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Toll-Like Receptors Are Temporally Involved in Host Defense

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Toll-like receptors (TLRs) are evolutionarily conserved proteins that recognize microbial molecules and initiate host defense. To investigate how TLRs work together to fight infections, we tested the role of TLRs in host defense against the Gram-negative bacterial pathogen, *Salmonella*. We show that TLR4 is critical for early cytokine production and killing of bacteria by murine macrophages. Interestingly, later on, TLR2, but not TLR4, is required for macrophage responses. Myeloid differentiation factor 88, an adaptor protein directly downstream of TLRs, is required for both early and late responses. TLR4, TLR2, and myeloid differentiation factor 88 are involved in murine host defense against *Salmonella* in vivo, which correlates with the defects in host defense observed in vitro. We propose a model where the sequential activation of TLRs tailors the immune response to different microbes. ***The Journal of Immunology*****, 2004, 172: 4463–4469.**

Toll-like receptors (TLRs)[2] participate in host defense against bacteria, viruses, fungi, and parasites (1, 2). These receptors signal in response to conserved microbial components such as LPS, bacterial lipoproteins (BLP), and dsRNA via TLR4, TLR2, and TLR3, respectively. Thought to be critical components of the immune response, TLRs recognize microbes, initiate the innate immune response, and modulate the adaptive immune system.

TLRs are one-pass transmembrane receptors that contain extracellular leucine-rich repeats and a characteristic intracellular Toll/IL-1R homology domain. Several adaptor molecules act directly downstream of TLRs (3–7). Among these adaptors, myeloid differentiation factor 88 (MyD88) is critical for TLR-mediated activation of the transcription factor NF-κB and hence the induction of proinflammatory cytokines such as TNF-α (8).

It is not known whether TLRs work together in host defense. The response of individual TLRs to their specific activators is well described (1). However, the relative roles of different TLRs in response to bacteria, which contain several TLR activators, are not clear. Furthermore, it is not known how differences in expression of TLRs contribute to host defense. Different cell types express varying combinations of TLRs, and TLR expression can be modulated based on the activation state of a cell. For example, resting mouse macrophages express TLR4, but extremely low levels of TLR2 and little or no TLR3 (9). Upon activation, TLR4 is down-regulated, and TLR2 and TLR3 are up-regulated (9).

All bacteria have TLR activators. Additionally, pathogenic bacteria make specialized proteins called virulence factors that target specific functions in the host cell. Here, we use infections with the enteric pathogen *Salmonella* as a model to study the role of TLRs during bacterial infection. *Salmonella* species cause enteric infections and typhoid fever and are a serious public health problem. *Salmonella typhi* infects ~17,000,000 people per year and is responsible for 600,000 deaths (10). *Salmonella* is acquired orally and invades the small intestine where it encounters resident tissue macrophages (11). If *Salmonella* evades the macrophages, it can spread to the mesenteric lymph nodes, eventually reaching the bloodstream, spleen, and liver. The inflammation induced by endotoxin, which contains LPS and BLP, is thought to be a major cause of the symptoms of salmonellosis. However, the relative contributions of LPS and BLP are not known.

Here, we show that TLR4 and TLR2 are required sequentially for efficient macrophage function in *Salmonella* infections. Using knockout mice, we show that TLR4, TLR2, and MyD88 are involved in the host response against *Salmonella* in vivo. We propose a model where the temporal involvement of TLRs is required for an effective immune response to different classes of pathogens.

Materials and Methods

Bacterial strains

Wild-type *Salmonella typhimurium* SL1344 (12) and the *Salmonella* pathogenicity island 1 (SPI1)-deficient mutant, *hilA*::mTn5Km2 (representing strain P4H2 from pools of signature tagged mutants) (13), were grown standing overnight in high salt Luria broth (0.3 M NaCl) supplemented with 200 μg/ml streptomycin.

