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Original article

Determinant role for Toll-like receptor signalling in acute mycobacterial infection in the respiratory tract

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Abstract

Toll-like receptors (TLRs) are a vital component of the innate branch of the immune system in its battle against mycobacterial infections. Extensive in vitro studies have demonstrated a role for both TLR2 and TLR4 in recognition of mycobacterial components, whereas the in vivo situation appears less clear, with results depending on the infection model. In the present work, the importance of TLR signalling in the course of mycobacterial infection was investigated in a human-like infection model using TLR-knockout mice. TLR2$^{-/-}$ and TLR4$^{-/-}$ mice infected with *Mycobacterium tuberculosis* by aerosol, or for the first time, intranasally with *Mycobacterium bovis* bacillus Calmette–Guérin (BCG), displayed increased susceptibility at an early stage of infection in the respiratory tract, while at a later stage of infection, the TLR deficiency appeared to be overcome. The higher susceptibility was correlated to impaired pro-inflammatory responses to BCG components, and reduced induction of antibacterial activity by infected macrophages from TLR2$^{-/-}$ mice, and to a lesser extent from TLR4$^{-/-}$ mice. These findings demonstrate a role for TLR signalling in protection against mycobacterial infection specifically in the respiratory tract at the acute phase, whereas the TLR deficiency can be compensated at a later stage of infection.

Keywords: Bacillus Calmette–Guérin (BCG); Lung; Toll-like receptor; Infection

1. Introduction

Mycobacterium tuberculosis, the causative agent of infectious tuberculosis, is an intracellular pathogen capable of surviving and persisting within host mononuclear cells. An efficient immune response against the pathogen is critically dependent on rapid detection of the invading organism by the innate immune response and the activation of the adaptive immune response. Most humans are resistant to tuberculosis, presumably because they are able to generate a successful immediate immune response against the pathogen, when early recognition of *M. tuberculosis* by the innate immune system plays an important role. Thus, an effective protective immune response to *M. tuberculosis* relies on both innate and adaptive branches of the immune system [1].

Toll-like receptors (TLRs) play a central role in innate immunity by recognising conserved motifs predominantly found in microorganisms, and thereby discriminating between pathogens and self [2–4]. Stimulation of TLRs induces production of antimicrobial peptides and proinflammatory cytokines through NF-κB and other signalling pathways, thereby linking innate and adaptive immunity [4–6]. Signal transduction by most TLRs, with the exception of TLR3, requires the adapter molecule myeloid differentiation factor 88 (MyD88) [7–9], which links TLR recognition with activation of the interleukin (IL)-1 receptor/IL-1 receptor-associated kinase (IRAK)

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pathway and tumour necrosis factor (TNF) receptor-associated factor, translocation of NF-κB, and gene transcription [10]. Mice deficient for MyD88 fail to generate proinflammatory responses when stimulated through TLRs [7,8,11] and demonstrate high susceptibility to several infectious agents, including mycobacteria [12–16].

M. tuberculosis has a particular tropism for the lungs, pulmonary tuberculosis being the most common form of disease [17]. Aerogenic infection of the lungs is exceedingly pathogenic, probably due to evasion of adequate innate defences in the respiratory mucosa. Progression of disease is contained by local innate and adaptive immune responses, culminating in the formation of granuloma around the infective loci [18]. Alveolar macrophages are the first cells in the lungs that become infected with *M. tuberculosis*, and macrophages respond to infection by initiating the inflammatory cascade needed for the recruitment of leukocytes to sites of infection and for their own activation [19]. TLR2, TLR4 and, more recently, TLR1 or TLR6 that heterodimerise with TLR2, have been implicated in the recognition of mycobacterial antigens [20,21]. A predominant role for TLR2 in immune recognition of *M. tuberculosis* has been demonstrated. Whole bacteria, bacterial cell wall, or components such as lipomannan, lipoarabinomanan, 19-kDa lipoprotein and phosphatidylinositol mannosides activate macrophages and dendritic cells through TLR2 and TLR4 [22–26]. Moreover, TLR2 activation leads to killing of intracellular tubercle bacilli in both human and mouse macrophages and induction of proinflammatory responses [1,27,28]. Proinflammatory cytokines such as TNF are required for the control of bacillary growth and the protective granulomatous response [29–31]. The importance of interferon (IFN)-γ to protect against tuberculosis through its capacity to activate macrophages has been well established in the murine system [32,33], but knowledge about the IFN-γ activation of anti-mycobacterial activity in human macrophages is more limited. However, the enhanced susceptibility to mycobacterial infections in individuals functionally deficient in IFN-γ signalling provides strong evidence that this cytokine also plays a significant role in defence against *M. tuberculosis* in humans [34,35].

Several in vitro studies have demonstrated an active role of TLR2 in mycobacterial recognition and immune responses [25,26,36]; however, in vivo studies have revealed diverse results [27,37,38]. Moreover, the contribution of TLR4 to host defence in vivo appears to differ, depending on the route of infection and size of the inoculum [39–41].

In this work we have investigated the role of TLRs in the early and late stages of infection in the respiratory tract using *M. tuberculosis* and *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) as infectious agents. Our results show that TLR knockout mice are more susceptible than wild-type mice at an early stage of infection and that $TLR2^{-/-}$ mice are more susceptible than $TLR4^{-/-}$ or wild-type mice. TLR4 signalling was also shown to play a role in protection against mycobacterium infection, although not to the same degree as TLR2. The higher susceptibility was correlated to impaired pro-inflammatory responses to BCG and BCG components and reduced induction of anti-bacterial activity by infected macrophages.

2. Materials and methods

2.1. Mice

The studies were performed using C57BL/6 (Taconic, Ejby, Denmark), $TLR2^{-/-}$ mice [16], and $TLR4^{-/-}$ mice [42] with a C57BL/6 background, 7–12 weeks of age. Breeding pairs of the TLR-knockout mice, which had been backcrossed at least six times to C57BL/6 mice, were obtained from Karolinska Institute, Sweden, with the permission of S. Akira (Osaka University, Japan), and kept at the Animal Department at the Arrhenius Laboratories, Stockholm University, Sweden. All experiments performed were in accordance with the relevant ethical committee in Stockholm. Mice were supervised every day, and sentinel animals were used to check specific-pathogen-free conditions in the facility.

