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# Toll-Like Receptor 2-Deficient Mice Succumb to *Mycobacterium tuberculosis* Infection

Michael B. Drennan,* Delphine Nicolle,† Valerie J. F. Quesniaux,† Muazzam Jacobs,* Nasiema Allie,* Joseph Mpagi,* Cécile Frémond,† Hermann Wagner,‡ Carsten Kirschning,‡ and Bernhard Ryffel†

*From the Department of Immunology,* University of Cape Town, Cape Town, South Africa; Centre National de la Recherche Scientifique (CNRS),† GEM2358, Orleans, France; and the Institute for Medical Microbiology, Immunology and Hygiene,‡ Technical University of Munich, Munich, Germany*

**Recognition of *Mycobacterium tuberculosis* by the innate immune system is essential in the development of an adaptive immune response. Mycobacterial cell wall components activate macrophages through Toll-like receptor (TLR) 2, suggesting that this innate immune receptor plays a role in the host response to *M. tuberculosis* infection. After aerosol infection with either 100 or 500 live mycobacteria, TLR2-deficient mice display reduced bacterial clearance, a defective granulomatous response, and develop chronic pneumonia. Analysis of pulmonary immune responses in TLR2-deficient mice after 500 mycobacterial aerosol challenge showed increased levels of interferon-$\gamma$, tumor necrosis factor-$\alpha$, and interleukin-12p40 as well as increased numbers of $CD4^+$ and $CD8^+$ cells. Furthermore, TLR2-deficient mice mounted elevated Ag-specific type 1 T-cell responses that were not protective because all deficient mice succumb to infection within 5 months. Taken together, the data suggests that TLR2 may function as a regulator of inflammation, and in its absence an exaggerated immune inflammatory response develops. *(Am J Pathol 2004, 164:49–57)***

The global resurgence of tuberculosis has intensified research efforts directed at identifying the role of immune cell activation by *Mycobacterium tuberculosis*. Protective cell-mediated type 1 immune responses play a critical role in host defense against intracellular *M. tuberculosis* infection. The secretion of type 1 cytokines by Ag-specific T cells play an important role in protective granuloma formation and stimulates the anti-microbial activity of infected macrophages, allowing intracellular microbial killing.[1,2]

The discovery of the Toll-like receptor (TLR) protein family and its importance in mediating immune responses has provided novel insights into mechanisms linking innate and adaptive immunity. The number of members of human and mouse TLR thought to participate in innate immunity has grown, and evidence suggests a role for TLRs in the activation of immune cells by *M. tuberculosis*.[3,4] *M. tuberculosis*-induced tumor necrosis factor (TNF)-$\alpha$ production was found to be primarily TLR2-dependent, however, other TLRs can contribute to proinflammatory signaling.[3–5] The immunostimulatory responses to *M. tuberculosis* 19-kd lipoprotein, lipoarabinomannan, and mannosylated phosphatidylinositol are mediated by TLR2,[6–8] whereas a heat-labile factor associated with *M. tuberculosis* activates cells through TLR4.[8] Direct anti-microbial activity has been shown to be TLR2-dependent as activation by 19-kd lipoprotein resulted in the killing of *M. tuberculosis* in both mouse and human macrophages.[9] Although TLR2-dependent activation of murine macrophages led to nitric oxide-dependent growth inhibition of intracellular *M. tuberculosis*, human monocyte cultures displayed anti-microbial activity dependent on TLR2 but independent of both nitric oxide and TNF-$\alpha$. Additionally, maturation of dendritic cells is mediated by microbial lipopeptide signaling through TLR2,[10,11] a process that results in the stimulation of naïve T cells and the development of an adaptive immune response. *M. tuberculosis* 19-kd protein mediates the inhibition of MHC class II expression and antigen processing through TLR2,[12] a mechanism that may allow intracellular bacilli to evade immune surveillance, thereby promoting chronic infection. Abel and colleagues[13] showed previously TLR4 dependence of macrophage activation and clearance of mycobacteria. By contrast, Reiling and colleagues[14] suggested that TLR2 and TLR4 are redundant to control *M. tuberculosis* infection and only at extremely high infectious doses (2000 CFU) was reduced survival reported in TLR2-deficient ($TLR2^{-/-}$) mice.

To determine whether TLR2 contributes to the host response to *M. tuberculosis* infection, we aerogenically infected $TLR2^{-/-}$ mice with the virulent strain *M. tuberculosis* H37Rv. We demonstrate a critical role for TLR2 in the host response to mycobacterial infection. $TLR2^{-/-}$

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Address reprint requests to Michael B. Drennan, Department of Immunology, Old Main Building H53, Groote Schuur Hospital, University of Cape Town, Cape Town, South Africa. E-mail: mdrennan@uctgsh1.uct.ac.za.

mice have up-regulated MHC class II expression, an increased proinflammatory response, and T-cell recruitment, but lack functional granulomas during the chronic stages of infection, and develop fatal chronic pneumonia.

## Materials and Methods

### Mice

TLR2$^{-/-}$[15] and TNF$^{-/-}$[16] mice backcrossed five times to C57BL/6 were obtained from the Institute Transgenose, Centre National de la Recherche Scientifique, Orleans, France. For experiments, adult (>7 weeks old) animals were housed in individually ventilated cages under specific pathogen-free conditions at the University of Cape Town (Cape Town, South Africa) animal facility, or after infection in individually ventilated cages in a biohazard level 3 physical containment facility. All experiments performed were in accordance with the guidelines of the Animal Research Ethics Board of the University of Cape Town.

### Bacteria and Infections

*M. tuberculosis* H37Rv[13] was grown in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with Middlebrook oleic acid albumin dextrose catalase (OADC) enrichment medium (Life Technologies, Gaithersburg, MD), 0.002% glycerol, and 0.05% Tween 80. Pulmonary infection with *M. tuberculosis* H37Rv of either 100 or 500 live bacteria was performed using a Glas-Col inhalation exposure system, model A4224. Inoculum size was checked 24 hours after infection by determining the bacterial load in the lungs of infected mice.