Reagents

Highly purified *Salmonella minnesota* LPS was from List Biologicals (Campbell, CA) and synthetic $Pam^3CysSerLys^4$ (BLP) was from Boehringer-Mannheim Biochemica (Indianapolis, IN). Murine TNF-α ELISA was from R&D Systems (Minneapolis, MN). Anti-mouse TLR4/MD-2^{PE} and anti-mouse $TLR2^{FITC}$ were from eBioscience (San Diego, CA).

Mice

Mice were bred under specific pathogen-free conditions at New York University Medical Center (New York, NY) or the Bundesinstitut für Risikobewertung (Berlin, Germany). Mice were housed in filter-top cages and provided with sterile water and food ad libitum. $TLR4^{-/-}$ (14), $TLR2^{-/-}$ (15), and $MyD88^{-/-}$ (8) mice were described previously. We generated $TLR4^{-/-}TLR2^{-/-}$ mice by crossing $TLR4^{-/-}$ and $TLR2^{-/-}$ mice. C57BL/6 mice were from Taconic (Germantown, NY) or the Bundesinstitut für Risikobewertung. C3H/HeN and C3H/HeJ mice were purchased from Charles River Breeding Laboratories (Wilmington, MA).

Bone marrow-derived macrophages

Bone marrow-derived macrophages were prepared as described (16). Briefly, bone marrow was collected from the femur and tibia of mice. Bone marrow cells were plated on sterile petri dishes and incubated in DMEM containing 10% FCS, 5% horse serum, 10 mM HEPES, 1 mM pyruvate, 10

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[2] Abbreviations used in this paper: TLR, Toll-like receptor; BLP, bacterial lipoprotein; MyD88, myeloid differentiation factor 88; RSV, respiratory syncytial virus; MOI, multiplicity of infection; SPI1, *Salmonella* pathogenicity island 1.

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mM L-glutamine, and 20% M-CSF-conditioned medium. M-CSF-conditioned medium was collected from an L929 M-CSF cell line. Bone marrow cells were incubated at 37°C and 7% CO_2, and macrophages were harvested after 6 days. All assays were performed in standard tissue culture plates at 37°C and 7% CO_2 in similar medium excluding horse serum and M-CSF-conditioned medium.

Macrophage cytotoxicity assays

Ninety-six-well plates were seeded with 50,000 macrophages/well. Cells were allowed to adhere overnight and then washed the following day. Assays were performed in 200 μl of medium. Different amounts of bacteria were added, and plates were spun for 10 min at 850 $\times$ *g*. Gentamicin (100 μg/ml) was added at 30 min postinfection. At 6 and 24 h postinfection, 50 μl of supernatant were collected from each sample, and the amount of LDH was detected using a Cytotox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI) according to the manufacturer's specifications.

Bacterial killing assays

Twenty-four-well plates were seeded with 200,000 macrophages/well. Cells were allowed to adhere overnight and washed the following day. Assays were performed in 500 μl of medium. Bacteria were added at time zero, and plates were spun for 10 min at 850 $\times$ *g*. Gentamicin (100 μg/ml) was added at 30 min postinfection. Triplicate samples were collected at 30 min, 6 h, and 24 h postinfection. To determine the number of intracellular bacteria, medium was removed, and cells were lysed by adding 50 μl of 10% Triton X-100 for 10 min before addition of 450 μl of cold sterile PBS. Appropriate dilutions were made, and samples were plated on Luria agar plates containing 200 μg/ml streptomycin. Colonies were counted the next day.

TLR expression

Macrophages were treated with LPS (100 ng/ml) or left unstimulated. Cells were harvested at either 6 or 24 h and stained with anti-murine TLR4 or anti-murine TLR2 Abs, and staining was quantified using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ).

Measurement of TNF-α production

Supernatants from samples in the bacterial killing assays were frozen and later assayed by ELISA for determination of TNF-α concentrations according to the manufacturer's specifications. For experiments using LPS and BLP, 96-well plates were seeded with 50,000 macrophages per well. Cells were allowed to adhere overnight and then washed the following day. Assays were performed in 200 μl medium. LPS or BLP (100 ng/ml) were prepared and added to macrophages in medium supplemented with 0.05% sterile human serum albumin. Supernatants were collected and frozen until assayed by ELISA.

