CUTTING EDGE

# Cutting Edge: Toll-Like Receptor (TLR)2- and TLR4-Mediated Pathogen Recognition in Resistance to Airborne Infection with *Mycobacterium tuberculosis*[1]

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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 signaling contributes to innate resistance against *M. tuberculosis* in borderline situations, its function, and that of CD14 and TLR4, in initiating protective responses against naturally low-dose airborne infection is redundant. *The Journal of Immunology*, 2002, 169: 3480–3484.**

Human 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-$\gamma$ and TNF (5) stimulate the antimicrobial activity of infected macrophages (6). Although TNF can activate macrophages in an autocrine loop, the release of IFN-$\gamma$ 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[4]; Refs. 8 and 9). Specifically, CD14 was reported to be engaged by lipoarabinomannan from *M. tuberculosis*, although subsequent studies questioned this finding (10, 11). In addition, analysis of transfected chinese hamster ovary fibroblasts suggested that live *M. tuberculosis* may use both Toll-like receptor (TLR)2 and TLR4 proteins for intracellular signaling (11). On a functional level, the interaction of mycobacterial components with TLRs may be a critical early step of macrophage activation in the course of infection. Thus, stimulation of TLR2 with the *M. tuberculosis*-derived 19-kDa lipoprotein induced mycobacteriostasis in both human and murine macrophages (12). Because TLR signaling is believed to be essential for the initial production of IL-12p40 and TNF by APCs after infection with *M. tuberculosis*, it is likely critical also for the induction of a protective cell-mediated immune response (13). In the absence of endogenous IL-12p40 or TNF, mice are severely compromised in terms of type 1 cytokine production, granuloma formation, and protection during mycobacterial infection (14, 15). Therefore, current thinking predicts that deficiencies in PRRs, such as TLR2 or TLR4, will be accompanied by greatly diminished innate immune responses resulting in early exacerbation of *M. tuberculosis* infection (16–18).

However, the role of TLR-mediated pathogen recognition for initiating the immune response against *M. tuberculosis* has thus far 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.

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[4] Abbreviations used in this paper: PRR, pattern recognition receptor, TLR, Toll-like receptor; iNOS, inducible NO synthase.



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## Materials and Methods

### *Mice and macrophages*

TLR2- (TLR2$^{-/-}$; Ref. 19) and CD14-deficient (CD14$^{-/-}$) mice (20) were at least sixth generation backcrosses 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, $0.5 \times 10^6$ macrophages were infected with $0.5–50 \times 10^6$ 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 $\mu$m). 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. 1*a*). Likewise, TLR4-defective C3H/HeJ (Fig. 1*b*) and TLR2$^{-/-}$ mice (Fig. 1*c*) 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 TNFRp55 (TNFR 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 TNFRp55$^{-/-}$ 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 aerogenic 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 bronchioalveolar 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

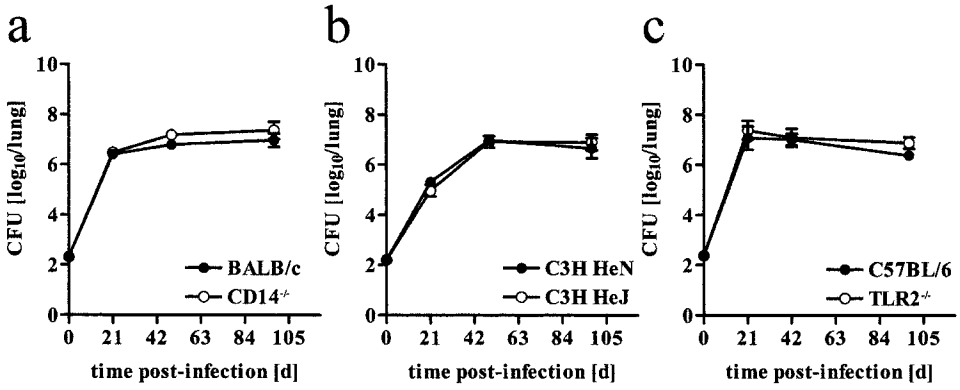


**FIGURE 1.** *M. tuberculosis* CFU counts in the lungs of aerosol-infected mice deficient in CD14, TLR4, or TLR2 function. Control (●) and deficient mice (○) were aerogenically infected with 100 CFU *M. tuberculosis*. CFU counts were determined at indicated time points. Lungs were taken from BALB/c 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.