The *M. tuberculosis* aerosol-infection experiments were performed using specific-pathogen-free, 6- to 8-week-old, C57BL/6 mice, obtained from Harlan Iberica S.L. (Sant Feliu de Codines, Catalonia, Spain), and $TLR2^{-/-}$ and $TLR4^{-/-}$ mice. The animals were kept under controlled conditions in a BL3 high security facility with sterile food and water ad libitum and weighed once a week. They were supervised every day under a protocol paying attention to weight loss, apparent health (bristled hair and wounded skin) and behaviour (signs of aggressiveness or isolation). Animals were euthanised with halothane (Fluothane, Zeneca Farma, Madrid, Spain) overdose to avoid any suffering. All experimental proceedings were approved and supervised by the Animal Care Committee of "Germans Trias i Pujol" University Hospital in agreement with the European Union Laws for protection of experimental animals.

2.2. Experimental infections

M. bovis BCG (Pasteur strain) was genetically tagged with the green fluorescent protein from the jellyfish *Aequorea victoria*. The organisms, prepared by previously described genetic manipulation procedures for mycobacteria [43], were obtained from R. Reljic, Guy's Hospital Campus of Kings College, London. The bacteria were cultured in Middlebrook 7H9 medium over a period of 7–10 days. Seed lots of bacteria were stored at −70 °C. Mice were infected with 10^6 colony-forming units (CFU) of BCG in 30 μl PBS through the intranasal (i.n.) route or in 50 μl PBS through the intravenous (i.v.) route of infection, under anaesthesia with isofluorane (Baxter Medical AB, Kista, Sweden). At weeks 1, 3 and 8 after infection, mice were killed, and the numbers of viable bacteria in the lungs or spleen were determined. The lungs and spleens from individual mice were homogenised in 2 or 3 ml, respectively of PBS supplemented with 0.15 M NaCl and 0.05% Tween-80 (v/v). Serial dilutions of the tissue homogenates were plated on Middlebrook 7H11 agar plates. The numbers of CFU were determined after 3–4 weeks of incubation at 37 °C.

M. tuberculosis H37Rv was grown in Proskauer Beck medium containing 0.01% Tween-80 to mid-log phase and stored at −70 °C. Mice were placed in the exposure chamber of an airborne infection apparatus (Glas-col Inc., Terre Haute, IN,

USA). The nebuliser compartment was filled with 7 ml of the bacillar suspension at a previously calculated concentration to provide an approximate uptake of 20 viable bacilli within the lungs. The numbers of viable bacteria in the left lung, and spleen homogenates on weeks 3 and 8 were followed by plating serial dilutions on nutrient Middlebrook 7H11 agar and counting bacterial colonies after 21 days incubation at 37 °C.

2.3. Antigens

M. bovis BCG, obtained from Serum Statens Institute (SSI, Copenhagen, Denmark), was grown on Middlebrook 7H11 agar for 2 weeks at 37 °C. To produce BCG components, the cells were scraped from the plates; one part of them was collected, autoclaved for 20 min at 121 °C, diluted in PBS and aliquoted (heat-killed BCG, (hkBCG)). From the rest of the cells, non-covalently attached lipids and glycolipids were extracted first with chloroform/methanol (1:2, v/v), and then with chloroform/methanol (2:1, v/v), under continuous stirring for 2 days for each extraction. Pooled organic extracts, containing the lipids, were partitioned with chloroform/methanol/water (8:4:2, v/v/v); the organic phase was separated and evaporated to dryness. This lipidic fraction was diluted in PBS [44] and aliquoted (cell wall fraction; CW). The bacterial residue was resuspended in PBS and aliquoted under sterile conditions (delipidated BCG; dBCG). All the antigens were kept at −70 °C until use. Purified protein derivative (PPD) was obtained from Serum Statens Institute (SSI, Copenhagen, Denmark).

2.4. Generation of bone marrow-derived macrophages

Bone marrow-derived macrophages were obtained as previously described [45,46]. Briefly, mice were euthanised, and the femur and tibia of the hind legs were dissected. Bone marrow cavities were flushed with cold, sterile PBS. The bone marrow cells were washed and resuspended in DMEM containing glucose and supplemented with 10% FCS, 20% L929 cell-conditioned medium (as a source of M-CSF), 100 μg/ml streptomycin, and 100 U/ml penicillin. Bone marrow cells were plated in 24-well plates and incubated for 7 days at 37 °C, 5% CO_2, with replacement of medium every second day. Before use, bone marrow-derived macrophage cultures were washed vigorously to remove non-adherent cells.

2.5. In vitro restimulation assay

Mononuclear cells were collected from lungs 1 and 3 weeks after i.n. BCG infection. Briefly, the lungs were homogenised in balanced salt solution using a glass homogeniser. The cell suspension was thereafter subjected to gradient separation using Lympholyte M (Cederlane Laboratories, Ontario, Canada). After centrifugation at 1500 × *g* for 20 min at 24 °C, the interface was collected and the cells washed twice in balanced salt solution. Spleens were removed from BCG-infected mice, 1 and 3 weeks after infection, and single cell suspensions were prepared. Lung mononuclear cells (2×10^5 cells/well) and splenocytes (5×10^5 cells/well) were cultured in 96-well plates for 48 h at 37 °C, under stimulation with BCG (2:1) or with hkBCG, dBCG, CW, and PPD (5 μg/ml). Supernatants were thereafter collected and analysed for cytokine production.