Infections

Age- and sex-matched mice were used for all experiments. Mice were starved for 14–16 h. Mice were then infected orally with 200 μl of the indicated number of *Salmonella* in sterile PBS using a gavage needle, and the food was replaced. For i.p. infections, mice were injected with the indicated number of *Salmonella* in 500 μl of sterile PBS.

For survival experiments, groups of 12–15 mice were infected and monitored each day, and survival was recorded. For bacterial colonization experiments, tissues were collected on the indicated days and then weighed and homogenized in 1 ml of sterile PBS. Serial dilutions were plated on Luria agar plates supplemented with 200 μg/ml streptomycin and CFU per

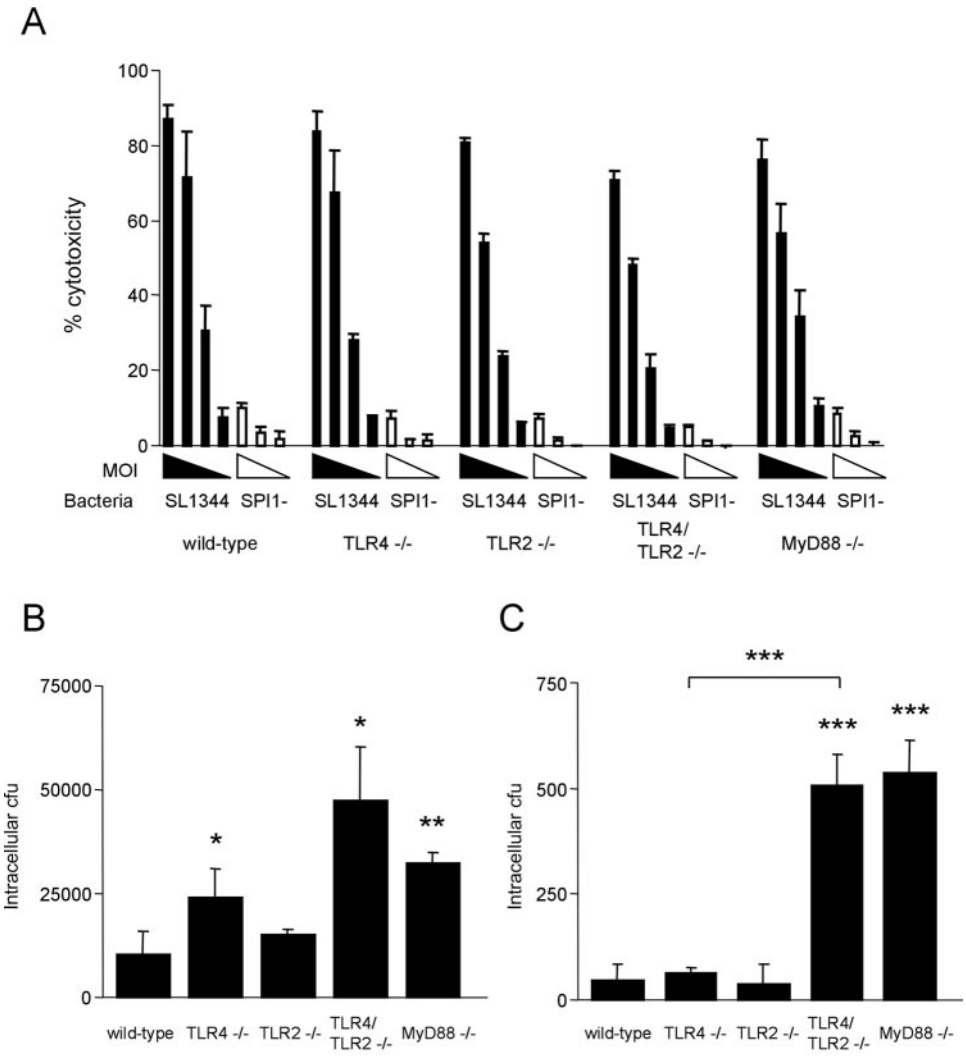

FIGURE 1. TLRs contribute to macrophage bactericidal activity. *A*, Bone marrow-derived macrophages were infected with wild-type *Salmonella* SL1344 at MOIs of 50:1, 15:1, 5:1, and 1:1 or with noncytotoxic SPI1 *Salmonella* at MOIs of 50:1, 15:1, and 1:1. Supernatants were collected in triplicate at 6 h postinfection and assayed for LDH, and the percent cytotoxicity was calculated. The data are representative of three independent experiments. *B* and *C*, Bone marrow-derived macrophages were infected with wild-type *Salmonella* at a MOI of 1:1, and cell lysates from triplicate samples were collected at 6 (*B*) and 24 (*C*) h postinfection. Levels of intracellular bacteria, or CFU, were quantified. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$ as compared with wild type. The data are representative of four independent experiments.

gram of tissue was calculated. The CFU/g value from each mouse was plotted on the graph, and the geometric mean of each group of mice is represented by a bar.

Statistics

Statistical significance was calculated using Student's *t* test for bacterial killing and TNF-α production assays, the χ^2 test for mouse survival experiments, and the Mann-Whitney *U* test for bacterial colonization experiments. Unless otherwise stated, all analyses compare knockout macrophages or mice with the wild-type control. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

Results

TLRs contribute to macrophage bactericidal activity