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

| Strain | Day[a] | IL-12p40[b] (ng/lung) | | TNF[b] (ng/lung) | | IFN-γ[b] (ng/lung) | |
|---|---|---|---|---|---|---|---|
| | | Control | Defective | Control | Defective | Control | Defective |
| CD14 | 21 | 3.48 ± 0.24 | 3.00 ± 0.41 | 1.07 ± 0.31 | 1.25 ± 0.25 | 0.78 ± 0.28 | 0.69 ± 0.16 |
| | 42 | 3.69 ± 0.49 | 3.52 ± 0.07 | 1.04 ± 0.12 | 1.22 ± 0.45 | 0.65 ± 0.19 | 0.54 ± 0.04 |
| TLR2 | 21 | 1.73 ± 0.25 | 1.66 ± 0.15 | 2.14 ± 0.50 | 1.78 ± 0.53 | 2.68 ± 0.77 | 1.99 ± 1.35 |
| | 42 | 2.17 ± 0.46 | 2.02 ± 0.64 | 2.19 ± 0.53 | 2.65 ± 0.49 | 4.40 ± 0.37 | 4.11 ± 0.69 |
| TLR4 | 21 | 1.74 ± 0.89 | 1.61 ± 0.50 | 0.96 ± 0.26 | 1.07 ± 0.37 | 0.84 ± 0.15 | 0.77 ± 0.34 |
| | 42 | 1.63 ± 0.40 | 1.70 ± 0.41 | 0.77 ± 0.04 | 0.79 ± 0.17 | 0.66 ± 0.19 | 0.67 ± 0.18 |

[a] 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.

[b] 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).

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* argue 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).

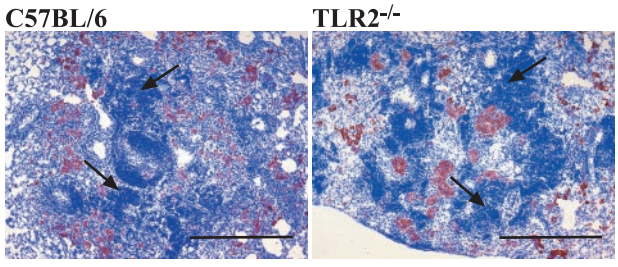


**FIGURE 2.** Granuloma formation in the lungs of *M. tuberculosis*-infected TLR2$^{-/-}$ mice. Sections (2–3 μm) were prepared from formalin-fixed lungs taken from TLR2$^{-/-}$ and C57BL/6 mice 49 days after aerosol infection with 100 CFU *M. tuberculosis*. Immunohistological staining was performed with a polyclonal rabbit anti-mouse iNOS antiserum. Shown are representative results of four mice per group obtained in three independent experiments. Arrows indicate lymphocyte aggregates; brown staining shows iNOS-positive epithelioid macrophages. Bar, 0.5 mm.

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. 3*a*, 3 day). In contrast, in TLR2$^{-/-}$ macrophages, TNF and IL-12p40 production were drastically reduced after stimulation with *M. tuberculosis* (Fig. 3, *b* and *e*). Although TNF production was not significantly decreased, IL-12p40 levels

