Cutting Edge: Toll-Like Receptor (TLR)2- and TLR4-Mediated Pathogen Recognition in Resistance to Airborne Infection with Mycobacterium tuberculosis

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Innate resistance against Mycobacterium tuberculosis is thought to depend critically on engagement of pattern recognition receptors on macrophages. However, the relative contribution of these receptors for containing M. tuberculosis infection has remained unexplored in vivo. To address this issue, we infected mice defective in CD14, TLR2, or TLR4 with M. tuberculosis by aerosol. Following infection with 100 mycobacteria, either mutant strain was as resistant as congenic control mice. Granuloma formation, macrophage activation, and secretion of proinflammatory cytokines in response to low-dose aerosol infection were identical in mutant and control mice. However, high-dose aerosol challenge with 2000 CFU M. tuberculosis revealed TLR2-, but not TLR4-defective mice to be more susceptible than control mice. In conclusion, while TLR2 and TLR4, in initiating protective responses against naturally low-dose airborne infection is redundant.

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uman tuberculosis caused by Mycobacterium tuberculosis is the most prevalent and deadly bacterial infectious disease world-wide (1). Incomplete understanding of the molecular nature of protective immune responses has hampered the development of more effective vaccines and therapies.

Animal models of low-dose aerosol infection with M. tuberculosis are believed to reflect the typical infection that occurs when humans inhale only a few virulent bacteria aerosolised in the course of an infected individual’s coughing (2, 3). Aerosol infection in mice has been instrumental in defining the prominent features of the cell-mediated immune response now known to be critical in host defense. In particular, CD4+ T cells play an important role in protective granuloma formation by secreting type 1 cytokines (4). Especially IFN-γ and TNF (5) stimulate the antimicrobial activity of infected macrophages (6). Although TNF can activate macrophages in an autocrine loop, the release of IFN-γ by NK and TH1 cells is triggered by IL-12, which is produced by APCs upon infection with mycobacteria (7).

Microbes express pathogen-associated molecular patterns capable of activating APCs following engagement of pattern recognition receptors (PRRs; Refs. 8 and 9). Specifically, CD14 was shown to be capable of activating APCs following engagement of pattern recognition receptors (PRRs). Although TNF can activate macrophages in an autocrine loop, the release of IFN-γ by NK and TH1 cells is triggered by IL-12, which is produced by APCs upon infection with mycobacteria (7).

Innate immunity, consisting of natural killer cells, NK and TH1 cells is triggered by IL-12, which is produced by APCs upon infection with mycobacteria (7).

We infected mice defective in CD14, TLR2, or TLR4 with M. tuberculosis to determine the role of TLR-mediated pathogen recognition for initiating the immune response against M. tuberculosis. We found that M. tuberculosis infection in mice deficient in CD14, TLR2, or TLR4 remained unexplored in vivo. To address this issue, we analyzed the course of aerosol M. tuberculosis infection in mice deficient in CD14, TLR2, or TLR4.

Abbreviations used in this paper: PRR, pattern recognition receptor; TLR, Toll-like receptor; iNOS, inducible NO synthase.
Materials and Methods

Mice and macrophages

TLR2 (TLR2−/−; Ref. 19) and CD14-deficient (CD14−/−) mice (20) were at least sixth generation backcrossed onto a C57BL/6 or BALB/c background, respectively. TLR4-defective C3H/HeJ (21) mice and the following congenic control mice were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany): C57BL/6 (TLR2+/+), BALB/c (CD14+/+), and C3HHeN (TLR4-competent). Bone marrow-derived macrophages were generated as previously described (22).

Bacteria and infection

M. tuberculosis (H37Rv) was grown and prepared for all experiments as described (14). For in vitro experiments, 0.5 × 10⁶ macrophages were infected with 0.5–50 × 10⁶ CFU M. tuberculosis. As a control stimulus, LPS was used at 10 ng/ml (22).

Pulmonary infection of experimental animals with M. tuberculosis with a natural dose of 100 CFU/lung or a high dose of 2000 CFU M. tuberculosis per lung was performed as described (14). Inoculum size was confirmed 24 h postinfection by determining the bacterial load in the lungs of infected mice.

 Colony enumeration assay

At different time points after infection with M. tuberculosis, lungs of sacrificed animals were removed aseptically and weighed, and one lobe per mouse was homogenized in PBS containing a proteinase inhibitor mixture (Roche Diagnostics, Mannheim, Germany). For colony enumeration, 10-fold serial dilutions of organ homogenates were plated in duplicates and processed as described (14).

Immunohistological analysis

One lung lobe per mouse was fixed in 4% formalin-PBS, set in paraffin blocks, and sectioned (2–3 μm). For immunohistology, tissue sections were prepared and stained with a polyclonal rabbit anti-mouse inducible NO synthase (iNOS) antiserum (Biomol, Hamburg, Germany) as previously described (23).

