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IMMUNOLOGY ORIGINAL ARTICLE

Toll-like receptor 4 signalling through MyD88 is essential to control *Salmonella enterica* serovar Typhimurium infection, but not for the initiation of bacterial clearance

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Summary

Toll-like receptor-4 (TLR4) is important in protection against lethal *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) infection. Control of the early stages of sublethal *S.* Typhimurium infection in mice depends on TLR4-dependent activation of macrophages and natural killer (NK) cells to drive an inflammatory response. TLR4 signals through the adapter proteins Mal/MyD88 and TRIF-related adaptor molecule (TRAM)/TIR-domain-containing adaptor-inducing interferon-b (TRIF). In the mouse typhoid model we showed that TLR4 and MyD88, but not Mal or TRIF, are essential for the control of exponential *S.* Typhimurium growth. $TRIF^{-/-}$ mice have a higher bacterial load in comparison with wild-type mice during a sublethal infection because TRIF is important for bacterial killing during the first day of systemic disease. Minimal pro-inflammatory responses were induced by *S.* Typhimurium infection of macrophages from $TLR4^{-/-}$, $MyD88^{-/-}$ and $TRIF^{-/-}$ mice *in vitro*. Pro-inflammatory responses from $Mal^{-/-}$ macrophages were similar to those from wild-type cells. The pro-inflammatory responses of $TRIF^{-/-}$ macrophages were partially restored by the addition of interferon-γ (IFN-γ), and $TRIF^{-/-}$ mice produced markedly enhanced IFN-γ levels, in comparison to wild-type mice, probably explaining why bacterial growth can be controlled in these mice. $TLR4^{-/-}$, $MyD88^{-/-}$, $TRIF^{-/-}$ and $Mal^{-/-}$ mice all initiated clearance of *S.* Typhimurium, suggesting that TLR4 signalling is not important in driving bacterial clearance in comparison to its critical role in controlling early bacterial growth in mouse typhoid.

Keywords: Mal; MyD88; *Salmonella*; toll-like receptor; TLR4; TRIF

Introduction

Systemic infection of mice with *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) causes a disease similar in many respects to typhoid fever in humans.[1] The immune responses to systemic sublethal *S.* Typhimurium infection are well characterized and this is therefore a valuable model for using to understand how different immune mechanisms contribute to the control of *Salmonella* infections and for studying how an immune response to infection develops *in vivo*. In a systemic infection the bacteria reside and replicate within phagocytes.[2] During the first few hours of systemic disease there is a decrease in bacterial numbers that is dependent on bacterial killing by neutrophil and macrophage nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activity in the context of prevention of bacterial growth through natural resistance-associated macrophage protein (NRAMP)-1/Slc11a1 activity.[2,3] During the next few days of systemic infection, exponential growth of *S.* Typhimurium occurs, the rate of which depends on the virulence of the bacterial strain and the genetic background of the host. This growth may, in turn,

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be controlled by macrophages and natural killer (NK) cells releasing cytokines to induce an inflammatory response.[2] This control of S. Typhimurium growth has been referred to as the plateau phase.[4] Initiation and expansion of T helper 1 (Th1)-type T-cell immunity is required for the late phase of control and eventual clearance of the infection.[2] Pro-inflammatory cytokines that are produced by the host in response to systemic S. Typhimurium infection are therefore critical for the control of bacterial growth.[2]

Initial host recognition of S. Typhimurium infection depends on pattern recognition receptors (PRRs), in particular during systemic infection, on toll-like receptor (TLR)4;[5–9] however, TLR2 is important in the later stages of lethal infection models.[10] Lipopolysaccharide (LPS) expressed by S. Typhimurium is recognized by TLR4, in association with MD2 and CD14.[6,11] TLR4 recognition of S. Typhimurium activates macrophages, initiating the oxidative burst and inducing cytokine production. TLR4 signals via recruitment of at least four adapter proteins: MyD88, Mal, TRIF-related adaptor molecule (TRAM) or TIR-domain-containing adaptor-inducing interferon-b (TRIF).[12] TLR4/Mal/MyD88 signalling induces cytokines such as tumour necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-12p40, whereas signalling through TLR4/TRAM/TRIF also induces Type I interferon (IFN)-dependent proteins such as IFN-β, regulated on activation, normal, T-cell expressed, and secreted (RANTES) and IL-12p35.[12] Cytokines are required for granuloma formation and for activation of inducible nitric oxide synthase (iNOS)-dependent antibacterial functions of macrophages during the plateau phase.[2]

TLR2 has a limited role in the early control of S. Typhimurium infection,[6–9] but is important in protection against lethal sepsis driven by both Gram-positive[13] and Gram-negative[10] bacteria. The roles of TLR4 and TLR2 in protecting mice against lethal infection with S. Typhimurium are clear,[5,8,10,14,15] but much less is known about how these receptors and their signalling pathways are involved in the immune responses that control and clear *Salmonella* infections. In LPS-resistant mice (C3H/HeJ; with mutated, non-signalling TLR4) the plateau phase does not occur, indicating that control of bacterial growth is dependent on the inflammatory response induced by TLR4 recognition of LPS.[2,5,8,9] It is critical to understand the immune mechanisms involved in the control and clearance of bacteria if successful management of invasive salmonellosis is to be achieved.

Here we have studied how TLR2, TLR4, MyD88, Mal and TRIF influence plateau formation during the sublethal infection of mice with S. Typhimurium. We showed that TLR4 and MyD88, but not TLR2, Mal or TRIF, are essential for plateau formation, whereas TRIF is involved in the early phase of bacterial killing. By comparison, whilst TLR4 is critical for driving the plateau phase in S. Typhimurium growth, TLR4-dependent signalling seems to be much less important in initiating the clearance phase of this bacterial infection.

Materials and methods

Mouse strains

Mice were bred under specific pathogen-free conditions at Harlan, Loughborough, UK or in the Department of Veterinary Medicine, the University of Cambridge (Cambridge, UK). Mice were housed in isolators or in filter-top cages and were provided with sterile water and food *ad libitum*. TLR4$^{-/-}$,[16] TLR2$^{-/-}$,[17] TLR2$^{-/-}$ TLR4$^{-/-}$,[8] MyD88$^{-/-}$,[18] Mal$^{-/-}$,[19] and TRIF$^{-/-}$[20] mice on a C57BL/6 background were as described previously. C57BL/6 mice were purchased from Harlan, UK.

Bacterial strains and ligands

Infection studies *in vivo* were performed using S. Typhimurium M525P, a strain that establishes sublethal infection in immunocompetent C57BL/6 mice, but is virulent enough to cause rapidly lethal infections in animals with impaired innate resistance and/or adaptive responses.[7,21] To determine whether effective Th1 responses can be generated in TLR4$^{-/-}$ and MyD88$^{-/-}$ mice we used S. Typhimurium SL3261, which is an attenuated mutant that causes slowly progressive lethal infections if the animals fail to mount T-cell immunity.[22] In the macrophage experiments, virulent, wild-type S. Typhimurium SL1344 was used for all experiments. S. Typhimurium strain SL1344 LPS was a generous gift from Anjam Khan (University of Newcastle, Newcastle, UK).