2.6. Cytokine ELISA

Quantification of IFN-γ (Mabtech, Stockholm, Sweden) and TNF levels (R&D Systems, Minneapolis, MN, USA) in supernatants collected from cell cultures was assayed by ELISA, according to the manufacturer's recommendations, with some modification. Streptavidin conjugated to alkaline phosphatase, instead of horseradish peroxidase, was used at a 1:1000 dilution. The enzyme-substrate reaction was developed using *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO, USA). Optical density was read in a multiscan plate reader at 405 nm and concentrations were determined after background value correction.

2.7. Measurement of in vitro macrophage function

To assess macrophage responses, cultured bone-marrow-derived macrophages were stimulated with heat-killed BCG or infected with BCG with a multiplicity of infection of 2:1 or 5:1, for 4 h. After vigorous washing of the cells to remove all free bacteria, fresh medium was added. Culture supernatants were collected after 24 h of stimulation or infection and assayed by ELISA for production of TNF-α, according to the previously described assay (Cytokine ELISA). Levels of intracellular growth of bacteria in BCG-infected macrophages were analysed by lysing cells with 0.01% Triton X-100, 48 and 72 h after infection, and plating cell lysates on Middlebrook 7H11 agar plates to determine bacterial CFU.

2.8. Histology and morphometry

Procedures have been described in previous works [47]. Briefly, two right lung lobes from each mouse were fixed in buffered formalin and subsequently embedded in paraffin. Every sample was stained with haematoxylin-eosin. For histometry, 5-μm thick sections from each specimen were stained with haematoxylin–eosin and photographed at 6× using a Stereoscopic Zoom SMZ800 microscope (Nikon, Tokyo, Japan) and a Coolpix 990 digital camera (Nikon). Sections of eight lung lobes were studied in each case. A sequence of appropriate software programs was used: Scion Image (Scion Corp., Frederick, MA, USA) and Photoshop 5.0 (Adobe Systems Inc., San José, CA, USA), to determine the area of each single lesion and the total tissue area on photomicrographs at each time point. Sections were blindly evaluated in order to get a more objective measurement.

2.9. mRNA quantification

The procedures are described elsewhere [48]. In short, total RNA from the middle right lobe of the lungs of aerosol-infected mice was extracted with a commercial phenol-chloroform

method, RNAzol (Cinna/Biotecx, Friendswood, TX, USA). After a DNase treatment with DNA-free kit (Ambion, Woodward Austin, TX, USA), a denaturing agarose gel was used to assess the stability of RNA. Five micrograms of RNA was reverse transcribed using a Superscript RT kit (Gibco BRL, Grand Island, NY, USA) following the manufacturer's recommendations to obtain cDNA. The quantitative analysis for IFN-γ, regulated on activation, normal T-cell expressed and secreted (RANTES), inducible nitric oxide synthase (iNOS) and TNF was performed using a LightCycler™ System (Roche Biochemicals, Idaho Falls, ID, USA). Real-time PCR was carried out in glass capillaries to a final volume of 10 μl in the presence of 1 μl of 10× reaction buffer (Taq Polymerase, dNTPs, $MgCl_2$, SYBRGreen, Roche Biochemicals), 1 μl of cDNA (or water as negative control, which was always included), $MgCl_2$ to a final concentration of 2–5 mM, and primers to a final concentration of 0.5 μM. A single peak was obtained for each PCR product by melting-curve analysis and only one band of the estimated size was observed on the agarose gel. Hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA expression was analysed for every target sample to normalise for efficiency in cDNA synthesis and RNA loading. A ratio based on the HPRT mRNA expression was obtained for each sample.

2.10. Statistical analysis

Differences between TLR knockout mice and wild-type mice were compared by Mann–Whitney *U*-test. The level of significance was set at $P < 0.05$.

3. Results

3.1. Impaired control of intranasal BCG infection in TLR2$^{-/-}$ mice during the early phase of infection

TLRs are believed to play an essential role in the innate and adaptive immune response to mycobacteria, and triggering of different TLRs induces various anti-bacterial activities.

We first investigated the role of TLR signalling for control of bacterial growth after infection with BCG via the respiratory tract using TLR-knockout mice. Mice were infected i.n. with 10^6 CFU of BCG, and bacterial burden in the lungs was assessed. Our results revealed a significantly higher ($P < 0.01$) bacterial load in the lungs of infected TLR2$^{-/-}$ mice compared to wild-type mice during the early phase of infection, 1 week and 3 weeks post-infection (Fig. 1). No difference between those two experimental groups was seen at the later phase of infection, week 8 after infection. The role of TLR4 signalling appears less important for control of bacterial growth in the lungs after BCG infection, both during early and late stages of infection, since TLR4$^{-/-}$ mice displayed bacterial loads similar to those of wild-type mice at the two time points analysed (Fig. 1). To assess whether the respiratory tract is more dependent on innate immunity, and TLR signalling for protection against mycobacteria infection, we investigated the outcome using the i.v. route of infection. Viable bacterial counts in lungs and spleen after i.v. infection with BCG revealed no differences between TLR2$^{-/-}$ and wild-type mice, either in the lungs or spleens, at any time point investigated (Fig. 2). The same results were also obtained for TLR4$^{-/-}$ mice (data not shown).

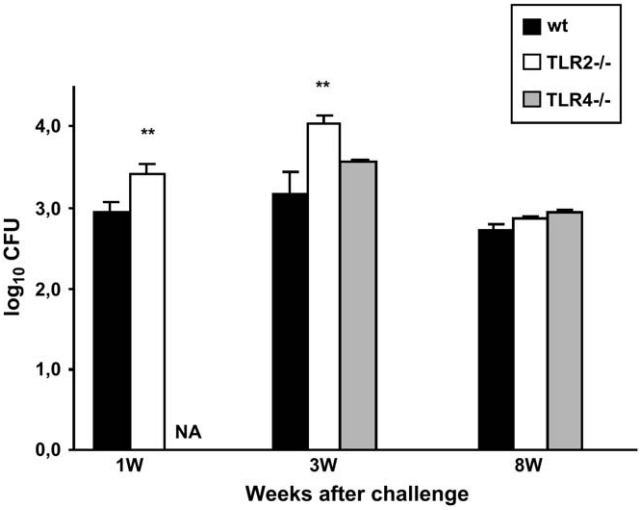

Fig. 1. Impaired control of BCG infection in TLR2$^{-/-}$ mice after i.n. infection. Bacterial load in the lungs of infected mice was determined at different time points after i.n. infection. Results are expressed as mean $\log_{10}$ of CFU ± S.E.M. and were obtained from groups of six mice. $^{**}P < 0.01$ vs. wild-type mice. A representative of two different experiments is shown. NA, not available.