### Colony Enumeration Assay

Bacterial loads in the lung, liver, and spleen of infected mice were evaluated at different time points after infection with *M. tuberculosis* H37Rv. Organs were weighed and defined aliquots were homogenized in 0.04% Tween 80 saline. Tenfold serial dilutions of organ homogenates were plated in duplicates onto Middlebrook 7H10 agar plates containing 10% OADC and incubated at 37°C for 19 to 21 days. Colonies on plates were enumerated and results are expressed as $\log_{10}$ CFU per organ.

### Microscopic Investigation of the Lungs

For analysis, mice were sacrificed by carbon dioxide inhalation. Organs were weighed and fixed in 4% phosphate-buffered formalin and paraffin-embedded. Sections (2 to 3 $\mu$m) were stained with hematoxylin and eosin and a modified Ziehl-Nielsen method as described.[17] Microscopic alterations such as thickening of alveolar septae, alveolar exudates, granulomas, bronchitis, and pleuritis were graded semiquantitatively using a score from 0 to 3 (no difference, moderate, distinct/severe lesions). The remaining ventilated airspace was assessed using a simplified morphometric test and expressed as a percentage of the total lung cross-section. For the immunostaining formalin-fixed paraffin-embedded sections were deparaffinized and rehydrated and stained with rabbit anti-mouse antibody specific for inducible nitric oxide synthase (iNOS) as described.[18] The sections were then washed in phosphate-buffered saline (PBS) and incubated for 30 minutes at room temperature with the biotinylated secondary antibody. The sections were incubated with avidin-biotin complexes (ABC vector kit) for 30 minutes, washed, and incubated with diaminobenzidine substrate (DAKO, Glostrup, Denmark).

### Lung Homogenate Preparations and Fluorescence-Activated Cell Sorting Analysis of Cell Surface Markers

Whole lungs were removed from infected mice at different time points and were homogenized in 1 ml of 0.04% Tween 80 saline and supernatants were collected after low-speed centrifugation, aliquoted, and frozen at −80°C. Isolated lung cells were obtained by collagenase and DNase treatment as described previously.[19] The cells were counted and incubated with antibodies against CD3 (anti-CD3 PE, clone 145. 2C11), CD4 (anti-CD4 FITC, clone H129.19), CD8 (anti-CD8 FITC, clone 53–6.7), CD11a (anti-CD11a PE, clone M17/4), CD44 (anti-CD44 PE, clone IM7), CD11c (anti-CD11c FITC, clone HL3), Ly-6G (anti-Ly6G FITC, clone RB6-8C5), I-A/I-E (anti-I-A/I-E PE, clone M5/114.15.2), CD16/32 (clone 2.4G2). All staining procedures were performed in PBS containing 0.1% bovine serum albumin and 0.1% sodium azide (fluorescence-activated cell-sorting buffer) for 20 minutes at 4°C. All antibodies were used at 0.2 $\mu$g/$10^6$ cells and obtained from BD PharMingen (San Diego, CA). Cells were fixed with 4% paraformaldehyde for at least 1 hour and analyzed by flow cytometry. Cells were gated on the lymphocyte or monocyte population by forward and side scatter, and the data analyzed using CellQuest software (BD Systems, San Jose, CA).

### Preparation of Elicited Peritoneal Macrophages

Mice were injected with 1 ml of 4% thioglycolate (Difco). Five days later, peritoneal exudate cells were isolated from the peritoneal cavity by washing with ice-cold Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Life Technologies). Cells were cultured overnight and washed with DMEM/10% FCS to remove nonadherent cells. Adherent monolayer cells were used as peritoneal macrophages. Peritoneal macrophages ($5 \times 10^5$cells/ml) were cultured in DMEM/10% FCS and stimulated with either lipopolysaccharide (LPS) (*Escherichia coli*, serotype O111:B4, 100 ng/ml; Sigma) or *M. tuberculosis* H37Rv (multiplicity of infection, 2:1). Supernatants were harvested after 4 hours.

### *Antigen-Specific Interferon (IFN)-γ Production*

For measuring antigen-specific production of IFN-γ, single-cell suspension of splenocytes were prepared from wild-type and TLR2$^{-/-}$ mice 4 weeks after infection with 500 CFU *M. tuberculosis* H37Rv. Cells were resuspended in DMEM supplemented with 10% FCS, penicillin, and streptomycin (100 U/ml and 100 μg/ml; Life Technologies). Splenocytes were cultured at $5 \times 10^5$ cells/well in 96-well round-bottom microplates (Nunc, Naperville, IL), and stimulated with either 5 μg/ml Con A (Sigma) or live *M. tuberculosis* H37Rv (multiplicity of infection, 2:1) at 37°C and 5% $CO_2$. Supernatants were harvested after 3 days.

### *CD4$^+$ and CD8$^+$ T-Cell Enrichment*

Peripheral lymph nodes were obtained 4 weeks after infection with 500 CFU *M. tuberculosis* H37Rv. Single-cell suspensions were prepared in DMEM supplemented with 10% FCS, and penicillin and streptomycin (100 U/ml and 100 μg/ml; Life Technologies). Enrichment of CD4$^+$ and CD8$^+$ T cells was performed by negative selection with magnetic mouse anti-B220-specific Dynabeads (Dynal; Robbins-Scientific, Mountain View, CA). Negatively enriched CD3$^+$ T-cell suspensions contained >90% CD3 cells, as determined by flow cytometry analysis.[20]

### *Functional CD4$^+$ and CD8$^+$ T-Cell Assay*

*In vitro* responses to mycobacterial Ag were measured as described previously,[21] with slight modifications: $4 \times 10^5$ CD3 -enriched lymph node cells obtained from C57BL/6-infected mice were cultured with $2 \times 10^4$ peritoneal macrophages pulsed with $2 \times 10^5$ *M. tuberculosis* H37Rv or 1 μg of PPD for 4 hours in antibiotic-free DMEM. Resident peritoneal macrophages were obtained 1 day before the experiment from peritoneal lavages of uninfected C57BL/6 and TLR2$^{-/-}$ mice and incubated in 96-well round-bottom microplates (Nunc) in complete DMEM. CD3$^+$ T cells and pulsed macrophages were incubated for 96 hours at 37°C and 5% $CO_2$. To determine mycobacterial Ag-specific IFN-γ production, 100 μl of supernatants from cultures were taken and kept frozen at −80°C.