Infection of mice

Bacteria were grown overnight at 37° as a static culture in Luria–Bertani (LB) medium. Bacteria were washed and diluted in phosphate-buffered saline (PBS) to obtain 5 x 10^3 colony-forming units (CFU)/ml. Unless otherwise stated, 200 µl (10^3 CFU) of this bacterial suspension was injected into the tail vein. Appropriate dilutions of the inoculum were plated onto LB agar for precise enumeration of the number of viable bacteria given to the mice. At each time-point after infection, mice were killed by cervical dislocation and spleens and livers were removed. The organs were placed into 10 ml of sterile distilled water and homogenized in a Colworth Stomacher (Sewerd Ltd, Worthing, UK) for subsequent determination of viable bacterial counts by plating out appropriate dilutions of the homogenates onto LB agar. Viable CFU isolated per liver for individual mice are shown.

Preparation of livers for histological analysis

Livers were fixed in 10% formalin and then prepared for standard paraffin embedding and sectioning. Three-

micrometer sections of liver from each mouse were stained with haematoxylin and eosin for histological analysis. Analyses were performed by one investigator (KH) who was blinded to the genotype of the mouse and to the experimental conditions. Quantitative assessment of liver lesions was undertaken by counting the number of liver inflammatory lesions per 10 high-power fields (×400 magnification) of hepatic parenchyma. Qualitative evaluation of lesion architecture was also undertaken.

Infection of bone marrow macrophages

Primary bone marrow-derived macrophages (BMDMs) were isolated from femurs and tibiae of mice killed by cervical dislocation, then cultured in BMDM medium [RPMI-1640 medium supplemented with 10 % (v/v) fetal calf serum, 2 mM glutamine, 5 % (v/v) horse serum, 1 mM sodium pyruvate and 10 μg/ml of gentamicin], in petri dishes. To isolate BMDM from MyD88 mice, femurs from knockout and wild-type mice were shipped on ice from Dr K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA, USA). For maintenance of BMDMs in culture this medium was further supplemented with 20% (v/v) supernatant taken from L929 cells (a murine macrophage colony-stimulating factor producing cell line).[6,23] For experiments, cells were plated onto 96-well plates at a plating density of 2×10^5 cells per well, in the absence of gentamicin.

S. Typhimurium SL1344 in mid-log phase was added to the cells at multiplicities of infection (MOI) of 1 or 10. Following a 2 hr incubation, the cells were incubated in BMDM medium containing 50 μg/ml of gentamicin for 1 hr to kill extracellular *Salmonella*. Cells were then incubated in BMDM medium containing 10 μg/ml of gentamicin until the end of the experiment.

We used the *S.* Typhimurium strain SL1344 for our *in vitro* experiments to ensure that our data were comparable with other published data on *S.* Typhimurium infections *in vitro*.[8,24–26] In our preliminary analysis comparing the *in vitro* response of SL1344 and M525P strains in wild-type, TLR4$^{-/-}$ and MyD88$^{-/-}$ BMDMs we saw similar levels of TNF-α and slightly lower levels of nitric oxide (NO) in response to SL1344. The effect of the gene knockouts on the macrophage response was identical for both bacterial strains.

Measurement of TNF-α production

To determine cumulative TNF-α production, supernatants were obtained 7 hr after infection (cumulative 3–7 hr after infection) and stored at $-80°$ until analysed using the Duoset® enzyme-linked immunosorbent assay (ELISA) development system (R&D Systems, Abingdon, Oxfordshire, UK).

Measurement of NO production

iNOS activity was determined indirectly using samples of supernatant taken 22 hr after infection and stored at $-20°$. Nitrite accumulation was measured using the Griess reaction as an indication of iNOS activity.[27]

Splenocyte cytokine measurements

Splenocytes were isolated from mice infected with *S.* Typhimurium. The spleens were removed aseptically and passed through a 40-μm nylon strainer to yield splenocytes. These were washed, then red blood cells were lysed by incubation with Gey's solution for 3 min, and plated at 1×10^6 cells/well in a 96-well plate in RPMI-1640 containing 10% (v/v) fetal calf serum (FCS), 1 mM HEPES, 5 mM L-glutamine, 2×10^{-5} M β-mercaptoethanol, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Splenocytes were stimulated with *S.* Typhimurium strain M525P grown to mid-log phase at an MOI of 1:1 or stimulated with LPS (1 μg/ml; from *S.* Typhimurium SL1344). Samples of supernatant were taken 6 and 24 hr later and assayed for TNF-α, or were taken 48 hr later and assayed for IFN-γ. Samples were stored at $-80°$ until assayed using the Duoset® ELISA development system (R&D Systems) for IFN-γ or TNF-α.

Statistical analysis

Statistical analysis was performed using a two-way analysis of variance (ANOVA) followed by Bonferonni post-testing. A Mann–Whitney *U*-test was performed on data pooled from two or more *in vivo* infections. Histological analysis was compared using a Student's *t*-test. Statistical differences are given by asterisks as follows: $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

Results

TLR4, but not TLR2, is required to control the growth of a sublethal systemic infection of S. Typhimurium *in vivo*

Mice were infected with 200 μl (10^3 CFU) of *S.* Typhimurium M525P and bacterial counts were measured in the livers and spleens from these animals. In wild-type mice, as expected, there was a drop in bacterial numbers of approximately half a log within the first day of infection.[4] After this, the growth of *S.* Typhimurium in liver and spleen was exponential until it plateaued at day 3 postinfection (Fig. 1, spleen data not shown). The plateau was sustained for the duration of the short-term experiment. In TLR4$^{-/-}$ mice, higher bacterial numbers were seen on day 1 postinfection and then, similarly to what is seen in C3H/HeJ mice,[5] bacterial growth increased more rapidly

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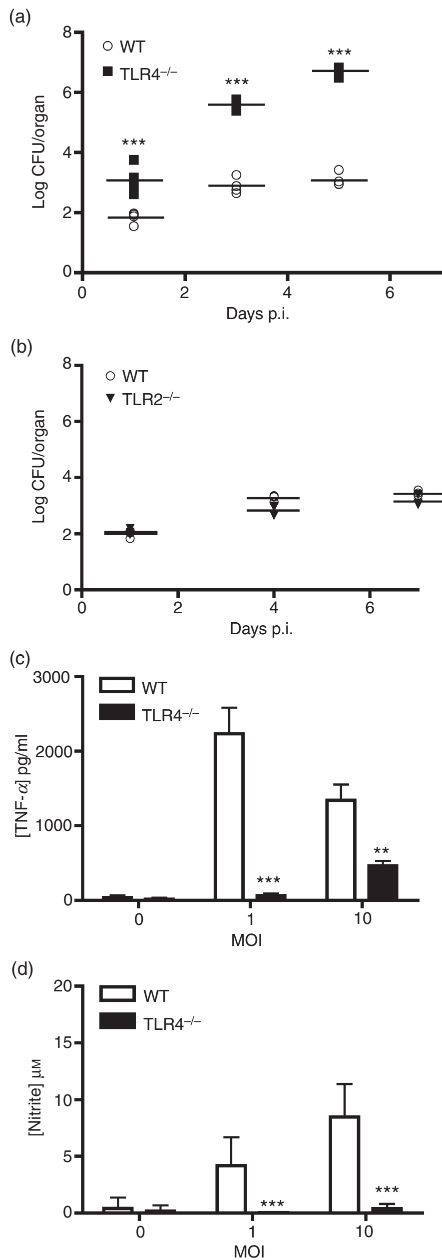