3.2. BCG-infected TLR2$^{-/-}$ mice displayed impaired proinflammatory responses

The finding of a higher bacterial load in the lungs of TLR2$^{-/-}$ mice may be a consequence of an impaired adaptive immune response in these mice. To test this, isolated mononuclear cells from the lungs of infected mice were assessed for their ability to produce IFN-γ and TNF after in vitro stimulation. The results showed that TLR2$^{-/-}$ cells had a significantly reduced production of IFN-γ when stimulated with heat-killed BCG ($P < 0.01$) and BCG ($P < 0.05$) 1 week after infection (Fig. 3A). Three weeks after infection, these differences between wild-type and TLR2$^{-/-}$ mice were sustained. Analysis of TNF production revealed the same pattern. TNF production by TLR2$^{-/-}$ cells was significantly reduced compared to wild-type cells at both 1 week ($P < 0.01$), and 3 weeks ($P < 0.05$) after infection, using both stimuli (Fig. 3B).

To further test the possibility of impaired adaptive immune responses in TLR$^{-/-}$ mice, and to determine whether this was a local or systemic phenomenon, we also performed an antigen-dependent splenocyte restimulation assay. The results of restimulation of splenocytes from infected mice, using BCG, heat-killed BCG, dBCG, CW and PPD as stimuli are listed in Table 1. Production of both IFN-γ and TNF by TLR2$^{-/-}$ splenocytes was undetectable or minimal. Additionally, TLR4$^{-/-}$ splenocytes demonstrated a reduced production of the cytokines, although the reduction was not as striking as for TLR2$^{-/-}$ splenocytes. The main reduction detected concerned IFN-γ production.

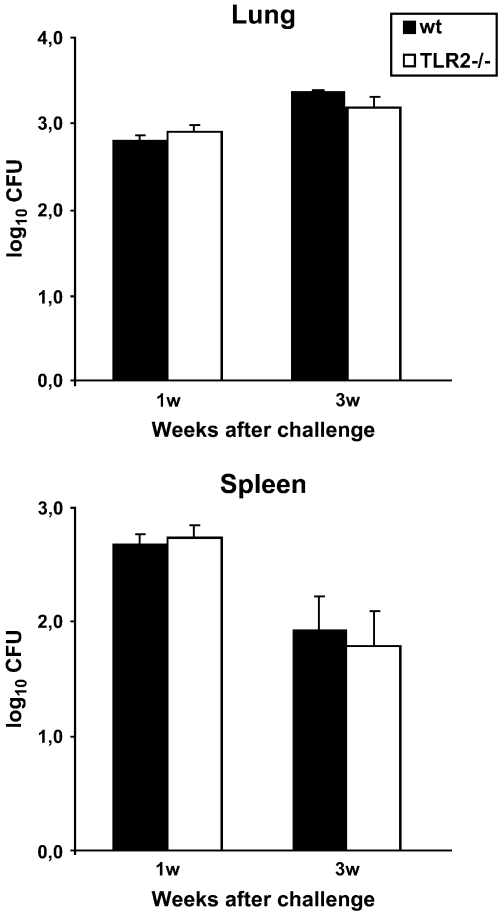

Fig. 2. Control of BCG infection in TLR2$^{-/-}$ mice using the i.v. route of infection. Bacterial counts were assessed in the lungs and spleens of infected mice at weeks 1 and 3 post-infection. Results are expressed as mean $\log_{10}$ of CFU ± S.E.M. and were obtained from groups of six mice. A representative of two different experiments is shown.

3.3. TLR2-mediated signalling confers anti-mycobacteria activity in macrophages

Macrophages are the preferred habitat of mycobacteria and play a key role in the control of infection. Production of cytokines by these cells is crucial to initiate cellular immune responses against mycobacteria. To test whether the higher bacterial load found in lungs of TLR2$^{-/-}$ mice could be a consequence of selective growth advantage of BCG in TLR2$^{-/-}$ macrophages, bone marrow-derived macrophages from wild-type and TLR-deficient mice were infected with BCG in vitro. Measurement of intracellular growth of BCG revealed a significantly increased ($P < 0.01$) growth of bacteria in TLR2$^{-/-}$ macrophages compared to wild-type macrophages at both time points investigated (Fig. 4A). TLR4$^{-/-}$ macrophages appeared more capable of controlling intracellular growth of BCG than TLR2$^{-/-}$ macrophages. Yet TLR4$^{-/-}$ macrophages displayed significantly higher ($P < 0.05$) intracellular bacterial growth 48 h after infection compared to wild-type macrophages, whereas by 72 h after infection no difference between TLR4$^{-/-}$ and wild-type macrophages could be detected.

To further delineate the mechanistic details behind the reduced resistance to BCG infection and impaired proinflammatory response in TLR2$^{-/-}$ mice, bone marrow-derived macrophages from knockout, and wild-type mice where compared for their production of TNF in vitro. Upon infection with BCG, or stimulation with heat-killed BCG, macrophages from TLR2$^{-/-}$ mice produced significantly lower ($P < 0.01$) levels of TNF compared to wild-type mice (Fig. 4B). The same result was obtained for TLR4$^{-/-}$ macrophages, although the levels of production were higher compared to TLR2$^{-/-}$ macrophages. Moreover, preliminary results have revealed a reduced iNOS expression in TLR2$^{-/-}$ macrophages upon BCG infection when compared with wild-type macrophages (data not shown).