### *Cytokine Enzyme-Linked Immunosorbent Assay*

Supernatants were harvested and assayed for cytokine content using commercially available enzyme-linked immunosorbent assay reagents for TNF-α, IFN-γ, and interleukin (IL)-12p40 (R&D Systems, Abingdon, UK, and BD PharMingen, San Diego, CA).

### *Nitrite Measurements*

Nitrite concentrations in freshly obtained lung homogenate supernatants were determined using the Griess reagent (3% phosphoric acid, 1% *p*-aminobenzene-sulfonamide, 1% *n*-1-napthylethylenediamide) as described.[22]

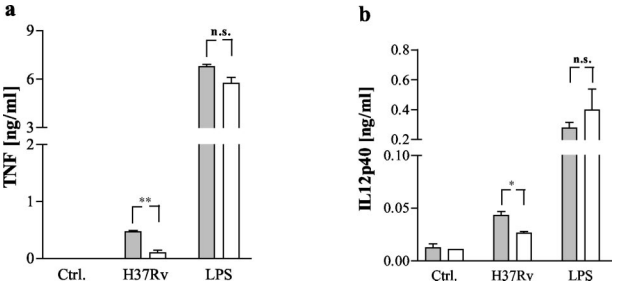


**Figure 1.** Reduced proinflammatory cytokine production in murine-elicited peritoneal macrophages from TLR2$^{-/-}$ mice. Elicited peritoneal macrophages from TLR2$^{-/-}$ (**open bars**) as well as control mice (**filled bars**) were incubated with either LPS (100 ng/ml) or viable *M. tuberculosis* (MOI = 2:1). Supernatants were harvested 4 hours after infection and measured for TNF-α (**a**) and IL-12p40 (**b**) concentrations, respectively. Data from one representative experiment of two are shown. Each point indicates the means + SD (error bars) of triplicate values. *, $P \leq 0.01$; **, $P \leq 0.001$; n.s., not significant.

### *Statistical Analysis*

Data were analyzed by comparison of C57BL/6 and TLR2$^{-/-}$ mice at each time point, with four to six mice per time point. Analysis was performed using Student's *t*-tests and values of $P \leq 0.05$ were considered significant. Each experiment was repeated at least once to ensure reproducibility.

## *Results*

### *TLR2 Dependence of* M. tuberculosis*-Induced Cytokine Response in Macrophages*

TNF-α and IL-12 play important roles in the control of local immune responses to intracellular organisms such as *M. tuberculosis*. We therefore investigated the ability of elicited TLR2$^{-/-}$ peritoneal macrophages to secrete TNF-α and IL-12p40 in response to infection with *M. tuberculosis in vitro*. The concentrations of both TNF-α and IL-12p40 in the supernatant secreted by TLR2$^{-/-}$ macrophages were significantly lower compared to wild-type controls (Figure 1, a and b), whereas TNF-α and IL-12p40 secretion in response to LPS stimulation was comparable for wild-type and TLR2$^{-/-}$ macrophages. In view of the reduced production of proinflammatory cytokines, we predicted that TLR2$^{-/-}$ mice would have reduced resistance to *M. tuberculosis* infections, as described for TNF$^{-/-}$ mice.[23]

### *TLR2$^{-/-}$ Mice Initially Control* M. tuberculosis *Infection, but Succumb during the Chronic Stages of Infection*

TLR2$^{-/-}$ mice infected with a standard dose (100 CFU/mouse) of virulent *M. tuberculosis* experienced no change in clinical status and survived the duration of the 6-month experimental period (Figure 2a), although they exhibited 1 $\log_{10}$ higher bacterial loads in the lungs compared to wild-type controls at 5 months after infection (Figure 2b). The heightened pulmonary infection was accompanied