Figure 1. Bacterial counts after intravenous infection of mice with 1×10^3 colony-forming units (CFU)/mouse of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) M525P. Mice were killed by cervical dislocation, bacterial loads were determined in the liver and the results are expressed as log CFU per organ for each mouse with the mean marked by a bar. Wild-type (WT), toll-like receptor (TLR)$4^{-/-}$ ($n = 4$) (a) or TLR$2^{-/-}$ ($n = 3$) (b) mice were infected and the results shown are representative of at least two separate experiments. Bone marrow-derived macrophages (BMDMs) from WT and TLR$4^{-/-}$ mice were infected with *S.* Typhimurium strain SL1344 at a multiplicity of infection (MOI) of 0:1, 1:1 or 10:1 (bacteria : cell). Supernatant samples were analysed by enzyme-linked immunosorbent assay (ELISA) for tumour necrosis factor-α (TNF-α) 7 hr after infection ($n = 4$) (c) and for nitrite accumulation by the Griess reaction 22 hr after infection ($n = 6$) (d). The results are expressed as mean ± standard deviation, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$. p.i., postinfection.

than in wild-type controls, at approximately 1 log per day, until the experiment was stopped because an overwhelming bacterial load had caused the mice to become terminally ill (Fig. 1) [Analysis of the combined results from two separate experiments using the Mann–Whitney *U*-test found that bacterial burdens in the TLR$4^{-/-}$ mice were significantly higher at all the time-points shown ($P < 0{\cdot}01$ at day 1 and $P < 0{\cdot}001$ at days 3 and 5 post-infection)]. TLR4 is therefore essential for both the control of the initial growth rate of *S.* Typhimurium and for plateau formation to occur. The growth of *S.* Typhimurium in TLR$2^{-/-}$ mice was identical in both liver and spleen to what is seen in the wild-type mice (Fig. 1, spleen data not shown) (No significant increase in bacterial burdens were found in the TLR$2^{-/-}$ mice when the data from two experiments were pooled). We infected mice with higher numbers of bacteria (10^4 CFU) to see if this would reveal any role for TLR2 in controlling *S.* Typhimurium *in vivo*, but bacterial growth was again similar to that seen in wild-type animals, suggesting that TLR2 does not play a role in controlling *S.* Typhimurium growth in sublethal infection (data not shown). In a preliminary experiment, where we infected TLR$2^{-/-}$ TLR$4^{-/-}$ double knockout mice, there was no difference in bacterial counts from those seen during infection of TLR$4^{-/-}$ mice, suggesting that TLR2 is unimportant, in comparison to TLR4, for the control of sublethal bacterial growth in the spleen and liver (data not shown).

The macrophage pro-inflammatory response to *S.* Typhimurium infection is absent in cells from TLR$4^{-/-}$ mice

The generation of a pro-inflammatory response to *S.* Typhimurium is required in order for the host to control bacterial growth. Activation of TLR4 by *S.* Typhimurium induces the production of pro-inflammatory cytokines, such as TNF-α, and inflammatory proteins, such as iNOS.[6] To confirm that TLR4 activation by *S.* Typhimurium was driving a pro-inflammatory response, we infected BMDMs from wild-type and TLR$4^{-/-}$ mice with this bacterium and measured TNF-α production after 7 hr and NO production at 22 hr. Wild-type BMDMs produced a robust inflammatory response to infection, but the TLR$4^{-/-}$ BMDMs, as expected, produced little TNF-α or NO (Fig. 1c,d).

MyD88, but not Mal or TRIF, is essential for establishing the plateau phase in controlling the growth of sublethal infection of mice with *S.* Typhimurium

The essential role of TLR4 in controlling bacterial growth led us to investigate the contribution of both the MyD88 and TRIF signalling pathways in mouse typhoid. In MyD$88^{-/-}$ mice, exponential bacterial growth continued

unchecked, in a similar manner to that seen in the $TLR4^{-/-}$ animals, and the mice were unable to mount an effective response to control the growth of *S.* Typhimurium (Fig. 2a) [a significantly higher ($P < 0{\cdot}01$) bacterial burden was observed in the $MyD88^{-/-}$ mice on days 3 and 5 when the combined results from two experiments were analysed]. MyD88 is therefore essential for establishment of the plateau phase of bacterial growth.

The importance of TLR4 and MyD88 in controlling bacterial growth suggested that TLR4 signalling through the TRIF pathway was likely to be less important in driving host responses to *S.* Typhimurium. $TRIF^{-/-}$ mice infected with *S.* Typhimurium, however, showed higher bacterial counts by day 1 in comparison to infected wild-type mice (Fig. 2b). We studied bacterial growth over a short time course and found that this difference in bacterial counts was apparent [and statistically significant ($P < 0{\cdot}001$) when pooling results from two separate experiments] by 4 hr (Fig. 2d). After day 1, however, the rates of bacterial growth in livers and spleens were very similar to those seen in the wild-type animals. This meant that the $TRIF^{-/-}$ animals had a consistently higher bacterial burden, at each time-point, of 0·4–1·2 log CFU/spleen or of 0·8–1·6 log CFU/liver (Fig. 2b) [values determined from data pooled from five separate experiments, which found that the bacterial burdens in the $TRIF^{-/-}$ mice were significantly higher ($P < 0{\cdot}001$) than in the wild-type mice on days 1, 4 and 7 postinfection]. $TRIF^{-/-}$ mice are therefore able to control sublethal infection with *S.* Typhimurium, but achieve the plateau phase at a higher bacterial burden than the wild-type mice.

Finally, $Mal^{-/-}$ mice were infected with *S.* Typhimurium and, surprisingly, unlike $TLR4^{-/-}$ and $MyD88^{-/-}$ mice, the $Mal^{-/-}$ mice controlled the infection in a manner similar to that of wild-type mice (Fig. 2c). By 7 days postinfection the bacterial load in the $Mal^{-/-}$ mice was significantly higher than in the wild-type mice. The difference between $Mal^{-/-}$ and wild-type bacterial burdens at these time-points was $< 0{\cdot}5$ log CFU/organ. Biologically, the relevance of this increase in CFU is small when compared with the increase of approximately 4 log CFU/organ observed in the $TLR4^{-/-}$ and $MyD88^{-/-}$ mice by day 5.