3.4. TLR2 and TLR4 contribute to the control of M. tuberculosis *infection in lungs at the early stage of infection*

After demonstrating a role for TLR2 in protective immune responses against the attenuated mycobacterium BCG, we wanted to investigate whether TLR signalling is important for control of infection with virulent *M. tuberculosis*. TLR-knockout and wild-type mice were aerogenically infected with virulent *M. tuberculosis*. Bacterial growth in lungs and spleens was assessed and there proved to be significantly higher ($P < 0.01$) CFUs in the lungs of infected TLR2$^{-/-}$ and TLR4$^{-/-}$ mice, at the early stage, 3 weeks post-infection, compared to wild-type mice (Fig. 5A). Similarly to the results from BCG-infected mice, no differences between the groups could be detected in the lungs at the later stage of infection, by week 8. The impaired control of bacterial growth found in the TLR-deficient mice was restricted to the lungs, since bacterial counts in the spleens were similar among all experimental groups at both time points investigated (Fig. 5B).

3.5. Histological analysis

Since the granulomatous response plays a critical role in controlling mycobacterial dissemination, we examined the granulomatous infiltration in the lungs of *M. tuberculosis*-infected mice. By week 3 post-infection, no significant differences between the mouse strains were demonstrated (Fig. 6). However, a difference in granulomatous infiltration was shown by week 8 post-infection, where both TLR2$^{-/-}$ and TLR4$^{-/-}$ mice displayed significantly higher ($P < 0.05$) percentage of infiltration than wild-type mice. Analysis of the nature of the granulomatous infiltration in the lungs revealed no qualitative differences between the experimental groups (data not shown).

3.6. Comparable expression of protective mediators in the lungs of M. tuberculosis*-infected TLR2$^{-/-}$, TLR4$^{-/-}$, and wild-type mice*

To assess whether the increased susceptibility to *M. tuberculosis* infection initially seen in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice was associated with impaired cytokine production in the lungs,

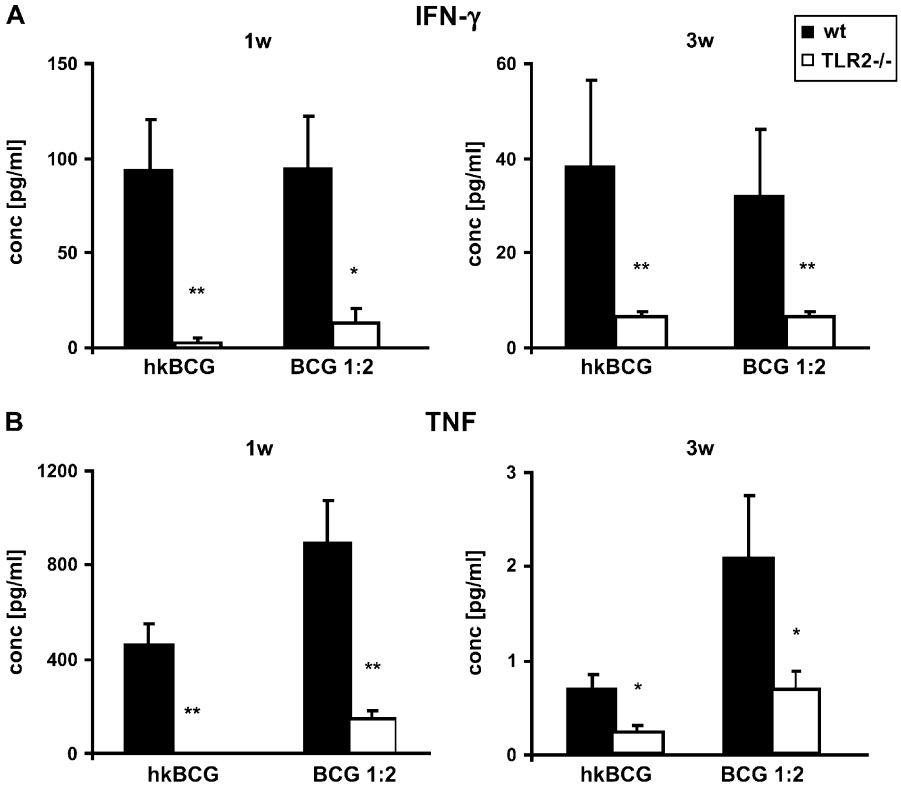

Fig. 3. Reduced IFN-γ and TNF production in the lungs of TLR2$^{-/-}$ mice after BCG infection. Mononuclear cells were isolated from the lungs 1 and 3 weeks after infection and analysed for their ability to produce IFN-γ (A) and TNF (B) upon stimulation with live BCG and heat-killed BCG in vitro using ELISA. Data are expressed as mean ± S.E.M. from six mice per group. *$P < 0.05$, **$P < 0.01$ vs. wild-type mice. A representative of two different experiments is shown.

we compared mRNA expression of IFN-γ, TNF, RANTES, and iNOS from lung tissues. The results revealed no differences between the knockout mice and wild-type mice at either experimental time point (Fig. 7).

4. Discussion

Tuberculosis is primarily a pulmonary infectious disease, and efficient immune responses in the lungs play an important role for protection against infection [49]. Emerging evidence supports the concept that innate immune responses are vital for the host defence against *M. tuberculosis* [50]. TLRs represent critical pathogen-recognition receptors whose signals lead to generation of important effector responses, including Th1 responses [11]. In this study we show a role for TLR signalling in the acute phase of infection in the respiratory tract, using *M. tuberculosis* and BCG as infectious agents in infection models closely resembling natural infection.