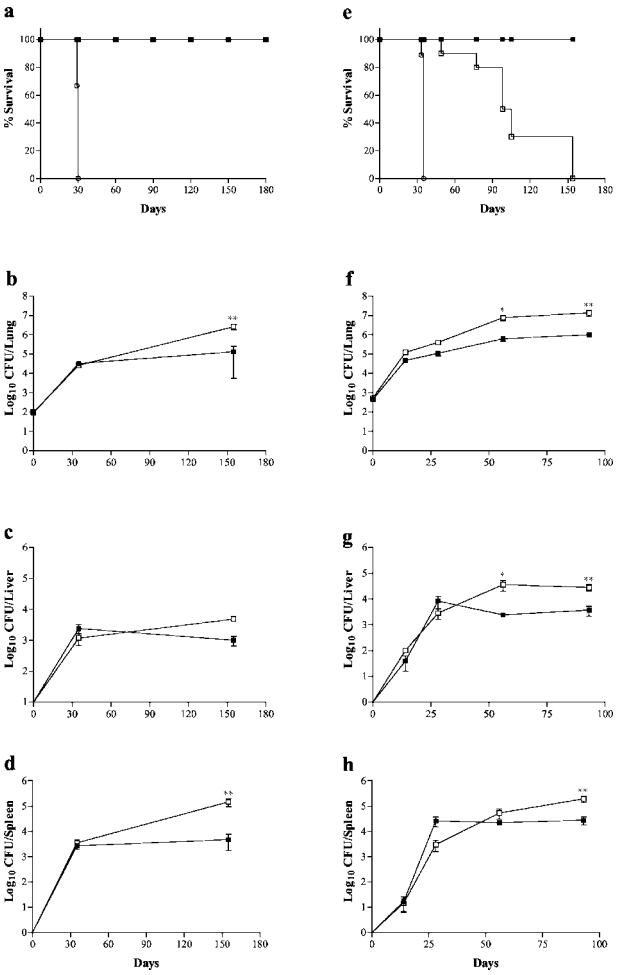


**Figure 2.** TLR2$^{-/-}$ mice are unable to control a *M. tuberculosis* infection. Control (■), TLR2$^{-/-}$ (□), and TNF$^{-/-}$ (○) mice were aerogenically infected with a standard (**a–d**; 100 CFU/mouse) and high dose (**e–h**; 500 CFU/mouse) of *M. tuberculosis*. C57BL/6, TLR2$^{-/-}$, and TNF$^{-/-}$ mice (**a** and **e**; 10 mice per group) were monitored for survival. Moribund mice were sacrificed. Survival rates of C57BL/6 and TLR2$^{-/-}$ mice were significantly different (**e**; $P < 0.0001$). CFU counts were determined at days 35 and 155 after infection (**b–d**), as well as days 14, 28, 56, and 98 after infection (**f–h**). Lungs (**b** and **f**), livers (**c** and **g**), and spleens (**d** and **h**) were taken from C57BL/6 and TLR2$^{-/-}$ mice. Data represent the means ± SD of four mice. One experiment representative of two performed is shown. *, $P \leq 0.05$; **, $P \leq 0.01$.

by bacterial dissemination into the liver and spleen (Figure 2, c and d). To exacerbate the clinical consequences of the defective bacterial control, TLR2$^{-/-}$ mice were infected with a higher dose (500 CFU/mouse) of *M. tuberculosis*. Infected TLR2$^{-/-}$ mice appeared healthy at 4 weeks, but became morbid and emaciated with initial deaths occurring between weeks 7 and 11. At 15 weeks after infection, 50% of TLR2$^{-/-}$ mice succumbed to infection and all mice were dead by week 22 (Figure 2e). Wild-type mice controlled the same infectious dose and appeared healthy at 10 months after infection at which point the experiment was terminated. TNF$^{-/-}$ mice used as a positive control strain for susceptibility in both standard and high-dose experiments succumbed to infection within 1 month after infection (Figure 2, a and e). The number of viable mycobacteria recovered from the lungs of TLR2$^{-/-}$ was 1 $\log_{10}$ higher at 8 weeks after infection,

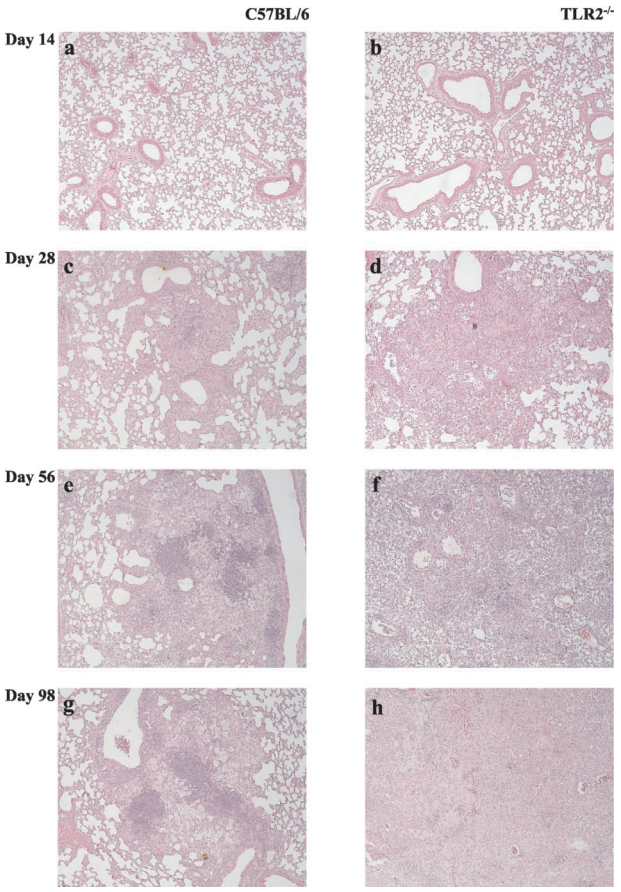


**Figure 3.** TLR2$^{-/-}$ mice exhibit an exaggerated inflammatory response during chronic *M. tuberculosis* infection. Sections (2 to 3 μm) were prepared from formalin-fixed lungs taken from TLR2$^{-/-}$ and C57BL/6 mice 14 (**a** and **b**), 28 (**c** and **d**), 56 (**e** and **f**), and 98 (**g** and **h**) days after aerosol infection with 500 CFU *M. tuberculosis*. Shown are representative results of four mice per group obtained in two independent experiments. Original magnifications, ×40.

and persisted through to week 13 when compared to wild-type mice (Figure 2f). An increased pulmonary load of viable mycobacteria in the TLR2$^{-/-}$ mice was associated with concomitant dissemination of bacilli into the liver at week 8 (Figure 2g) and the spleen at week 14 (Figure 2h).