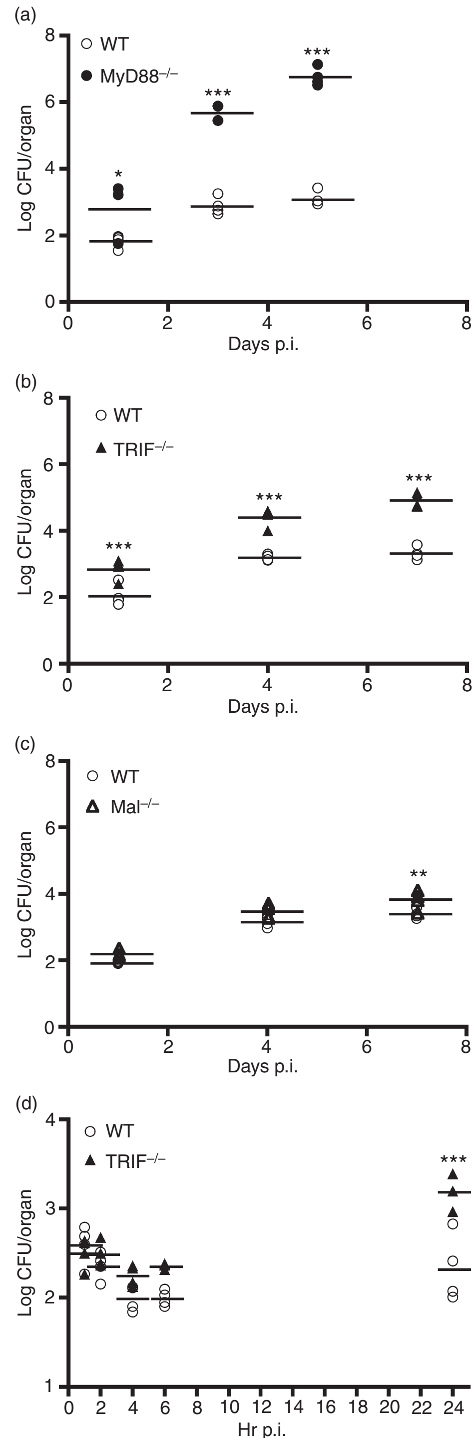

Figure 2. Bacterial counts after intravenous infection of mice with 1×10^3 colony-forming units (CFU) per mouse of *Salmonella enterica* serovar Typhimurium (S. Typhimurium) strain M525P. Mice were killed by cervical dislocation, bacterial loads were determined in the liver and the results are expressed as log CFU per organ for each mouse with the mean marked by a bar. Wild-type (WT), $MyD88^{-/-}$ ($n = 3$) (a), TIR-domain-containing adaptor-inducing interferon-$b^{-/-}$ ($TRIF^{-/-}$) (b and d) ($n = 4$) and $Mal^{-/-}$ ($n = 4$) (c) mice were infected and the graphs are representative of at least two separate experiments, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$. p.i., postinfection.

TNF-α and NO production in response to *S.* Typhimurium infection are deficient in $MyD88^{-/-}$ and $TRIF^{-/-}$ macrophages, but not in $Mal^{-/-}$ macrophages

BMDMs from wild-type, $MyD88^{-/-}$, $Mal^{-/-}$ and $TRIF^{-/-}$ mice were infected with *S.* Typhimurium, and TNF-α production and iNOS activity were measured. *S.* Typhimurium infection stimulated the production of TNF-α from wild-type BMDMs, whilst the levels of this cytokine were very low in BMDMs from $MyD88^{-/-}$ or $TRIF^{-/-}$ mice (Fig. 3a). NO production was also negligible in

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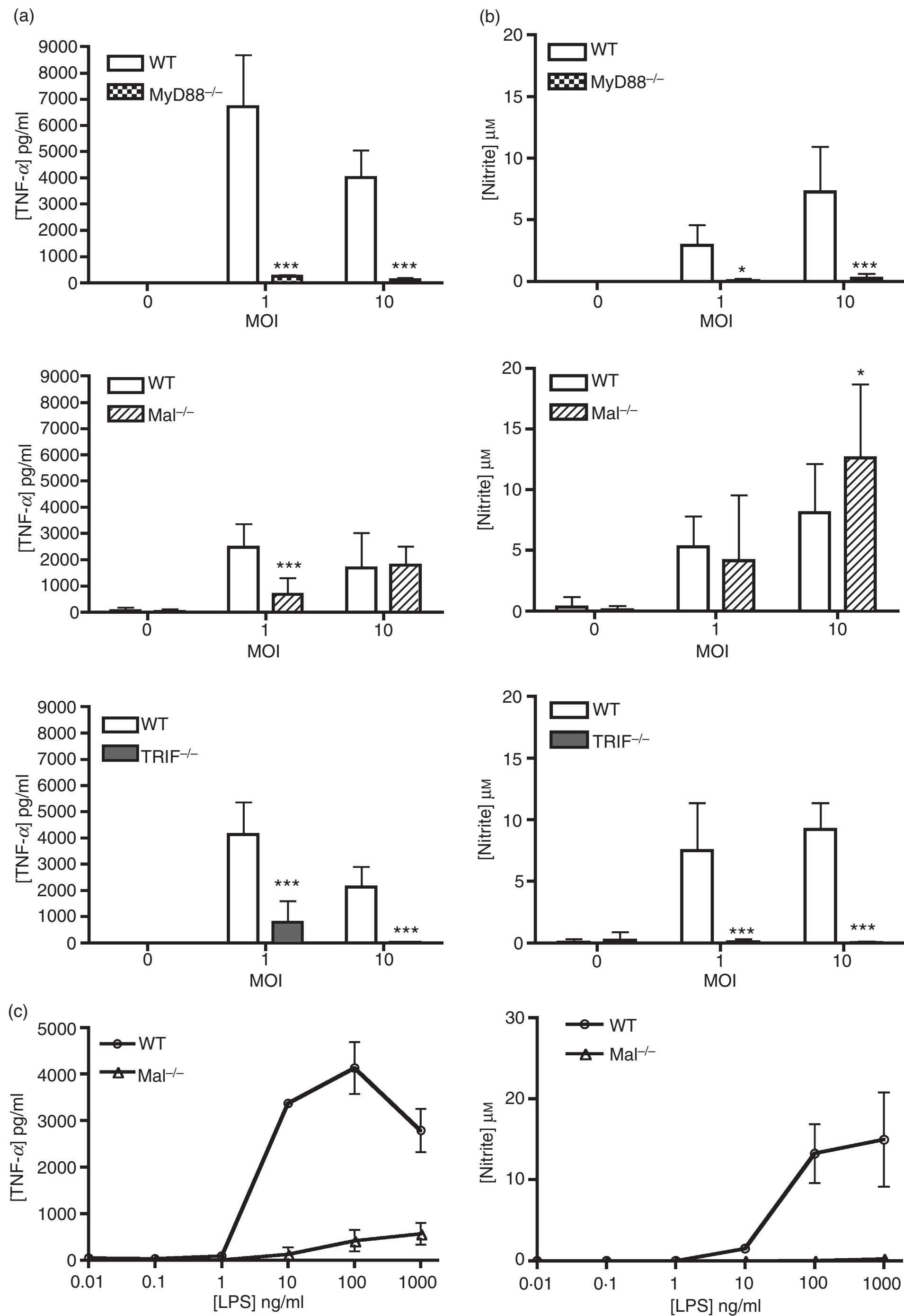