Salmonella can either kill or be killed by macrophages. *Salmonella* kills macrophages using virulence factors encoded in SPI1 (17). We first investigated whether TLR signaling contributes to *Salmonella*-induced macrophage cytotoxicity. *Salmonella* killing of macrophages derived from wild-type, TLR4, TLR2, TLR4/TLR2, or MyD88 knockout mice was similar at MOIs (multiplicity of infection) ranging from 1:1 to 50:1, at both 6 h (Fig. 1*A*) and 24 h postinfection (data not shown). Under these conditions, killing of macrophages was dependent on *Salmonella* virulence genes encoded in SPI1, because a SPI1-deficient strain did not kill macrophages (Fig. 1*A*).

To investigate whether TLRs are involved in macrophage killing of *Salmonella*, subsequent experiments were done using a MOI of 1:1, an infectious dose that is not cytotoxic to macrophages (Fig. 1*A*). Thirty minutes postinfection, macrophages from all the strains tested harbored similar numbers of bacteria, demonstrating that *Salmonella* uptake by macrophages is independent of TLR4, TLR2, and MyD88 (data not shown). Six hours postinfection, TLR4$^{-/-}$, TLR4/TLR2$^{-/-}$, and MyD88$^{-/-}$, but not TLR2$^{-/-}$ macrophages contained more *Salmonella* than wild-type cells (Fig. 1*B*). Twenty-four hours postinfection, however, only TLR4/TLR2$^{-/-}$ and MyD88$^{-/-}$ cells harbored more bacteria than wild-type macrophages (Fig. 1*C*). These results demonstrate that TLR4 plays an important role in the early killing of *Salmonella*. Later, TLR4 and TLR2 may be redundant, because the TLR4/TLR2$^{-/-}$ as well as the MyD88$^{-/-}$ cells, but not the TLR4 or TLR2 knockout cells, have a defect in bacterial killing.

TLRs are temporally involved in macrophage responses to TLR agonists and Salmonella

The early and late functions of TLR4 and TLR2, respectively, in killing of *Salmonella* correlates with the timing of their mRNA expression in mouse macrophages. TLR4 mRNA is expressed in resting mouse macrophages and TLR2 mRNA is expressed later, upon macrophage activation (9). We observed that TLRs are expressed at low levels on the surface of macrophages, as previously reported (18). Resting wild-type macrophages expressed TLR4, and upon activation with LPS, TLR4 was down-regulated (Fig. 2*A*) (19, 20). In contrast, resting wild-type macrophages expressed a very low level of TLR2 compared with TLR2$^{-/-}$ macrophages. Upon LPS stimulation, TLR2 was up-regulated at 24 but not at 6 h.