We demonstrate for the first time increased susceptibility to i.n. BCG infection in TLR2$^{-/-}$ mice, compared to TLR4$^{-/-}$ or wild-type mice at the acute phase of infection. Our results indicate that deficiency in TLR2 signalling can be compensated for at later stages, possibly by signalling through other TLRs or related signalling pathways [51–54]. Infection with *M. tuberculosis* additionally revealed higher susceptibility to infection in TLR4$^{-/-}$ mice compared to wild-type mice [37,38,55]. Possible explanations for the different results obtained with *M. tuberculosis* and BCG could be due to the size of inoculums administered or the nature of the infectious agent. In agreement with our findings with BCG, the difference in susceptibility was more pronounced at the acute phase of *M. tuberculosis* infection. The fact that the bacterial load in the lungs of the TLR$^{-/-}$

Table 1
Cytokine production by splenocytes from BCG-infected mice 3 weeks post-infection, after restimulation in vitro

Stimulus	IFN-γ			TNF		
	WT	TLR2$^{-/-}$	TLR4$^{-/-}$	WT	TLR2$^{-/-}$	TLR4$^{-/-}$
BCG	29814 ± 6610	127 ± 104**	3606 ± 2868**	563 ± 220	32 ± 42**	125 ± 60*
hkBCG	3900 ± 225	N.D.**	818 ± 407**	986 ± 149	N.D.**	653 ± 60**
dBCG	2983 ± 422	5 ± 0**	268 ± 123**	410 ± 81	N.D.**	254 ± 64**
CW	1343 ± 448	N.D.**	8 ± 16**	360 ± 108	N.D.**	60 ± 63**
PPD	1026 ± 362	23 ± 39**	8 ± 12**	86 ± 36	8 ± 19**	N.D.**

Results are expressed in pg/ml of cytokine produced. The means ± S.E.M. were obtained from 5–6 mice per group. *$P < 0.05$, **$P < 0.01$ vs. wild-type mice using Mann–Whitney *U*-test. A representative of two different experiments is shown. N.D., not detected.

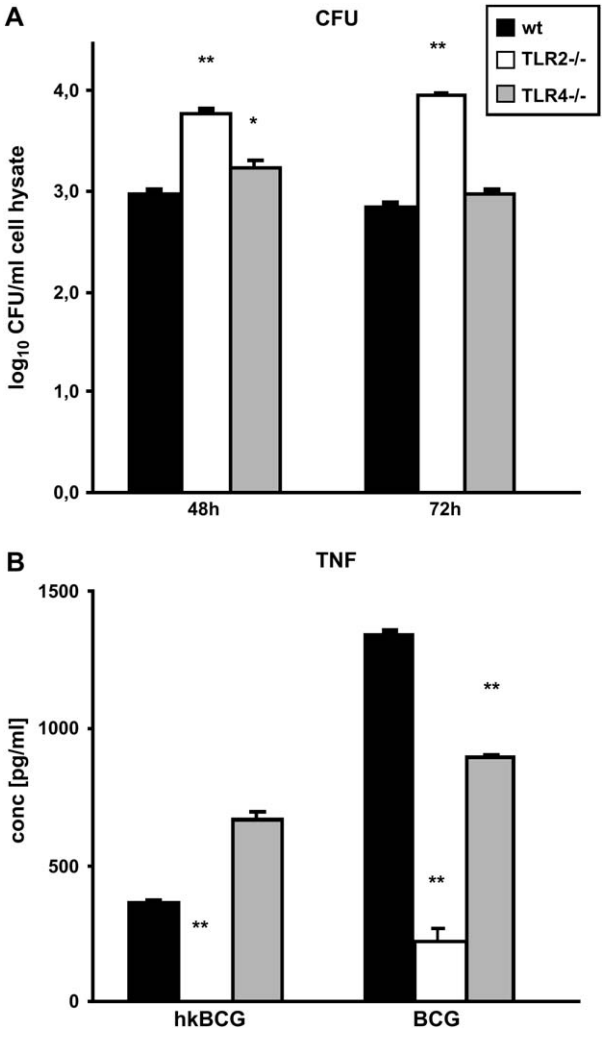

Fig. 4. Impaired induction of bactericidal activity and proinflammatory cytokine production in TLR2-deficient macrophages. Bone marrow-derived macrophages were analysed for their capability for intracellular killing of bacteria (A) and TNF production (B). (A) Macrophages (5×10^5 cells/well) were infected with live BCG (5:1), and lysates from infected cells were analysed for viable bacterial counts, 48 and 72 h after infection. Results are expressed as mean $\log_{10}$ of CFU ± S.E.M. from quadruple culture wells. (B) Alternatively, macrophages were infected with live BCG (2:1) or stimulated with heat-killed BCG, and supernatants were collected after 48 h and assessed for cytokine quantities. Data are expressed as mean values ± S.E.M. from triplicate culture wells. $^*P < 0.05$, $^{**}P < 0.01$ vs. wild-type mice. A representative of three different experiments is shown.