Figure 3. Tumour necrosis factor-α (TNF-α) and nitric oxide (NO) production from MyD88$^{-/-}$, Mal$^{-/-}$ and TIR-domain-containing adaptor-inducing interferon-b$^{-/-}$ (TRIF$^{-/-}$) macrophages. Bone marrow-derived macrophages (BMDMs) from wild-type, MyD88$^{-/-}$, Mal$^{-/-}$ or TRIF$^{-/-}$ mice were infected with *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) strain SL1344 at multiplicity of infections (MOIs) of 0:1, 1:1 or 10:1 (bacteria : cell). Supernatant samples were assayed by enzyme-linked immunosorbent assay (ELISA) for tumour necrosis factor-α (TNF-α) 7 hr after infection (a) or analysed using the Griess reaction for nitrite accumulation 22 hr after infection (b). The results are expressed as the mean ± standard deviation from at least four separate experiments, $^{*}P < 0{\cdot}05$, $^{***}P < 0{\cdot}001$. Wild-type and Mal$^{-/-}$ BMDMs were also stimulated with increasing doses of lipopolysaccharide (LPS) from *S.* Typhimurium SL1344, and TNF-α and NO production were assayed 7 and 22 hr after stimulation, respectively (c).

macrophages from the MyD88$^{-/-}$ or TRIF$^{-/-}$ mice after infection (Fig. 4b). BMDMs from Mal$^{-/-}$ mice showed minimal TNF-α and NO production in response to LPS, as expected (Fig. 3c), but in response to infection, the levels of these inflammatory mediators were mostly equivalent to those seen in infected wild-type BMDMs.

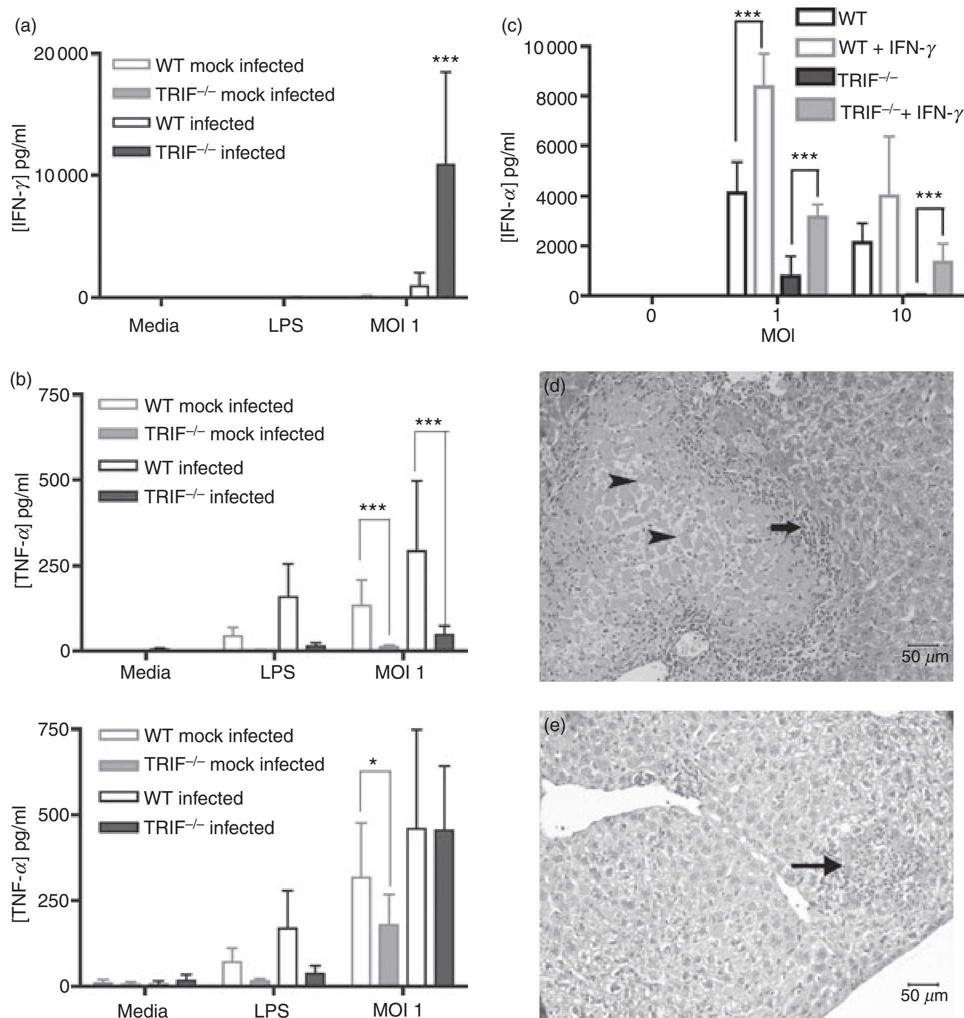

Figure 4. Interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) are up-regulated in TIR-domain-containing adaptor-inducing interferon-b$^{-/-}$ (TRIF$^{-/-}$) mice infected with *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). Wild-type or TRIF$^{-/-}$ mice were injected intravenously with 1×10^3 colony-forming units (CFU) per mouse of *S.* Typhimurium strain M525P. Four days after infection splenocytes were harvested and challenged *in vitro* with 1 μg/ml of lipopolysaccharide (LPS) from *S.* Typhimurium SL1344 or infected with *S.* Typhimurium M525P at a multiplicity of infection (MOI) of 1:1 (bacteria : cell). Supernatant samples were taken 48 hr later and assayed by enzyme-linked immunosorbent assay (ELISA) for IFN-γ (a), or 6 hr (upper panel) or 24 hr (lower panel) after stimulation and analysed by ELISA for TNF-α (b). The results are expressed as mean ± standard deviation (n = 6). Bone marrow-derived macrophages (BMDMs) from wild-type and TRIF$^{-/-}$ mice were pretreated with 10 IU/ml of IFN-γ for 1 hr before infection with *S.* Typhimurium SL1344 at MOIs of 0:1, 1:1 or 10:1 (bacteria : cell). Supernatant samples were assayed by ELISA for TNF-α 7 hr after infection. The results are expressed as mean ± standard deviation (n = 4), *$P < 0{\cdot}05$, ***$P < 0{\cdot}001$ (c). Wild-type or TRIF$^{-/-}$ mice were injected intravenously with 1×10^3 CFU per mouse of *S.* Typhimurium M525P. Seven days after infection the livers were fixed in formalin, prepared for histological analysis and stained with haematoxylin and eosin. In a liver from a wild-type mouse, a focus of necrotic hepatocytes is surrounded by a rim of large numbers of degenerate neutrophils, mixed with lymphocytes. Arrowheads = coagulative necrosis of hepatocytes. Arrow = neutrophilic infiltrate (predominantly degenerate neutrophils) (d). In the liver from an infected TRIF$^{-/-}$ mouse there is a small cluster of lymphocytes and viable and degenerate neutrophils surrounding a focus of individual necrotic hepatocytes (arrow). Bar = 50 μm (e).