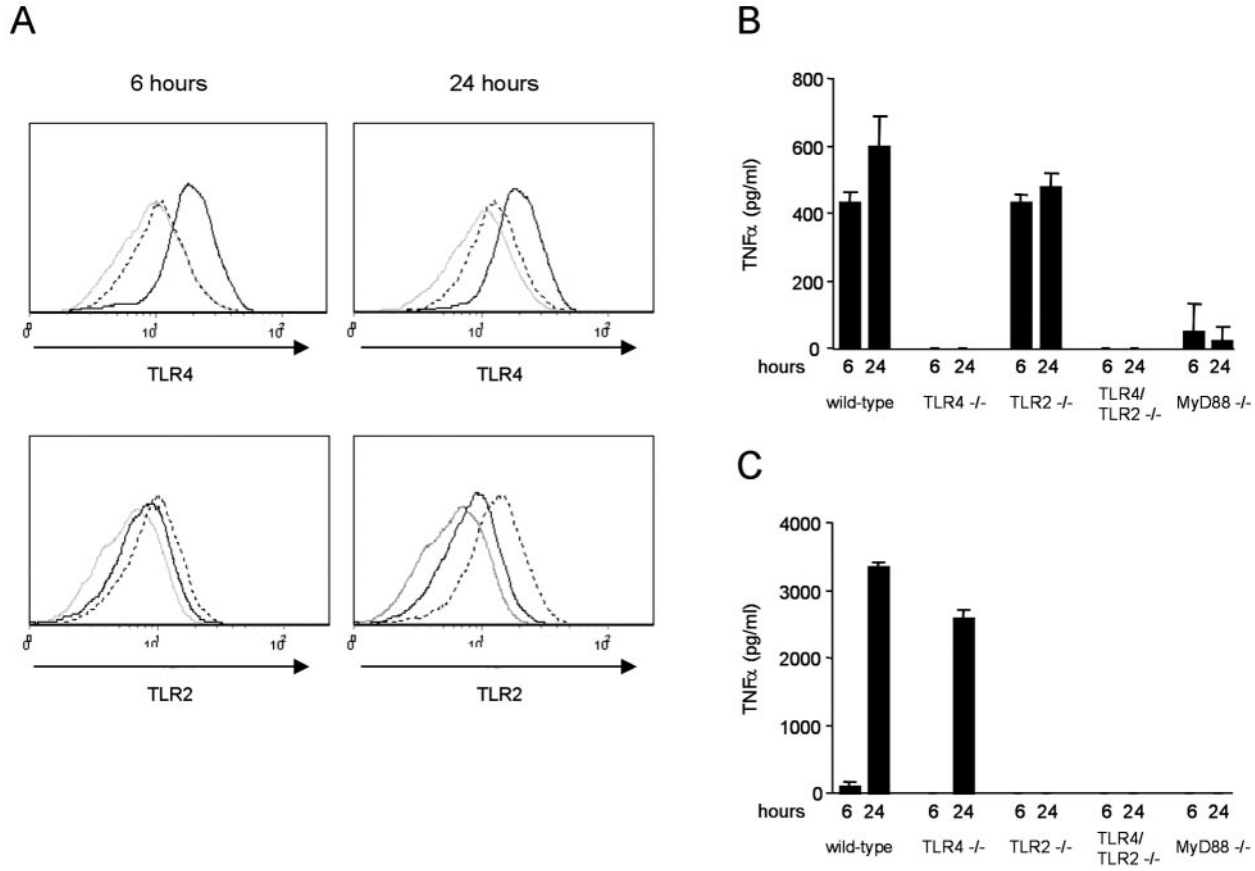

FIGURE 2. TLR4 is expressed and signals before TLR2 on macrophages. *A*, Unstimulated (solid black lines) or LPS-stimulated (dotted black lines) wild-type bone marrow-derived macrophages were collected at 6 and 24 h and stained with anti-TLR4 or anti-TLR2 Abs. Expression levels were quantified by FACS. Staining of negative control TLR4$^{-/-}$ (*top panels*) or TLR2$^{-/-}$ (*bottom panels*) macrophages is shown (gray lines). LPS (*B*) or BLP (*C*) was added to bone marrow-derived macrophages from the indicated mouse strains. Supernatants were collected at 6 and 24 h and assayed by ELISA for TNF-α concentration. Data are representative of three independent experiments.

These results support early and late roles for TLR4 and TLR2, respectively, in macrophage responses.

TLR signaling activates the production of TNF-α, a proinflammatory cytokine which is necessary to control *Salmonella* infection (21, 22). We investigated whether TLR4 and TLR2 are also sequentially involved in macrophage production of TNF-α in response to purified LPS and BLP, as well as *Salmonella*.

LPS induced the production of TNF-α by wild-type and TLR2$^{-/-}$ macrophages at 6 and 24 h (Fig. 2*B*). TLR4$^{-/-}$, TLR4/TLR2$^{-/-}$, and MyD88$^{-/-}$ cells did not produce TNF-α at either time point. The response to BLP in wild-type and TLR4$^{-/-}$ cells occurred only at 24 h and not at 6 h (Fig. 2*C*), in agreement with our data showing late TLR2 surface expression (Fig. 2*A*), and suggests that the low level of TLR2 expressed on resting macrophages is insufficient to induce TNF-α in response to