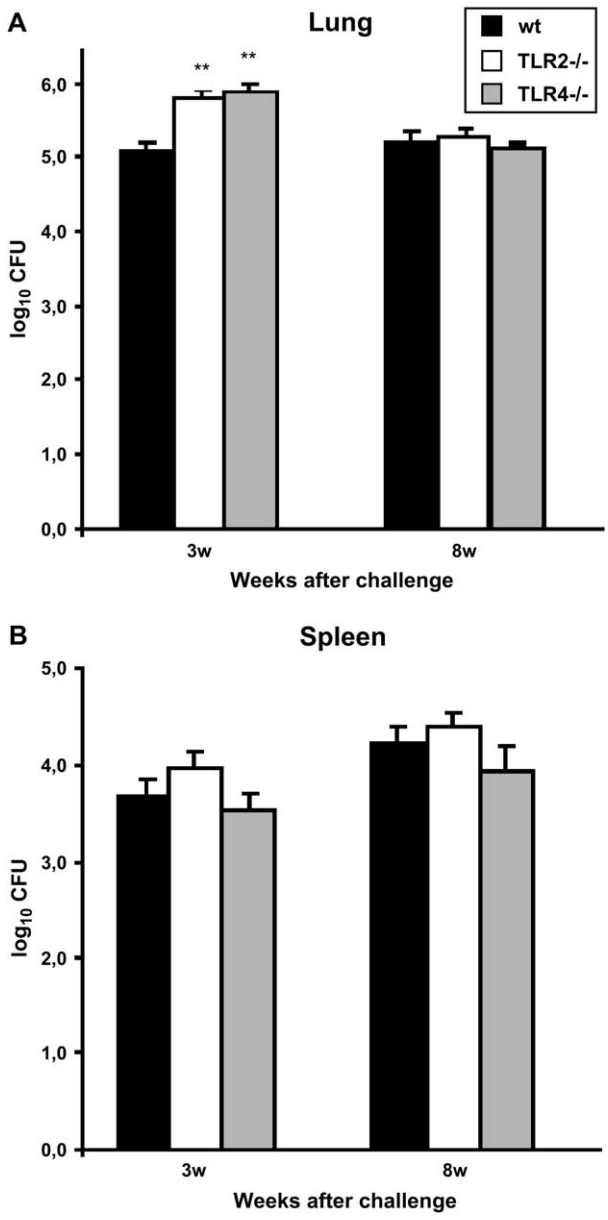

Fig. 5. TLR-deficient mice display increased susceptibility to aerosol infection with virulent *M. tuberculosis*. Bacterial counts were determined in the lungs (A) and spleens (B) of mice 3 and 8 weeks after aerosol infection with *M. tuberculosis*. Results are expressed as mean $\log_{10}$ of CFU ± S.E.M. obtained from groups of 4–5 mice. $^{**}P < 0.01$ vs. wild-type mice. A representative of two different experiments is shown.

mice decreased from early to late infection further, not seen in the wild-type mice, supports the concept of the importance of TLR signalling in the early phase of infection.

The use of the i.v. route of infection with BCG revealed indistinguishable bacterial counts in both lung and spleen between wild-type and $TLR^{-/-}$ mice. As opposed to the lungs, *M. tuberculosis* aerosol infection resulted in no detectable differences in viable bacterial counts in the spleen. These observations suggest that TLR-dependent effector mechanisms for control of bacterial growth are especially important in the respiratory tract. This may be due to differences in effector cell populations in the lung compared to the spleen. Support for this comes from Nicolle et al., who previously demonstrated a redundant role for both TLR2 and TLR4 for control of BCG infection when infecting i.v. [56]. Using the intraperitoneal route of infection, higher susceptibility to BCG infection in TLR2-deficient mice compared to wild-type mice has been reported, whereas the importance for TLR4 was only evident after increasing the inoculation dose [41].

The first encounter with mycobacterium in the lungs is made by alveolar macrophages, which provide an important cellular niche during infection. The higher susceptibility to mycobacteria infection observed in $TLR^{-/-}$ mice could be a consequence of defective macrophage activation, which represents the foremost effector cell of innate response during early infection [57],

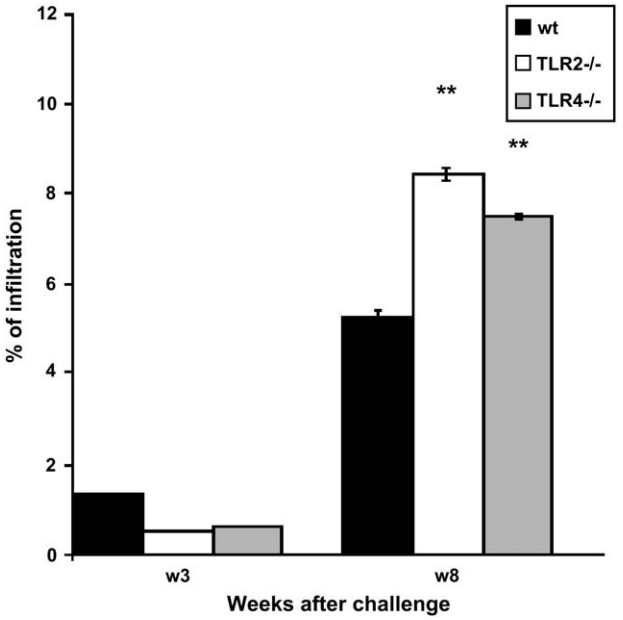

Fig. 6. Histological analysis of *M. tuberculosis*-infected mice. Granulomatous infiltration in the lungs of *M. tuberculosis*-infected mice was assessed by analysing two lung lobes from each mouse ($n = 5$). Data are expressed as percentages, calculated by dividing the granuloma-involved area by the total tissue area. *$P < 0.01$ vs. wild-type mice. A representative of two different experiments is shown.

leading to a selective growth advantage of BCG in TLR$^{-/-}$ macrophages. We detected reduced capability of controlling intracellular growth of BCG in macrophages derived from TLR2$^{-/-}$ mice compared to wild-type macrophages. Growth of BCG in TLR4$^{-/-}$ macrophages was not equally affected. This is in accordance with recent data demonstrating defective phagosome maturation into late endosomal and lysosomal stages in TLR-deficient macrophages [58]. TLR activation promotes the formation of critical anti-microbial mechanisms against intracellular pathogens, such as reactive oxygen species and reactive nitrogen species [25,36]. In accordance with this, we have preliminary data showing reduced expression of iNOS in BCG-infected TLR2-deficient macrophages compared to wild-type cells.

Defective capability of intracellular killing, preferentially in TLR2$^{-/-}$ macrophages, was correlated with impaired production of TNF, which is vital for containment of mycobacterial infections [29,30,59]. TNF production was completely abolished in BCG-infected MyD88-deficient macrophages (data not shown). Our results suggest that the majority of cytokine-inducing, and bactericidal activity in macrophages by viable BCG is dependent on TLR2 signalling, in line with data reporting TLR2 as the principal mediator of the proinflammatory signal induced by *M. tuberculosis* [26]. Moreover, our findings indicate that TLR2-mediated activity was transduced through MyD88; however, redundancy in the signalling pathway cannot be excluded [60,61]. The complete inhibition of TNF production by MyD88-deficient macrophages, as opposed to TLR2-deficient cells, infected with BCG, moreover, suggests involvement of other TLRs.