## *Chronic Pneumonia and Defective Granulomatous Response in TLR2$^{-/-}$ Mice*

Macroscopically the lungs of TLR2$^{-/-}$ mice were swollen, displayed pleural adhesions, and were significantly increased in weight within 8 weeks after 500 CFU infection (see Figure 7a). The lungs of both wild-type and TLR2$^{-/-}$ mice were examined histologically to assess the pattern of cellular responses and to assess the progression of the disease. Microscopically, both wild-type and TLR2$^{-/-}$ mice infected with 500 CFU *M. tuberculosis* exhibited comparable thickening of alveolar septae, with no discernable granulomatous structures at 2 weeks after infection (Figure 3, a and b). At 4 weeks after infection, wild-type mice had developed typical granulomatous lesions characterized by epithelioid macrophages accom-

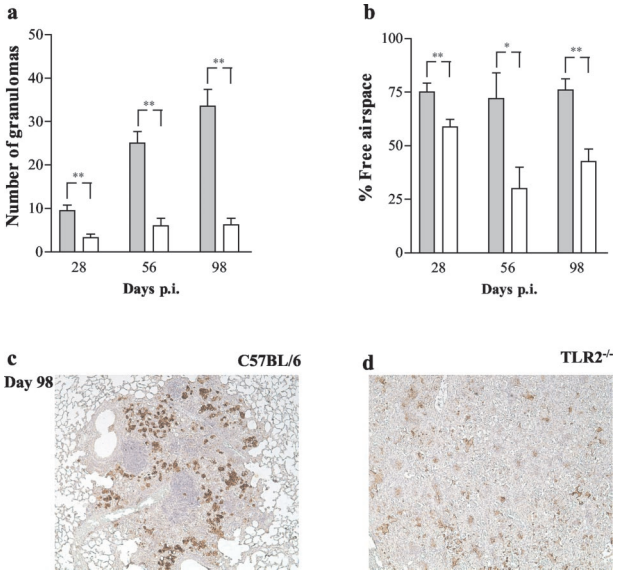


**Figure 4.** Reduced airway space, granuloma formation, and diffused iNOS expression in the lungs of *M. tuberculosis*-infected TLR2$^{-/-}$ mice. Sections (2 to 3 $\mu$m) were prepared from formalin-fixed lungs taken from TLR2$^{-/-}$ (**open bars**) and C57BL/6 (**filled bars**) mice after aerosol infection with 500 CFU *M. tuberculosis*. Granuloma formation (**a**) and free airspace (**b**) were assessed as described in Materials and Methods. Immunohistochemical staining (**c** and **d**) was performed with a polyclonal rabbit anti-mouse antibody. Brown staining shows iNOS-positive epithelioid macrophages. Shown are representative results of four mice per group obtained in two independent experiments. *, $P \leq 0.05$; **; $P \leq 0.01$. Original magnifications, ×40.

panied by lymphocytic perivascular and peribronchiolar cuffing (Figure 3c). TLR2$^{-/-}$ mice exhibited a diffuse recruitment of inflammatory cells with thickening of the alveolar septae and interstitial pneumonia (Figure 3d). Strikingly, markedly different lesions were visible at 8 weeks after infection. Although wild-type mice displayed dense infiltration of lymphocytes within compact epithelioid granulomas (Figure 3e), TLR2$^{-/-}$ mice showed massive coalescent inflammatory lesions, substantial perivascular cuffing accompanied by a few granulomatous structures, focal necrosis, and diffuse foamy macrophages (Figure 3f). At the late stages of infection, moribund TLR2$^{-/-}$ mice displayed chronic pneumonia, increased neutrophil infiltration, and pleuritis (Figure 3h). An identical pulmonary pathology was found in TLR2$^{-/-}$ mice infected with a standard dose of *M. tuberculosis* (100 CFU/mouse), albeit at a later stage. Here, chronic inflammation prevailed in TLR2$^{-/-}$ mice at 5 months after infection and was comparable to the high-dose pathology seen at 8 weeks (data not shown). Analysis of the number of granulomas and free airspace in the TLR2$^{-/-}$ mouse lungs revealed lower numbers of granulomas and dramatically reduced alveolar spaces when compared to the wild-type controls (Figure 4, a and b, respectively). Additionally, the induction and production of nitric oxide and related nitrogen intermediates (RNI) by macrophages is a major effector mechanism responsible for the anti-mycobacterial activity of IFN-$\gamma$ and TNF-$\alpha$.[1] We therefore investigated the extent of pulmonary macrophage activation in wild-type and TLR2$^{-/-}$ mice by iNOS immunostaining (Figure 4, c and d, respectively). Although iNOS was highly expressed in typical granulomas

**Table 1.** Production of RNI in C57BL/6 and TLR2$^{-/-}$ Mice after Aerosol MTB Infection

| Time after infection (days) | C57BL/6* ($\mu$M/g) | TLR2$^{-/-}$* ($\mu$mol/L/g) |
|---|---|---|
| 56 | 433.3 (44.6) | 303.1 (96.7) |
| 98 | 491.1 (43.1) | 273.8 (14.8)† |

*Total pulmonary nitrites in the lungs of wild-type and TLR2$^{-/-}$ mice infected with 500 CFU *M. tuberculosis* H37Rv. Total pulmonary nitrate was reduced by nitrite reductase to nitrite and measured by the Griess reagent. Mean ± SD ($n$ = 6).
†$P \leq 0.001$.

in the wild-type mice, a few scattered, but clearly iNOS-expressing macrophages were found in TLR2$^{-/-}$ mice.

Nitric oxide was assessed in the lung tissue homogenates and was reduced at 8 and 14 weeks after infection in TLR2$^{-/-}$ mice (Table 1.), a factor that may have contributed to the fatal outcome of infection. A poorly controlled pulmonary infection merited analysis of the local inflammatory cytokine response as well as the T cell activation status.