BLP. TLR2$^{-/-}$, TLR4/TLR2$^{-/-}$, and MyD88$^{-/-}$ macrophages did not respond to BLP (Fig. 2*C*). These results show that TLR4 signals before TLR2 on macrophages. Furthermore, the timing and specificity of the macrophage response to LPS and BLP correlate with the expression levels of TLR4 and TLR2, respectively.

As shown in Fig. 3, infection with live *Salmonella* induced TNF-α production by wild-type macrophages at 6 and 24 h. TLR4$^{-/-}$ cells produced TNF-α at 24 h but not at 6 h. TLR2$^{-/-}$ macrophages responded similarly as wild-type cells. TLR4/TLR2 knockout cells did not release TNF-α at 6 h and produced less TNF-α than wild-type cells at 24 h. MyD88$^{-/-}$ cells did not produce TNF-α at either time point. Interestingly, each type of macrophage reacted similarly to live SPI1- or heat-killed *Salmonella* as to live wild-type *Salmonella* (data not shown), indicating that TLR responses are not affected by *Salmonella* virulence factors. These results show that the initial induction of TNF-α by *Salmonella* is mediated through TLR4 and that TLR2 is involved in the later response because TLR4$^{-/-}$ and TLR4/TLR2$^{-/-}$ cells act differently at 24 h. Another MyD88-dependent receptor is likely to be involved in the late response, because the phenotype of the MyD88 knockout cells is more profound than that of the TLR4/TLR2 knockout cells. These data correlate with the sequential roles of TLR4 and TLR2 in macrophage killing of *Salmonella*. Taken together, the data show that hierarchically, TLR4 is more important than TLR2 in innate immune responses to *Salmonella*, yet both are required for optimal activation.