The defective pro-inflammatory response of TRIF$^{-/-}$ macrophages to *S.* Typhimurium infection *in vitro* is compensated for by IFN-γ production *in vivo*

TNF-α is one of the inflammatory proteins critical for successfully achieving a plateau in bacterial growth in sublethal *S.* Typhimurium infection of mice.[2] It is therefore surprising that the TRIF$^{-/-}$ mice controlled a sublethal *S.* Typhimurium infection despite their defective macrophage TNF-α response. IFN-γ is elevated during bacterial infections and enhances LPS-induced macrophage responses, including the production of TNF-α.[28] To determine whether IFN-γ priming was able to restore inflammatory responsiveness to TRIF$^{-/-}$ BMDM *in vitro*, the cells were pretreated with IFN-γ for 1 hr before infection with *S.* Typhimurium. TRIF$^{-/-}$ BMDM now produced levels of

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TNF-α and NO similar to those produced by unprimed wild-type BMDM (Fig. 4c and data not shown). Infected $TRIF^{-/-}$ mice showed higher bacterial burdens than the wild-type mice, and IFN-γ levels increased with increasing bacterial burden,[29] so we measured the levels of IFN-γ in these animals after infection. Splenocytes from day 4 of an infection in $TRIF^{-/-}$ mice showed enhanced levels of IFN-γ in comparison to control animals when re-infected *ex vivo* with *S.* Typhimurium (Fig. 4a). Therefore, the increased capability of splenocytes from infected $TRIF^{-/-}$ mice to produce IFN-γ may be sufficient to up-regulate cytokine production *in vivo* and allow the control of bacterial growth. As IFN-γ priming of $TRIF^{-/-}$ macrophages restored their ability to produce TNF-α in response to *Salmonella* infection *in vitro*, we measured TNF-α production from the splenocytes. Splenocytes from uninfected $TRIF^{-/-}$ mice produced significantly less TNF-α than wild-type splenocytes when re-infected *ex vivo*. Splenocytes from infected $TRIF^{-/-}$ mice were still deficient in TNF-α production 6 hr after re-infection, but by 24 hr after re-infection these cells were producing wild-type levels of TNF-α (Fig. 4b). These data suggest that IFN-γ restores the ability of the $TRIF^{-/-}$ cells to induce a pro-inflammatory response, probably explaining why these mice can control bacterial growth *in vivo*.

Neutrophil recruitment to lesions is defective in infected $TRIF^{-/-}$ mice

The reduced bacterial killing in the $TRIF^{-/-}$ mice, and the fact that TRIF has been linked to neutrophil recruitment in bacterial lung infections,[30–32] suggested that these animals may be defective in neutrophil recruitment during a *Salmonella* infection. Hepatic histological analysis on day 7, a time-point when the plateau is well established, showed that both wild-type and $TRIF^{-/-}$ mice had small, well-demarcated focal infiltrates of neutrophils and macrophages, interspersed by hepatocytes with pyknotic nuclei and scattered small deposits of fragmented nuclear debris. Some of the larger lesions had a central area of necrosis, surrounded by a rim of inflammatory cells (predominantly containing moderate numbers of neutrophils and lymphocytes and small numbers of macrophages) (Fig. 4d). The $TRIF^{-/-}$ mice, on qualitative assessment, had smaller lesions that appeared to be more frequent ($P = 0{\cdot}08$). By contrast, the wild-type animals had a smaller number of generally larger lesions, with a qualitatively higher proportion of neutrophils. These data suggest a deficit in neutrophil function in $TRIF^{-/-}$ mice.

Absence of signalling through TLR4 does not hinder the initiation of clearance of *S.* Typhimurium from the organs

Following control by the host of bacterial growth in a sublethal infection, *S.* Typhimurium will eventually be cleared predominantly through the activity of $CD4^{+}$ T cells. To study the clearance phase of infection in $TLR4^{-/-}$ mice requires the use of an attenuated bacterial strain because these animals are unable to control the growth of fully virulent *S.* Typhimurium. We therefore infected these mice with *S.* Typhimurium SL3261, a mutant that causes slowly progressing lethal infections if the animals fail to mount T-cell immunity.[22] Bacterial counts were 1–2 log higher in $TLR4^{-/-}$ mice compared with wild-type mice; hence, we decided to inoculate 10-fold fewer bacteria into the $TLR4^{-/-}$ mice than into the wild-type mice to achieve similar bacterial loads. Surprisingly, we found that $TLR4^{-/-}$ mice could initiate bacterial clearance, albeit at a higher bacterial load than in wild-type mice (Fig. 5a). Similar results were seen in $MyD88^{-/-}$ mice (Fig. 5b). The $Mal^{-/-}$ and $TRIF^{-/-}$ mice were able to control the growth of the virulent M525P strain, and therefore this strain was used for clearance studies in these mice. $Mal^{-/-}$ mice infected with M525P initiated clearance of *S.* Typhimurium (Fig. 5d). $TRIF^{-/-}$ mice were also able to start clearing the M525P strain of *S.* Typhimurium, although this process appeared to be defective compared with that in wild-type and $Mal^{-/-}$mice, as a large variation in the rate of clearance was observed between mice (Fig. 5c), and the $TRIF^{-/-}$ mice continued to have significantly higher ($P < 0{\cdot}01$) bacterial burdens than the wild-type mice after initiation of clearance (at days 21, 28 and 35), as determined from the combined results of two separate experiments.

Discussion

Here we showed that TLR4 signalling through MyD88, but not through Mal or TRIF, is critically required to protect mice in a typhoid model of *S.* Typhimurium infection. TRIF is required for the early control of bacterial growth during the first day of an intravenous infection and for the pro-inflammatory response of macrophages to infection. IFN-γ priming of $TRIF^{-/-}$ macrophages restores the ability of these cells to produce an inflammatory response to infection, and splenocytes from infected $TRIF^{-/-}$ mice produce high levels of this cytokine, which may explain why $TRIF^{-/-}$ mice can control bacterial growth *in vivo*. TLR2 plays little or no role in the control of bacterial growth in the sublethal mouse typhoid model. TLR signalling is not required for initiating the clearance phase of mouse typhoid, as shown by the presence of normal clearance patterns in $MyD88^{-/-}$ mice.