## *Analysis of Pulmonary Immune Responses in TLR2$^{-/-}$ Mice*

Down-regulation of cell surface expression of MHC class II molecules is one mechanism by which *M. tuberculosis* might inhibit recognition of macrophages by CD4$^+$ T cells. Recently, TLR2 has been implicated in inhibition of macrophage MHC class II expression by *M. tuberculosis* 19-kd lipoprotein,[12] thereby decreasing Ag recognition by T cells. Here, we examined whether the absence of TLR2 signaling affected the MHC class II surface expression of lung CD11c$^+$ and Ly-6G$^+$ cells, as such alterations might influence the outcome of CD4$^+$ T-cell differentiation.[24] At 2 weeks after infection, flow cytometric analysis of pulmonary CD11c$^+$ and Ly-6G$^+$ cells showed a significant increase in the expression levels of MHC class II in *M. tuberculosis*-infected TLR2$^{-/-}$ mice (Figure 5b). This was accompanied by significantly increased numbers of CD4$^+$ and CD8$^+$ cells in TLR2$^{-/-}$ mice at 4 and 8 weeks after infection (Figure 6, a and b). Addition-

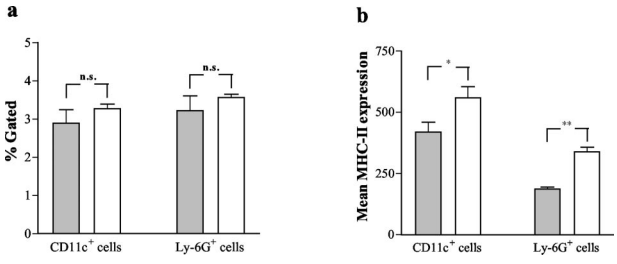


**Figure 5.** Analysis of cell-surface expression of MHC class II on CD11c$^+$ and Ly-6G$^+$ cells deficient in TLR2. C57BL/6 and TLR2$^{-/-}$ mice were aerogenically infected with 500 CFU of *M. tuberculosis*. Lung cells were obtained from control (**filled bars**) and TLR2$^{-/-}$ (**open bars**) mice at 2 weeks after infection; stained for CD11c, Ly-6G, and MHC class II; gated on monocyte population by size; and analyzed by flow cytometry. **a:** Percentages of CD11c$^+$ MHC-II$^+$ and Ly-6G$^+$ MHC-II$^+$ cells in monocyte gate from control and TLR2$^{-/-}$ mice. **b:** Expression of MHC class II on CD11c$^+$ and Ly-6G$^+$ cells in monocyte gate. Data represent the means ± SD of four mice per group. One experiment representative of two performed is shown. *, $P \leq 0.05$; **, $P \leq 0.0001$; n.s., not significant.

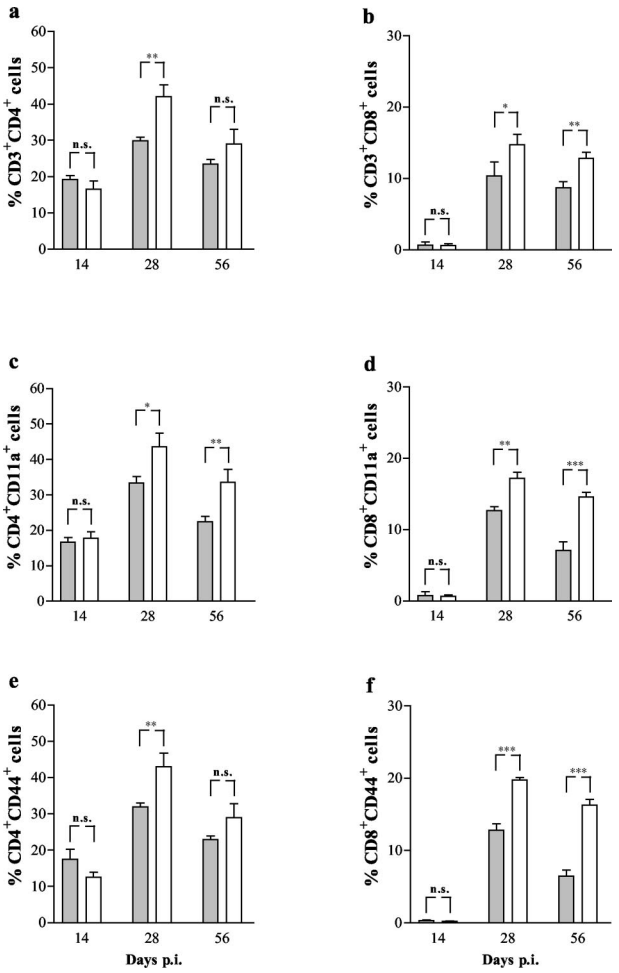


**Figure 6.** Recruitment of $CD4^+$ $CD8^+$ T cells in the lungs of wild-type and $TLR2^{-/-}$ mice during *M. tuberculosis* infection. Mice were infected with 500 CFU of *M. tuberculosis* via aerosol; lung cells were harvested 2, 4, and 8 weeks after infection; stained for CD3, CD4, CD8, CD11a, and CD44; gated on lymphocyte population by size; and analyzed by flow cytometry. **a** and **b:** Percentages of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ cells in lymphocyte gate from control (**filled bars**) and $TLR2^{-/-}$ (**open bars**) mice. **c–f:** Percentages of activated $CD4^+$ and $CD8^+$ cells in the lymphocyte gate. Data represent the means ± SD of four mice per group. One experiment representative of two performed is shown. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; n.s., not significant; days p.i., days after infection.

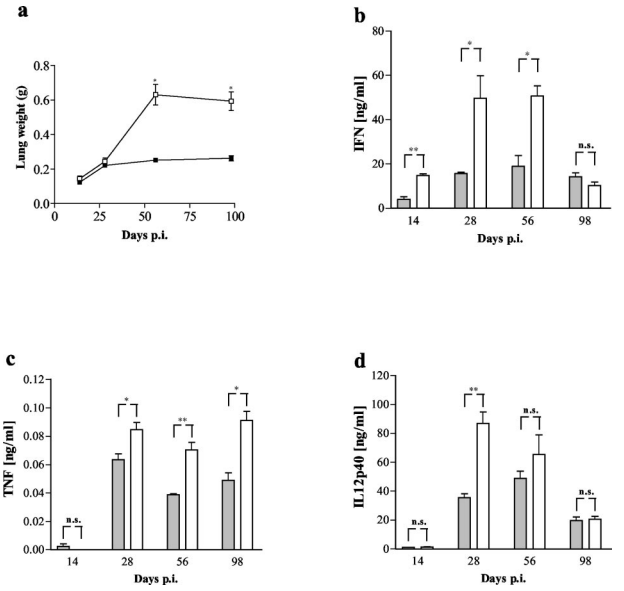


**Figure 7.** Lung weights and cytokine production in lung homogenates from aerogenically infected wild-type and $TLR2^{-/-}$ mice. Four mice per group were infected with 500 CFU of *M. tuberculosis*. Lung weights were recorded (**a**) and cytokine production (**b–d**) was measured in lung homogenates of control (■) and $TLR2^{-/-}$ (□) mice at the indicated days after infection. Concentrations of the indicated cytokines were determined by enzyme-linked immunosorbent assay. Data represent the mean ± SD of three mice per group per time point. One experiment representative of two performed is shown. *, $P \leq 0.01$; **, $P \leq 0.001$; n.s., not significant; days p.i., days after infection.