Quantification of IL-12p40, TNF, and IFN-γ by ELISA

Supernatants were collected at 24 and 96 h postinfection, and mouse TNF and IL-12p40-concentrations in the supernatants were measured by ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN and BD Bioscience, Heidelberg, Germany, respectively). Cytokine levels (TNF, IL-12p40, IFN-γ) in lung homogenates and serum from infected mice were analyzed in 3-fold serial dilutions by a sandwich ELISA (OptEia; BD Bioscience).

Statistical analysis

Data are expressed as the means of individual determinations and SD. Statistical analysis was performed using the Student t test or the log rank survival test.

Results and Discussion

Course of aerosol infection with 100 CFU M. tuberculosis in mice deficient in CD14, TLR2, or TLR4

During the course of aerosol infection with 100 CFU M. tuberculosis, the bacterial load in the lungs of BALB/c mice was almost identical with that found in CD14−/− mice (Fig. 1a). Likewise, TLR4-defective C3H/HeJ (Fig. 1b) and TLR2−/− mice (Fig. 1c) were as resistant to aerosol infection with M. tuberculosis as congenic control mice, respectively, refuting the hypothesis that TLR2 or TLR4 by themselves are of pivotal significance for innate resistance. In contrast, low-dose aerosol infection performed in parallel in mice deficient in iNOS or TNFp55 (TNF of 55 kDa), both known to be critical components of innate immunity, resulted in significantly increased pulmonary bacterial loads, in agreement with previously published data (Refs. 15 and 24; data not shown). All iNOS−/− and TNFp55−/− mice died around day 50, whereas control mice and all mice deficient in PRRs were still alive even at 14 wk postinfection. In summary, the presence of CD14, TLR4, or TLR2 is dispensable for mounting adequate innate resistance to aerosgenic infection with M. tuberculosis.

Analysis of immune responses in the lungs of M. tuberculosis-infected mice defective in CD14, TLR2, or TLR4

Because containment of bacterial replication is only one facet of an effective immune response, we analyzed whether other parameters indicative of inflammatory and protective processes might be altered in defective mice. IL-12p40, TNF, and IFN-γ were produced at comparably high levels independently of CD14−, TLR2−, or TLR4-mediated signaling in the lungs of M. tuberculosis-infected mice (Table I). There were significant differences in the absolute amount of cytokines detected in lung homogenates between the three groups of mice examined, likely reflecting the difference in their genetic backgrounds.

It was reported that M. tuberculosis-derived TLR2 agonists inhibit Ag processing and MHC class II expression in macrophages. However, flow cytometric analysis of bronchoalveolar macrophages at day 21 postinfection showed no significant difference in the expression levels of MHC class II in M. tuberculosis-infected TLR2-deficient vs congenic control mice (data not shown). This was paralleled by comparably increased numbers of CD4+, CD8+, and activated CD4+ cells in mediastinal lymph nodes (data not shown) as well as efficient epithelioid granuloma formation in all groups of mice (Fig. 2 and data not shown). In addition, iNOS

[FIGURE 1. M. tuberculosis CFU counts in the lungs of aerosol-infected mice deficient in CD14, TLR4, or TLR2 function. Control (●) and deficient mice (○) were aerogenically infected with 100 CFU M. tuberculosis. CFU counts were determined at indicated time points. Lungs were taken from BALB/c and CD14−/− (a), C3H HeN and TLR4-defective C3H/HeJ (b), and C57BL/6 and TLR2−/− (c) mice. Data represent means ± SD of four mice. One experiment representative of three performed is shown.]
TLR2, or TLR4 and congenic control mice (Fig. 2 and data not shown) for granulomatous lesions in lungs from mice defective in CD14, TLR2, or TLR4 and congenic control mice. Expression, a marker of macrophage activation, was similar in granulomatous lesions in lungs from mice defective in CD14, TLR2, or TLR4 and congenic control mice (Fig. 2 and data not shown).

Recently, signaling via TLRs was implicated in generating adaptive immune responses (25). Our data obtained in low-dose aerosol infection with M. tuberculosis suggest that development of adaptive immunity, as revealed by the generation of effector T cells and the prolonged containment of bacterial loads in compact granulomas, can also effectively proceed in a CD14-, TLR2-, or TLR4-independent fashion. A limitation of our study is that the observation period does not cover the entire life span of infected mice. Therefore, mechanisms active predominantly in controlling chronically persistent infection, e.g., release of perforin (26), may still be regulated by TLRs. Also, recent findings point to combinatorial actions of TLRs (27). It is therefore possible that the lack of more than one TLR would impair innate immunity to M. tuberculosis infection. Experiments in TLR2/TLR4 double-deficient mice and in mice lacking the TLR adaptor molecule MyD88 (28) are required to resolve this issue.