TLR4 and MyD88 are important for the survival of mice after lethal infection with *S.* Typhimurium.[8] Here we used well-described sublethal models of *S.* Typhimurium infection,[33] where the bacterial counts obtained are very consistent and show little variability, which allowed us to follow the course of prolonged infection. The immunological processes controlling bacterial growth in

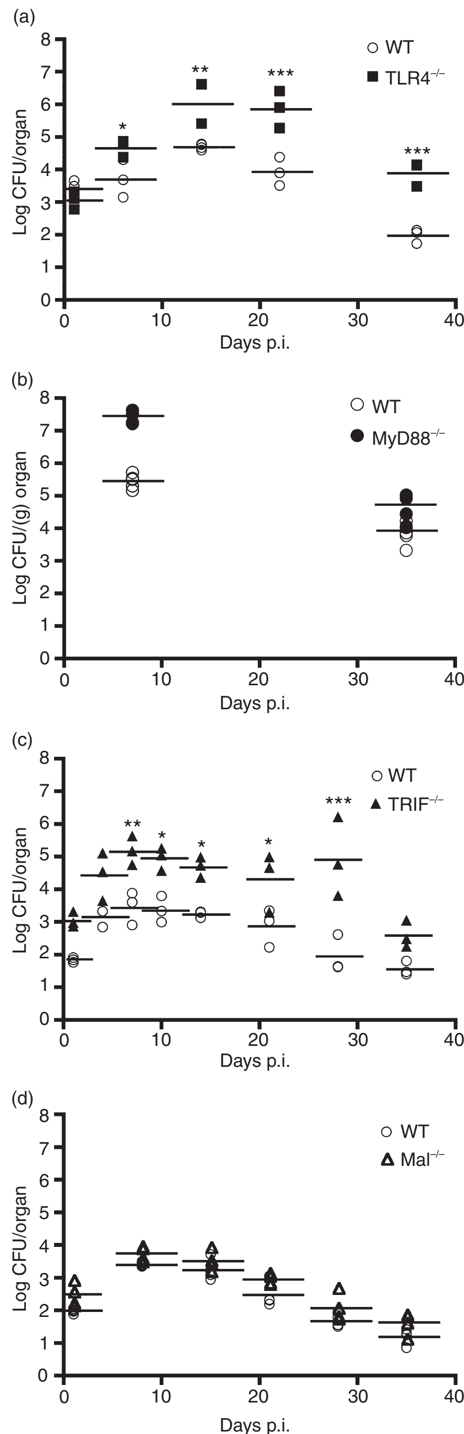

Figure 5. Bacterial counts after intravenous infection of wild-type (WT) mice [with 1×10^7 colony-forming units (CFU)/mouse] in comparison with toll-like receptor (TLR)$4^{-/-}$ ($n = 3$) (a) and MyD88$^{-/-}$ ($n = 4$) (b) mice (with 1×10^6 CFU/mouse) of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) SL3261, or intravenous infection of wild-type, TIR-domain-containing adaptor-inducing interferon-b$^{-/-}$ (TRIF$^{-/-}$) ($n = 3$) (c) and Mal$^{-/-}$ ($n = 4$) (d) mice with 1×10^3 CFU per mouse of *S.* Typhimurium M525P. Mice were killed by cervical dislocation, bacterial loads were determined in the liver and the results are expressed as log CFU per organ for each mouse with the mean marked by a bar *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$. p.i., postinfection.

these models are well understood.[2] We therefore explored whether TLR4 modifies the antibacterial immune response to *S.* Typhimurium infection. Bacterial growth from day 1 onwards continued exponentially and failed to plateau in TLR4$^{-/-}$ mice in comparison with wild-type mice. These results are similar to those seen in sublethal infection of the TLR4 mutant strain of mice, C3H/HeJ,[5] confirming a key role for TLR4 in controlling systemic infection with *S.* Typhimurium. In MyD88$^{-/-}$ mice, from day 1 postinfection onwards, the exponential bacterial growth was also unchecked, confirming that TLR4 signalling through MyD88 is vital for controlling *S.* Typhimurium growth *in vivo*. In both TLR4$^{-/-}$ and MyD88$^{-/-}$ macrophages, as expected,[6,8] the response to infection, as measured by TNF-α and NO production, was almost completely lost. These *in vitro* data are consistent with the failure of the TLR4$^{-/-}$ and MyD88$^{-/-}$ mice to establish a plateau phase in bacterial growth *in vivo*, which is dependent on the LPS-driven inflammatory response induced by *S.* Typhimurium.[5,34]

MyD88 is essential for signalling through other TLRs, including TLR2,[12] and in lethal sepsis models TLR2 helps to protect the host against infection,[10] but here, in the sublethal mouse typhoid model, TLR2$^{-/-}$ mice showed control of *S.* Typhimurium growth which was similar to that seen in wild-type mice. These data suggest that any role which TLR2 may play in protecting the host against systemic *Salmonella* infections is secondary to the role of TLR4. In survival studies, TLR2$^{-/-}$ mice showed a similar phenotype to wild-type mice, but lower bacterial loads were seen in tissues from these animals.[8] In our sublethal model, very similar spleen and liver bacterial loads were seen in wild-type and TLR2$^{-/-}$ mice, suggesting that TLR2 does not play a role in controlling this type of infection. Differences in the role of TLR2 in the host response to *Salmonella* infection seen in our sublethal study and in the lethal salmonellosis model used by us and by Weiss *et al.*,[8] are probably the result of a combination of elevated bacterial numbers seen in the lethal model (increasing the availability of TLR2 ligands produced by the bacteria) and the different routes of infection used, leading to activation of different cell types and/or different levels of TLR2 expressed.

To determine whether a role for TLR2 becomes apparent at higher bacterial loads, we infected the TLR2$^{-/-}$ mice with more bacteria, but again bacterial growth was controlled in a manner similar to what is seen in wild-type mice. We performed preliminary experiments in TLR2$^{-/-}$ TLR4$^{-/-}$ mice to see if this would reveal a role for TLR2 in sublethal *S.* Typhimurium infection, but the results were very similar to those seen in TLR4$^{-/-}$ mice. We concluded, therefore, that any role which TLR2 is playing in the immune response to sublethal *S.* Typhimurium infection is unlikely to be important in the control of bacterial growth.