ally, increased numbers of activated $CD4^+$ and $CD8^+$ cells were recruited to the lungs of $TLR2^{-/-}$ mice at weeks 4 and 8 after infection (Figure 6; c to f).

In sharp contrast with reduced TNF-$\alpha$ and IL-12p40 production using *in vitro* infected macrophages, pulmonary IFN-$\gamma$, TNF-$\alpha$, and IL-12p40 levels were elevated in $TLR2^{-/-}$ mice after infection, which preceded the increase in bacterial burden. The absolute amount of IFN-$\gamma$, TNF-$\alpha$, and IL-12p40 detected in lung homogenates of $TLR2^{-/-}$ mice was significantly elevated when compared to congenic control mice (Figure 7; b to d). Strikingly, pulmonary levels of IFN-$\gamma$, TNF-$\alpha$, and IL-12p40 were elevated in $TLR2^{-/-}$ mice while no significant difference in lung mass was observed during the first 4 weeks when compared to wild-type controls. The elevated levels of IFN-$\gamma$ observed in the lungs of $TLR2^{-/-}$ mice at 2 and 4 weeks after infection suggested an enhanced capacity to mount a mycobacterial Ag-specific T-cell response. Thus, the pathology and elevated bacterial burden observed in the lungs of $TLR2^{-/-}$ mice appears to be associated with an increased proinflammatory cytokine response.

## *Increased Antigen-Specific T-Cell Response in* M. tuberculosis*-Infected TLR2$^{-/-}$ Mice*

The increased pulmonary IFN-$\gamma$ production suggests an enhanced type 1 cell-mediated immune response in $TLR2^{-/-}$ mice. To confirm the immune competence of the $TLR2^{-/-}$ mice, their ability to mount comparable mycobacterial Ag-specific T-cell responses after aerosol *M. tuberculosis* infection was examined. Restimulation of $TLR2^{-/-}$ splenocytes with live *M. tuberculosis* showed significantly higher levels of IFN-$\gamma$ production when compared to control mice (Figure 8a). To confirm that T cells were responsible for IFN-$\gamma$ production in the *in vitro* splenocyte assay, T cells were purified from mediastinal lymph nodes of wild-type mice at 4 weeks after infection and co-cultured with either $TLR2^{-/-}$ or wild-type peritoneal macrophages. $TLR2^{-/-}$ peritoneal macrophages pulsed with either PPD or *M. tuberculosis* restimulated the wild-type T cells more efficiently than the congenic wild-type macrophages (Figure 8b). These results demonstrate an enhanced Ag-specific T-cell response in the absence of TLR2.

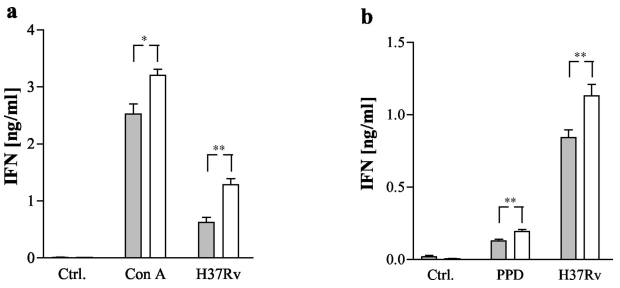


**Figure 8.** *In vitro* IFN-γ production by splenocytes and T cells from *M. tuberculosis*-infected wild-type and TLR2$^{-/-}$ mice. **a**: Control (**filled bars**) and TLR2$^{-/-}$ (**open bars**) were aerogenically infected with 500 CFU of *M. tuberculosis*. At 4 weeks after infection spleen cells were stimulated with medium alone, Con A, or live *M. tuberculosis*. Supernatants were collected 72 hours later. Data represent the means ± SD of four mice per group. **b:** Lymph nodes from C57BL/6 mice were obtained 4 weeks after aerosol infection with 500 CFU of *M. tuberculosis*. Purified T cells were stimulated *in vitro* using peritoneal macrophages from control (**filled bars**) and TLR2$^{-/-}$ (**open bars**) mice pulsed with either PPD or live *M. tuberculosis*. Supernatants were collected 96 hours later. Data represent the means ± SD of four mice per group. IFN-γ production was determined by enzyme-linked immunosorbent assay. One experiment representative of two performed is shown. *, $P \leq 0.05$; **, $P \leq 0.01$.

## *Discussion*

Microbial ligands have been shown to activate various mammalian TLRs, facilitating the transcription of genes that activate the adaptive immune response, including cytokines and various co-stimulatory molecules.[3–6,25] *M. tuberculosis*-induced TNF-α production was found to be primarily dependent on TLR signaling,[26] and subsequently TLR2 activation has been directly linked to killing of intracellular *M. tuberculosis in vitro*.[9] Here we examined the relative *in vivo* contribution of TLR2 to the generation of a protective immune response against *M. tuberculosis*. Our results demonstrate that TLR2$^{-/-}$ mice 1) are susceptible to *M. tuberculosis* infection and have reduced bacterial clearance, 2) develop a chronic pneumonia despite enhanced cell mediated immunity, 3) have an augmented production of proinflammatory cytokines, and 4) are unable to form mycobactericidal granulomas.