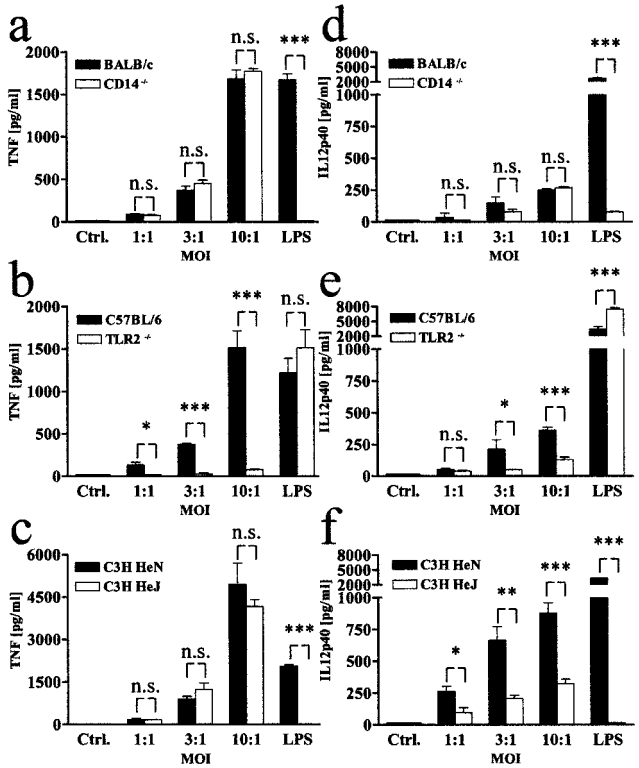


**FIGURE 3.** *M. tuberculosis*-induced TNF and IL-12p40 secretion in bone marrow-derived macrophages from CD14- or TLR2-deficient or TLR4-defective mice. Bone marrow-derived macrophages from CD14$^{-/-}$ (*a* and *d*), TLR2$^{-/-}$ (*b* and *e*), and TLR4-defective C3H/HeJ (*c* and *f*) as well as control mice were incubated with indicated multiplicities of infection (MOI) of viable *M. tuberculosis*. Supernatants were harvested 24 or 96 h after infection and measured for TNF and IL-12p40 concentrations, respectively. Data from one representative experiment of three are shown. Each point indicates the means + SD (error bars) of triplicate values. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.005$; n.s., not significant.

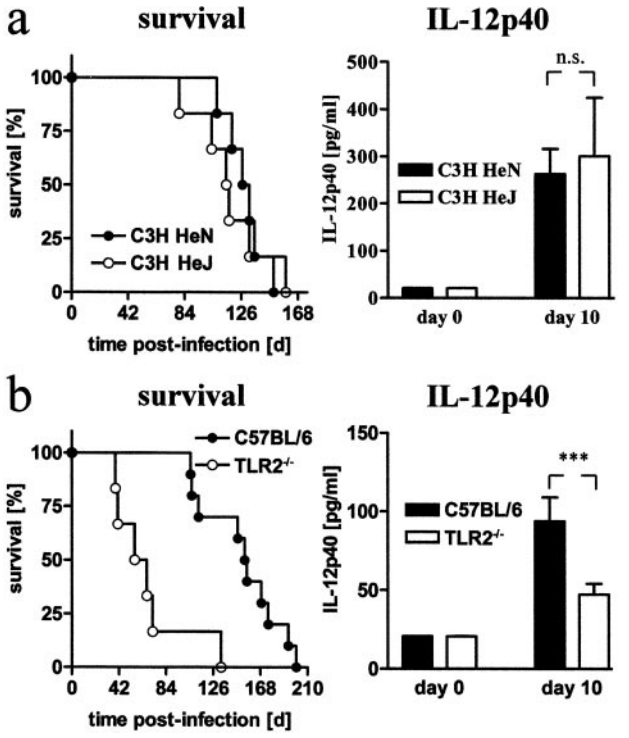


**FIGURE 4.** High-dose aerosol infection of TLR4-defective or TLR2-deficient mice with *M. tuberculosis*. Control (●) and deficient mice (○) were aerogenically infected with 2000 CFU *M. tuberculosis*. C3H/HeN and C3H/HeJ mice (*a*; 6 mice per group) or TLR2$^{-/-}$ and C57BL/6 mice (*b*; 10 mice per group) were monitored for survival. Moribund mice were sacrificed. Survival rates of C57BL/6 and TLR2$^{-/-}$ mice were significantly different ($p < 0.05$). After 10 days, a separate identically infected group of mice was sacrificed to determine IL-12p40 production in serum. ELISA data represent means ± SD of 4 mice per group. Differences in IL-12p40 production between C57BL/6 and TLR2$^{-/-}$ mice were statistically significant (∗, $p < 0.05$; n.s., not significant).

were ~65% lower in TLR4-defective C3H/HeJ macrophages when compared with simultaneously infected C3H/HeN macrophages (Fig. 3, *c* and *f*). Taken together, our in vitro infection experiments with primary macrophages corroborate earlier reports in transfected cells (11, 29) and show that engagement of TLR2 and TLR4 on macrophages indeed contributes to proinflammatory cytokine secretion in response to *M. tuberculosis*, whereas CD14 is not involved.

### *High-dose aerosol infection with* M. tuberculosis *in TLR4-defective and TLR2-deficient mice*

If TLR2- and TLR4-mediated signals significantly contribute to proinflammatory cytokine secretion in vitro, how is it possible that defective signaling in either of these molecules does not result in a discernible lack of resistance during aerosol infection in vivo? In gene-deficient mice, a distinct phenotype may often only become apparent if the missing molecule plays a nonredundant role. Additional insight may be gained from examining an experimental situation in which compensatory processes are minimized and in which even molecules that are not essential during a natural exposure 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 $1 \times 10^7$ CFU, representing an $LD_{50}$ for wildtype mice, was used (30).

To mimick 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. 4*a*), 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. 4*a*). In striking contrast, TLR2$^{-/-}$ mice were significantly more susceptible to high-dose aerosol *M. tuberculosis* infection than control mice (Fig. 4*b*). 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. 4*b*). 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., aerogenic 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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