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MyD88$^{-/-}$ mice are particularly susceptible to lethal infection with *S.* Typhimurium, probably because of the presence of a range of immune defects, including those in cytokine production, in the development of Th1-type responses, in NADPH oxidase activation, in phagocytic capacity, and in IL-1 and IFN-γ signalling.[8,35–37] In this study we have shown that, predictably, MyD88 plays a central role in host protection in a mouse typhoid model of infection. This is probably mostly through TLR4 stimulation, given that very similar data were seen in the MyD88$^{-/-}$ and TLR4$^{-/-}$ mice. The redundancy of Mal in the typhoid model is a particular surprise. Mal is required to bridge MyD88 and TLR4[38] and here we see a defective response of Mal$^{-/-}$ macrophages to LPS, but not to *S.* Typhimurium. Our studies *in vivo* showed that Mal$^{-/-}$ mice behaved like wild-type mice in response to *S.* Typhimurium infection. The lack of a role for Mal in the control of *S.* Typhimurium is similar to that seen in another study.[39] These authors suggested that mice lacking Mal accelerate the clearance phase of infection compared with wild-type mice. In our work, Mal played no role in the plateau or clearance phases of *S.* Typhimurium infection. It is unclear why Mal, which is important in some lung infection models,[30,40,41] is redundant in systemic *S.* Typhimurium infection. Potentially, Mal may be more important in controlling mucosal infections, rather than systemic infections or a *Salmonella* protein; for example, one of the newly identified Toll–interleukin-1 receptor domain proteins[42–44] may compensate for the lack of Mal. The lack of Mal-dependency in our data, except at low MOIs *in vitro*, is puzzling. It may be that at low MOIs Mal sensitizes the MyD88 signalling pathway, but at higher MOIs Mal is no longer required for full activation of MyD88-dependent signaling. *In vivo* our data would therefore suggest that there are sufficient numbers of bacteria present to drive a Mal-independent response. An alternative explanation for our data would be that a protein produced by *Salmonella* may compensate for the lack of Mal. At low MOIs of *Salmonella* the levels of this protein may be insufficient to compensate for the lack of Mal in terms of TNF-α production. At the higher MOI there may be enough of the *Salmonella* protein to allow full TNF-α production, similar to that seen from wild-type cells.

TLR4 activation of TRIF signalling is linked to the generation of a Th1 response to LPS.[35,45] In our study, TLR4 signalling through TRIF was required for controlling the early growth of *S.* Typhimurium *in vivo*, but despite the fact that macrophages from these mice are defective in inflammatory responses to infection, these mice control bacterial growth. Mice with elevated bacterial loads produce more IFN-γ[29] and in the present study we saw that infected TRIF$^{-/-}$ mice had elevated levels of this cytokine. In BMDMs from these mice, IFN-γ restored cytokine production in response to infection, and the enhanced production of IFN-γ in *S.* Typhimurium-infected TRIF$^{-/-}$ mice probably explains why they can control bacterial growth *in vivo*. IFN-γ signals via the JAK/signal transducer and activator of transcription [janus tyrosine kinase (JAK)/STAT] pathway, and STAT-binding sites have been recognized in the promoter regions of various inflammatory mediators, including iNOS.[46] LPS and IFN-γ synergize to increase the production of iNOS and TNF-α;[46,47] therefore, in the absence of TRIF-dependent TLR4 signalling, the IFN-γ driven activation of the STAT pathway could compensate for the lack of TRIF-driven IFN-β production, thus leading to full gene expression, even in the absence of this signalling protein. IFN-γ production in response to Gram-negative bacterial infection is TLR4 and MyD88 dependent[10] and will not be generated in the TLR4$^{-/-}$ and MyD88$^{-/-}$ mice. The elevated IFN-γ levels in TRIF$^{-/-}$ mice may be attributable to TRIF-dependent TNF receptor associated factor (TRAF)3 signalling. BMDMs from TRAF3-deficient mice lack IL-10 production in response to LPS stimulation.[48] IL-10 is a negative regulator of IFN-γ production[49] and therefore the lack of this cytokine may allow enhanced levels of IFN-γ to accumulate in the TRIF$^{-/-}$ mice.

TLR4 signalling through TRIF induces dendritic cell maturation and may modulate NADPH-oxidase activation.[12,50] *In vivo*, TRIF signalling contributes to the protection of mice against lung infections with *Pseudomonas aeruginosa* and *Escherichia coli*.[31,32] Infection of TRIF$^{-/-}$ mice with *S.* Typhimurium, similarly to what is seen in TLR4$^{-/-}$ mice, results in elevated bacterial numbers on day 1 postinfection in both liver and spleen in comparison to wild-type mice. The normal reduction in bacterial numbers on day 1 postinfection is dependent on bacterial killing by neutrophils and macrophages through complement and NADPH-dependent reactive oxidase activity.[2] TRIF is important for neutrophil recruitment in bacterial lung infections[31,32] and therefore it is likely that a failure in neutrophil recruitment may account, to some extent, for the elevated bacterial numbers seen in these mice. Histological analysis of tissue obtained on day 7 of a *S.* Typhimurium infection in TRIF$^{-/-}$ mice supports this hypothesis, with a smaller proportion of neutrophils present in the liver lesions compared with tissue from wild-type mice. TLR4 co-operates sequentially with the complement receptor C3 in neutrophil-driven killing of *Salmonella*.[51] It is therefore likely that the TLR4-dependent decrease in bacterial numbers seen by day 1 postinfection is driven, in part, by the TRIF pathway via a defect in both neutrophil and macrophage activities through a TLR4/C3 mechanism.

Our study also investigated whether PRRs are required to initiate clearance of *S.* Typhimurium infection, a process that is dependent on the activity of CD4$^+$ T cells.[1] TLRs have been linked to successful activation of CD4$^+$ T cells[52] and we expected to see a profound effect upon

bacterial clearance in mice lacking TLR4-dependent signalling. We found that bacterial clearance was initiated in $TLR4^{-/-}$, $MyD88^{-/-}$, $Mal^{-/-}$ and $TRIF^{-/-}$ mice if they were infected with a *Salmonella* strain that could be controlled by the host in the initial phase of infection. Our data are strikingly similar to early work in C3H/HeJ mice, which clear an *S.* Typhimurium *aroA* mutant and generate full protective immunity against virulent *S.* Typhimurium.[53] By contrast, MyD88, whilst also not being important for initiating bacterial clearance, is required for protective immunity against *S.* Typhimurium.[54] MyD88, in addition to being recruited to most TLRs, is an adapter in the signalling pathway for induction of IL-1β and IL-18,[12] and is important in IFN-γ signalling.[36] Protective immunity to *S.* Typhimurium may either be generated by a TLR other than TLR4 or by a nucleotide oligomerisation domain (NOD)-like receptor that induces IL-18 (a cytokine important in protection against this pathogen.[55]) Our data suggest that initiation of bacterial clearance, a process dependent on the successful activation of $CD4^{+}$ T cells, does not require TLR4- or MyD88-dependent signalling, yet protective immunity, which is independent of TLR4, does require MyD88. To reconcile these observations, we now therefore need to determine the contribution of PRRs to the different mechanisms of protective immunity against *S.* Typhimurium.

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Disclosures

The authors have nothing to disclose.

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