Higher susceptibility to BCG infection in the TLR2$^{-/-}$ mice was associated with defective mycobacterium-induced proinflammatory responses. By using different mycobacterial stimuli, known to contain various ligands to TLR2 and TLR4 (Table 2), an impaired production of IFN-γ and TNF

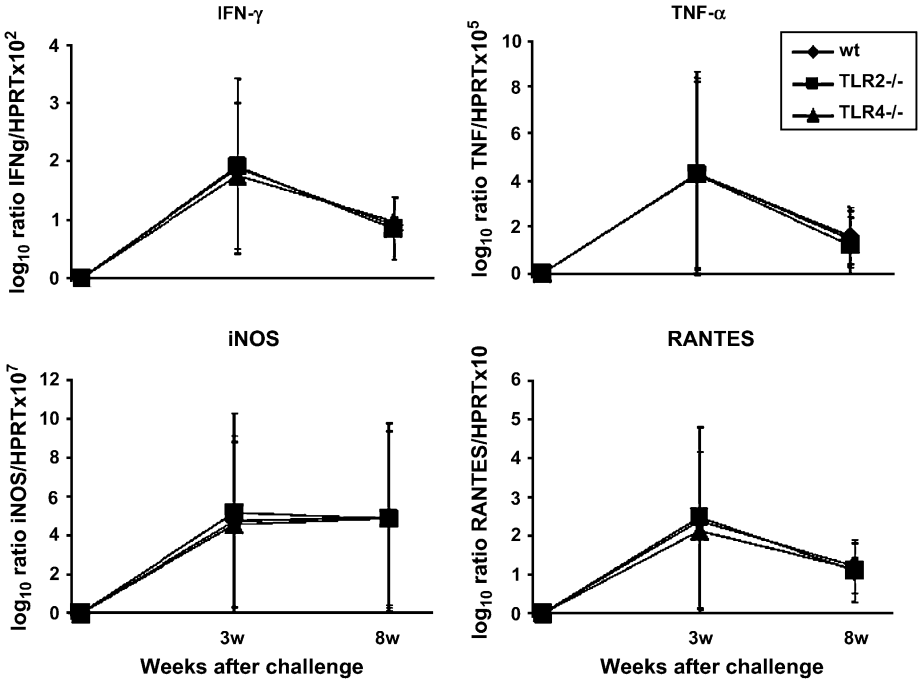

Fig. 7. Expression of protective mediators in lungs of infected mice. mRNA from the lungs of mice infected with *M. tuberculosis* was prepared and quantified by real-time PCR. Each expression level is normalised to HPRT mRNA expression. The mean ± S.E.M. of 4–5 individual mice is shown. A representative of two different experiments is shown.

Table 2
TLR ligands in mycobacterial components

Fraction	Antigen	TLR	Reference
dBCG	PGN	2, 4	[62]
	19 kDa lipoprotein	2	[36]
	LAM	2	[63]
	LM	2	[24,64,65]
CW	19 kDa lipoprotein	2	[36]
	PIM	2, 4	[23,39]

PGN, peptidoglycan; LAM, lipoarabinomannan; LM, lipomannan; PIM, phosphatidilinositol-mannoside.

in TLR2$^{-/-}$ mice was demonstrated, and to a lower extent in TLR4$^{-/-}$ mice also. Our data suggest that TLR2 signalling constitutes a major signalling pathway for live BCG and the other BCG products, whereas the strongest TLR4 ligands are contained in the PPD and CW fractions. At present we do not have any data on the restimulatory capacity of BCG recombinant antigens acting outside the TLRs, which could have provided more direct information on the status of the T cells. However, our data are compatible with less pronounced T-cell priming in the TLR2$^{-/-}$ mice.

In contrast to in vitro restimulation, ex vivo studies of the expression of protective mediators in the lungs of *M. tuberculosis*-infected mice revealed normalised levels of IFN-γ, TNF, RANTES and iNOS in TLR-knockout mice. A possible explanation may be that by week 3 post-infection, the impairment has been compensated by other mechanisms. Support for this idea comes from histological analysis, showing that the initially increased bacterial bulk in the lungs of the TLR-knockout mice triggered a higher granulomatous infiltration. At the later stage of infection, this infiltration was consequently higher in lungs of the knockout mice compared to wild-type mice, demonstrating the importance of an efficient immediate immune response to mycobacterial infections. Persistent TLR2 signalling can also induce anti-inflammatory responses, and rigorous pathology due to exaggerated proinflammatory immune responses has been observed in TLR2-knockout mice after infection with *M. tuberculosis* [37,66].

Moreover, innate recognition of pathogens also leads to changes in many cell types other than macrophages and dendritic cells which affect the generation of immune responses, such as NK cells, as well as stromal and endothelial cells. The epithelial layer provides the first line of defence against invading pathogens, and many TLRs are expressed on the epithelial cells [67,68]. Triggering of TLRs expressed by these cells leads to production of cytokines, chemokines and antimicrobial peptides [69,70]. It is possible that induction of anti-mycobacterial immune responses is more dependent on TLR signalling in the respiratory tract than systemically, due to differential expression of TLR molecules by tissue stromal cells and tissue-resident innate cells. Induction of costimulatory molecules on dendritic cells and other cell types is an essential control point for T-cell activation [71]. Presumably, signals from TLRs, primarily TLR2, are involved in the activation and maturation of antigen-presenting cells to allow priming of mycobacterium-specific T cells and the generation of proinflammatory cytokines that drive CD4^{+} T cells into a Th1 direction, essential for protection.

Collectively, our results provide evidence of a protective role for TLR2, and partly TLR4, in host defence against mycobacteria infection in the respiratory tract, particularly at the early stage. We furthermore report data on the functions of TLR2 and TLR4 in host resistance to infection with non-virulent BCG, which has not been as extensively investigated as infection with *M. tuberculosis*. Our results support the idea that the contribution of TLRs to host defence in vivo can differ, depending on the route of infection and size of inoculum.

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