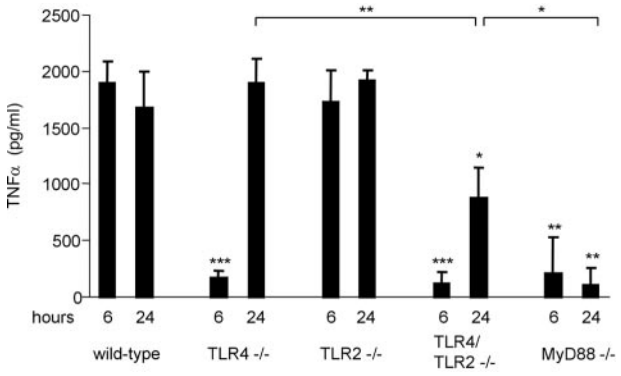

FIGURE 3. TLRs are sequentially involved in TNF-α production after *Salmonella* infection. Bone marrow-derived macrophages were infected with wild-type *Salmonella* at a MOI of 1:1. Supernatants were collected from triplicate samples at 6 and 24 h postinfection and assayed for TNF-α by ELISA. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$ as compared with wild-type. The data are representative of five independent experiments.

Susceptibility of knockout mice in vivo correlates with in vitro phenotypes

Because TLR4, TLR2, and MyD88 are involved in host defense against *Salmonella* in vitro, we tested their roles in in vivo infections. We first infected wild-type C57BL/6 and knockout mice i.p. This route of inoculation bypasses the initial intestinal infection and is a good model for the septicemic phase of the disease. In survival experiments, 50% of the wild-type mice died by day 8 postinfection (Fig. 4*A*). TLR4 knockout mice were more susceptible because 50% of the mice died by day 6 postinfection. TLR2$^{-/-}$ mice were similar to wild-type mice. TLR4/TLR2$^{-/-}$ mice were more susceptible than TLR4$^{-/-}$ mice, and MyD88$^{-/-}$ mice were the most susceptible, because 50% of the mice died by day 4.

In colonization experiments, spleens from TLR4 knockout mice contained >10-fold more bacteria than wild-type mice (Fig. 4*B*).

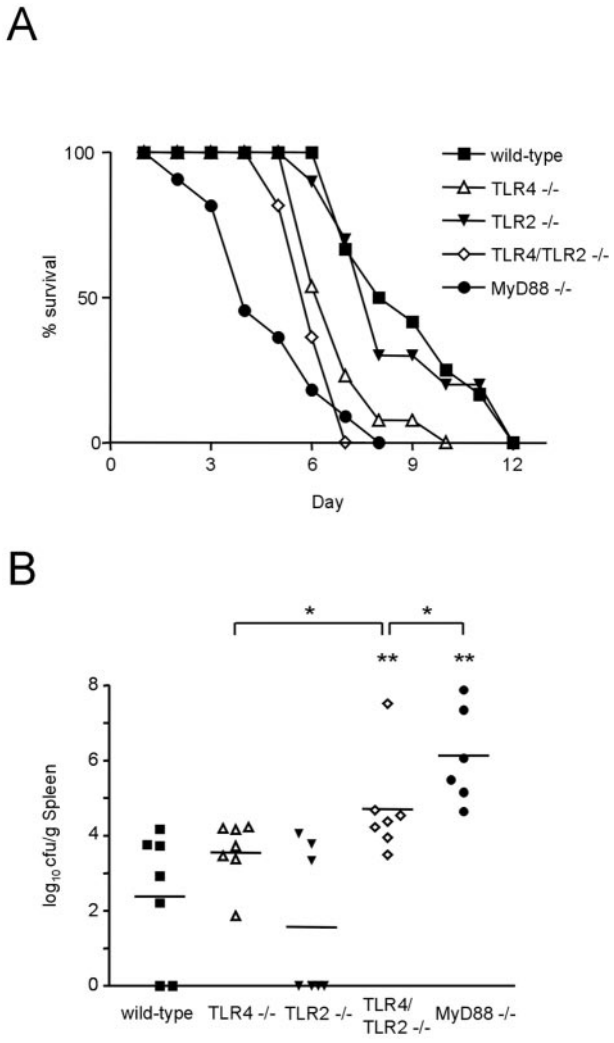

FIGURE 4. TLRs are required to control i.p. *Salmonella* infection. Mice were infected i.p. with 10 CFU *Salmonella* and assayed for survival (*A*) or bacterial colonization (*B*). *A*, Statistical significance as compared with wild-type mice: **, TLR4$^{-/-}$; ***, TLR4/TLR2$^{-/-}$; ***, MyD88$^{-/-}$. The difference between the TLR4$^{-/-}$ and the TLR4/TLR2$^{-/-}$ is statistically significant (*), as seen by pooling the results from two independent experiments. *B*, Spleens were collected on day 2 postinfection, and the number of bacteria per g of spleen was calculated. One MyD88$^{-/-}$ mouse died before organ collection on day 2. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$ as compared with wild-type. Data in *A* and *B* are representative of three independent experiments.

