TLR4 in the innate recognition of *M. tuberculosis*. We here demonstrate that TLR4 plays a protective role in host defense against pulmonary tuberculosis *in vivo*, as reflected by an increased mortality and mycobacterial load in the lungs of mice with a nonfunctional TLR4.

To our knowledge our study is the first to report on the role of TLR4 in the immune response to *M. tuberculosis in vivo*. Thusfar, the only investigation that examined the role of TLR4 in host defense against mycobacteria *in vivo* involved a study in which *M. bovis* BCG was injected intraperitoneally into TLR4 mutant and Wt mice (11). That study, reporting an enhanced mortality (9 of 20) of TLR4 mutant mice three days after the infection when compared with Wt mice (1/20), was somewhat compromised by the acute nature of the experiment and the lack of information about mycobacterial outgrowth and host defense mechanisms. Nonetheless, our present data are in line with
these earlier findings. Indeed, in two independent experiments, with follow ups of 15 and 6 weeks respectively, TLR4 mutant mice displayed an enhanced mortality when compared with Wt mice. Although in a third experiment, with a follow up of 23 weeks, the difference between TLR4 mutant and Wt mice was not significant, a trend in the same direction was seen. For comparison, mice that are devoid of IFN-γ or IL-12 activity were reported to display a profoundly reduced survival in models of mycobacterial infection (3, 4, 6), suggesting that the consequence of TLR4 deficiency on survival during lung tuberculosis is more modest. TLR4 mutant mice also demonstrated an increased outgrowth of M. tuberculosis 5 weeks after the infection. Although the difference was highly statistically significant (P=0.003), TLR4 mutant mice had on average just 3-fold more mycobacteria in their lungs than Wt mice. Together these data suggest that TLR4 plays a role, albeit limited, in protection of the host against mycobacterial infection.

TLR4 mutant mice tended to have a stronger inflammatory response in their lungs than Wt mice. Although at 5 weeks postinfection the higher mycobacterial load in TLR4 mutant may have contributed to this finding, an explanation for the difference at 2 weeks is less evident. Nonetheless, the histopathology of the lungs and the evaluation of leukocyte counts and differentials in whole lung cell suspensions clearly establish that TLR4 is neither required nor important for mounting an inflammatory response to pulmonary infection with M. tuberculosis. Furthermore, TLR4 is not indispensable for the recruitment of (activated) CD4+ or CD8+ T cells to the site of infection during lung tuberculosis.

IFN-γ is of crucial importance for an adequate immune response to mycobacteria (3, 4). TLR4 deficiency was associated with elevated levels of IFN-γ in lung homogenates at 5 weeks postinfection. Conceivably, this finding was the result of the higher mycobacterial load, providing a more potent stimulus of IFN-γ production, in combination with higher numbers of IFN-γ producing T cells in the lungs of TLR4 mutant mice. In contrast, the capacity of splenocytes to produce this prototypic type 1 cytokine upon aspecific stimulation with anti-CD3/CD28 or antigen-specific stimulation with PPD was reduced in TLR4 mutant mice. The reduced IFN-γ releasing capacity of splenocytes upon stimulation with a standardized (recall) antigen suggests that TLR4 may be involved in the generation of acquired T-cell mediated immunity to M. tuberculosis. Interestingly, a recent study also suggested a role for TLR signaling in the generation of an antigen specific type 1 T cell response. Indeed, mice with deficient TLR signaling were found to be incapable of mounting an ovalbumin specific T helper 1 response after
immunization with ovalbumin in the footpad (26). Additional research is warranted to further explore the role of TLRs in acquired T cell-mediated immunity.

If TLR4 only modestly influences the innate host response to *M. tuberculosis*, how then is this pathogen recognized by the immune system? All TLRs known to date share a common signaling component, myeloid differentiation protein 88 (MyD88) (8, 9). In the absence of MyD88 *M. tuberculosis* can not activate macrophages *in vitro*, strongly suggesting that signaling via TLRs is of major importance in the first step of immune activation by this microorganism (27). Means *et al.* have documented that viable *M. tuberculosis* bacilli are recognized by both TLR2 and TLR4 (10, 11). In line with these observations, Takeuchi *et al.* reported that tumor necrosis factor-α production by TLR4 deficient macrophages upon stimulation with *M. tuberculosis* lysates was reduced but not abolished, suggesting that besides TLR4 another receptor is involved in this response (12). Purified components and products of mycobacteria, such as AraLAM, culture supernatant soluble tuberculosis factor, mycolylarabinogalactan-peptidoglycan complex, the 19 kD antigen and total lipids, invariably utilize TLR2 as signaling receptor (10, 11, 13-18). Using transfected human dermal microvessel endothelial cells, Bulut *et al.* recently demonstrated that heterodimerization of TLR2 and TLR6 provides optimal signaling conditions for purified culture supernatant soluble tuberculosis factor (15). Together, these data suggest that *M. tuberculosis* is recognized by the host by a repertoire of different TLRs, among which TLR2, TLR4, TLR6 and possibly others. Investigations with mice that are deficient for one or more TLRs are required to resolve the individual and combined roles of different TLRs in the innate immune response to *M. tuberculosis*.

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