Our initial experiments revealed that in a standard-dose model of infection (100 CFU/mouse), TLR2$^{-/-}$ mice develop chronic pneumonia and have a reduced capacity to clear a mycobacterial infection, but survived the duration of the chronic infection for 6 months. Subsequent challenge with a higher dose (500 CFU/mouse) accelerated the progression of the disease development. Here TLR2$^{-/-}$ mice developed fatal chronic pneumonia with all of the animals succumbing to the infection within 5 months (Figure 2e). TLR2 therefore appears to play a crucial role in the host response to *M. tuberculosis* infection.

Conflicting data exists describing the role TLR4 may have in the development of an optimum immune response to *M. tuberculosis*,[13,14,26,27] while mycobacterial antigens have been reported to interact with TLR2 and a combination of additional TLRs including TLR1 and TLR6.[28,29] The critical role of TLRs in effecting an initial innate immune response has been shown in several disease models.[30–35] The fact that TLR2 has been directly implicated in effector responses of macrophages against *M. tuberculosis* expands its potential role beyond sensing and signaling. Subsequently, work done by Noss and colleagues[12] reported that *M. tuberculosis* inhibits MHC class II Ag processing of *M. tuberculosis* 19-kd lipoprotein via TLR2. Here, the TLR2-depedent down-regulation of MHC class II expression might be a mechanism used by *M. tuberculosis* to evade immune surveillance, thereby promoting chronic infection. Expanding on this observation we postulated that MHC class II expression could be elevated in the absence of TLR2. Analysis of MHC class II expression levels on CD11c$^+$ and Ly-6G$^+$ cells isolated from the lungs of TLR2$^{-/-}$ mice after an *M. tuberculosis* infection showed this to be the case (Figure 5b). This was accompanied by increased numbers of CD4$^+$, CD8$^+$, activated CD4$^+$, and activated CD8$^+$ cells in the lungs of TLR2$^{-/-}$ mice at 4 and 8 weeks after infection (Figure 6). An elevated type 1 cellular immune response was also observed after restimulation of TLR2$^{-/-}$ splenocytes (Figure 8a). Additionally, co-culturing purified T cells with *M. tuberculosis*-pulsed TLR2$^{-/-}$ peritoneal macrophages confirmed that T cells were responsible for the IFN-γ produced in the *in vitro* splenocyte assay (Figure 8b). Whether the elevated MHC class II expression levels present on TLR2$^{-/-}$ CD11c$^+$ cells is responsible for the increased numbers of CD4$^+$ and CD8$^+$ cells remains to be determined. However, locally, T cells recruited to the site of infection appear to be stimulated more by TLR2-deficient cells than T cells stimulated by their wild-type counterparts.

Although the contribution made by CD4$^+$ and CD8$^+$ cells and cytokines such as IFN-γ are critically important in the control of *M. tuberculosis* infection, TNF-α expression at the site of infection is also considered critical for determining the course of disease. TNF-α is essential for the control of murine tuberculosis *in vitro*[36–38] and *in vivo*.[23,39–41] Our *in vitro* results show that induction of TNF-α and IL-12p40 by peritoneal macrophages after infection with live *M. tuberculosis* is TLR2-dependent (Figure 1), and corroborates earlier reports in transfected cells.[8,26] Conversely, pulmonary levels of IFN-γ, TNF-α, and IL-12p40 were elevated in TLR2$^{-/-}$ mice infected with *M. tuberculosis* (Figure 7; b to d), not only during the chronic phase of infection but within the first 4 weeks as well. Thus, secretion of cytokines by resident macrophages cannot account for the elevated levels of TNF-α and IL-12p40 because their secretion by macrophages after *M. tuberculosis*-induced activation is TLR2-dependent. An alternative is that resident macrophages stimulate recruited T cells to produce more IFN-γ, as was shown to be the case *in vitro* (Figure 8b), whereas elevated TNF-α levels could be attributed to enhanced T-cell activation. Nevertheless, TNF-α has been associated with host pathology to *M. tuberculosis* infection and is a major factor in host-mediated destruction of lung tissue.[42,43] Recently Moreira and colleagues[44] showed that increased levels of pulmonary TNF-α resulted in increased pathology because of destructive inflammation. Although the bacillary load remained the same, an increase in the granuloma size in the lungs was observed. In human alveolar macrophages, induction of TNF-α has been shown to support bacterial multiplication,[45] thereby pro-

viding a mechanism for intracellular pathogens to contribute to virulence and the progression of infection.

Recently it was shown that TLR2 is not required to confer resistance to natural *M. tuberculosis* infection.[14] Our initial standard-dose aerosol infection revealed significantly elevated bacterial loads in TLR2$^{-/-}$ mice at 5 months after infection, a time point not reported on by Reiling and colleagues.[14] In terms of a high-dose *M. tuberculosis* aerosol infection, our results and those published by Reiling and colleagues show increased susceptibility in the absence of TLR2. Here, pathology was only apparent once the mycobacterial load increased in the TLR2$^{-/-}$ mice, a result observed by us in both standard and high-dose experiments.

In summary, the recognition of mycobacterial ligands by TLR2 facilitates the activation of antigen-presenting cells[46] and induction of accessory signals necessary for the optimum activation of T cells. Because of redundancy within the innate immune system's pattern recognition receptors (PRRs), it is probable that mycobacterial bacilli engage a multiligand activation pattern resulting in the differential activation of anti-bacterial effector pathways. To date, TLR2 has been shown to form heterodimers with TLR1 and TLR6, although the *in vivo* contribution of TLR1 and TLR6 required for optimal immunity of mice to *M. tuberculosis* remains to be determined. Whether TLR2 forms a heterodimer with other TLRs or forms a large receptor complex consisting of TLR1, TLR2, and TLR6 remains to be determined. Nevertheless, the present data demonstrate that TLR2 appears to play a more critical role in immunity to tuberculosis when compared to other members of the mammalian TLR family investigated thus far.

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