M. tuberculosis infection of bone-marrow derived macrophages from mice deficient in CD14, TLR2, or TLR4

The results from our aerosol infection in mutant mice is in striking contrast to the hypothesized critical role for PRRs in innate immune responses. However, current thinking is primarily based on results obtained from in vitro stimulation experiments performed on cell lines transfected with, e.g., CD14, TLR2, or TLR4 (11, 29).

To date, few data are available from primary macrophages addressing the roles of PRRs for initiating cytokine secretion in response to M. tuberculosis.

We found TNF and IL-12p40 production after infection of murine primary macrophages with live M. tuberculosis to be independent of CD14 (Fig. 3a, 3 day). In contrast, in TLR2−/− macrophages, TNF and IL-12p40 production were drastically reduced after stimulation with M. tuberculosis (Fig. 3c, d). Although TNF production was not significantly decreased, IL-12p40 levels

Table I. Cytokine production in lung homogenates from CD14−, TLR2−, and TLR4-defective mice after infection with M. tuberculosis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Day</th>
<th>Control</th>
<th>Defective</th>
<th>Control</th>
<th>Defective</th>
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<tr>
<td>CD14</td>
<td>21</td>
<td>3.48 ± 0.24</td>
<td>3.00 ± 0.41</td>
<td>1.07 ± 0.31</td>
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<td>42</td>
<td>3.69 ± 0.49</td>
<td>3.52 ± 0.07</td>
<td>1.04 ± 0.12</td>
<td>1.22 ± 0.45</td>
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<tr>
<td>TLR2</td>
<td>21</td>
<td>1.73 ± 0.25</td>
<td>1.66 ± 0.15</td>
<td>2.14 ± 0.50</td>
<td>1.78 ± 0.53</td>
<td>2.68 ± 0.77</td>
<td>1.99 ± 1.35</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>2.17 ± 0.46</td>
<td>2.02 ± 0.64</td>
<td>2.19 ± 0.53</td>
<td>2.65 ± 0.49</td>
<td>4.40 ± 0.37</td>
<td>4.11 ± 0.69</td>
</tr>
<tr>
<td>TLR4</td>
<td>21</td>
<td>1.74 ± 0.89</td>
<td>1.61 ± 0.50</td>
<td>0.96 ± 0.26</td>
<td>1.07 ± 0.37</td>
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<tr>
<td></td>
<td>42</td>
<td>1.63 ± 0.40</td>
<td>1.70 ± 0.41</td>
<td>0.77 ± 0.04</td>
<td>0.79 ± 0.17</td>
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<td>0.67 ± 0.18</td>
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</tbody>
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* Four mice per group were infected with 100 CFU M. tuberculosis via the aerosol route, and cytokine production was measured in lung homogenates at indicated days postinfection.

Results are representative of two independent experiments and expressed as means ± SD. Mean values of uninfected mice were 0.51 ng/lung (IL-12p40), 0.26 ng/lung (TNF), and 0.42 ng/lung (IFN-γ).
mice were infected with an inoculum of 2000 CFU by aerosol. The inoculum dose of 1 × 10^7 CFU, representing an LD_{50} for wildtype mice to i.v. infection need to be recruited to control the insult. In support of this reasoning, increased susceptibility of TLR2−/− mice to i.v. Staphylococcus aureus infection was only apparent when a very high inoculum dose of 1 × 10^7 CFU, representing an LD_{90} for wildtype mice, was used (30).

To mimic a similar situation in the M. tuberculosis model, mice were infected with an inoculum of 2000 CFU by aerosol. TLR4-defective C3H/HeJ mice still proved to be as resistant to high-dose infection with M. tuberculosis as C3H/HeN mice (Fig. 4a), confirming previous results from a high-dose i.v. infection model (31). Both groups of mice produced similar amounts of IL-12p40 following high-dose infection, providing a likely explanation for the similar outcome in these mice (Fig. 4a). In striking contrast, TLR2−/− mice were significantly more susceptible to high-dose aerosol M. tuberculosis infection than control mice (Fig. 4b). This enhanced susceptibility could be attributed to a significantly decreased proinflammatory response of TLR2−/− mice as evidenced by reduced serum levels of IL-12p40 10 days postinfection (Fig. 4b). These data clearly implicate TLR2, but not TLR4, in initiating antibacterial resistance in a borderline situation presented by a high inoculum.

Collectively, our findings may be interpreted as follows: during natural, i.e., aerosogenic and low-dose infection, M. tuberculosis triggers little, if any, response via CD14, TLR4, or TLR2. This may reflect the preferred mode of "surreptitious entry" for this highly pathogenic organism, causing as little inflammation as possible to establish infection in the lung and regional lymph nodes. Our results thus support the notion that a single deficiency in PRRs capable of detecting mycobacteria or mycobacterial components (such as CD14, TLR4, or TLR2) does not impair innate resistance to natural M. tuberculosis infection.

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References