Surprisingly, TLR2$^{-/-}$ mice had lower levels of bacteria than wild-type mice. TLR4/TLR2$^{-/-}$ mice had over 100-fold more bacteria, and MyD88$^{-/-}$ mice harbored >1000-fold more bacteria than wild-type mice (Fig. 4*B*). As expected, higher bacterial loads correlated with earlier mortality. Taken together, the survival and bacterial colonization data show that TLR4 plays a more important role than TLR2 in host defense against *Salmonella*. However, both receptors and MyD88 are required for a competent immune response. Because MyD88$^{-/-}$ mice have a more profound phenotype than TLR4/TLR2$^{-/-}$ mice, it is likely that another MyD88-dependent receptor is involved. The susceptibility of mice to *Salmonella* infection in vivo correlates with defects in macrophage bactericidal activity and TNF-α production in vitro.

Salmonella is naturally acquired orally, so we investigated the roles of TLR4, TLR2 and MyD88 using a *Salmonella* oral infection model. In survival experiments, TLR4 knockout mice died earlier than wild-type or TLR2 knockout mice (Fig. 5*A*). TLR4/TLR2$^{-/-}$ and MyD88$^{-/-}$ mice were more susceptible than wild-type mice. However, the differences among TLR4$^{-/-}$, TLR4/TLR2$^{-/-}$, and MyD88$^{-/-}$ mice were less pronounced than in i.p. infections.

Compared with wild-type mice, orally infected TLR4$^{-/-}$ mice had 10- to 100-fold more bacteria in the Peyer's patches (data not shown) and mesenteric lymph nodes (Fig. 5*B*) and similar levels in the spleen (Fig. 5*C*). TLR2$^{-/-}$ mice had either slightly lower (Fig. 5*B*) or similar (Fig. 5*C*) bacterial loads than did wild-type mice. Interestingly, TLR2$^{-/-}$ mice have lower bacterial levels than did wild-type mice after oral infection with *Yersinia enterocolitica* (23). TLR4/TLR2$^{-/-}$ and MyD88$^{-/-}$ mice harbored 100- to 1000-fold more bacteria than wild-type mice in all organs tested (Fig. 5, *B* and *C*). Thus, in mice infected by the natural route, and similar to the results from the i.p. infections, TLR4 has a greater role than TLR2 in host defense against *Salmonella*, but both receptors and MyD88 are required for a wild-type response to infection.

Requirement for TLRs in host defense depends on the acuteness of the infection

Salmonella infections are acute and progress rapidly. Even a short lag in initial host defense against *Salmonella* could seriously alter the outcome of the infection. The data presented above indicate that TLR4 triggers the early response to *Salmonella*. We hypothesized that in less acute, low dose *Salmonella* infections, the need for an immediate host response would be diminished, and thus TLR4 would not be required. Mice lacking TLR4 would therefore show wild-type levels of susceptibility to *Salmonella* infection, whereas only mice completely deficient in both the immediate and late TLR-mediated responses would show increased susceptibility. As predicted, at a low infectious dose, wild-type, TLR4$^{-/-}$, TLR2$^{-/-}$, and TLR4/TLR2$^{-/-}$ mice were equally susceptible to *Salmonella* infection (Fig. 5*D*). MyD88$^{-/-}$ mice, which are defective in both the early and late responses, were more susceptible than all other mice tested. These results demonstrate that the requirement for TLRs in host defense depends on the acuteness of the infection.

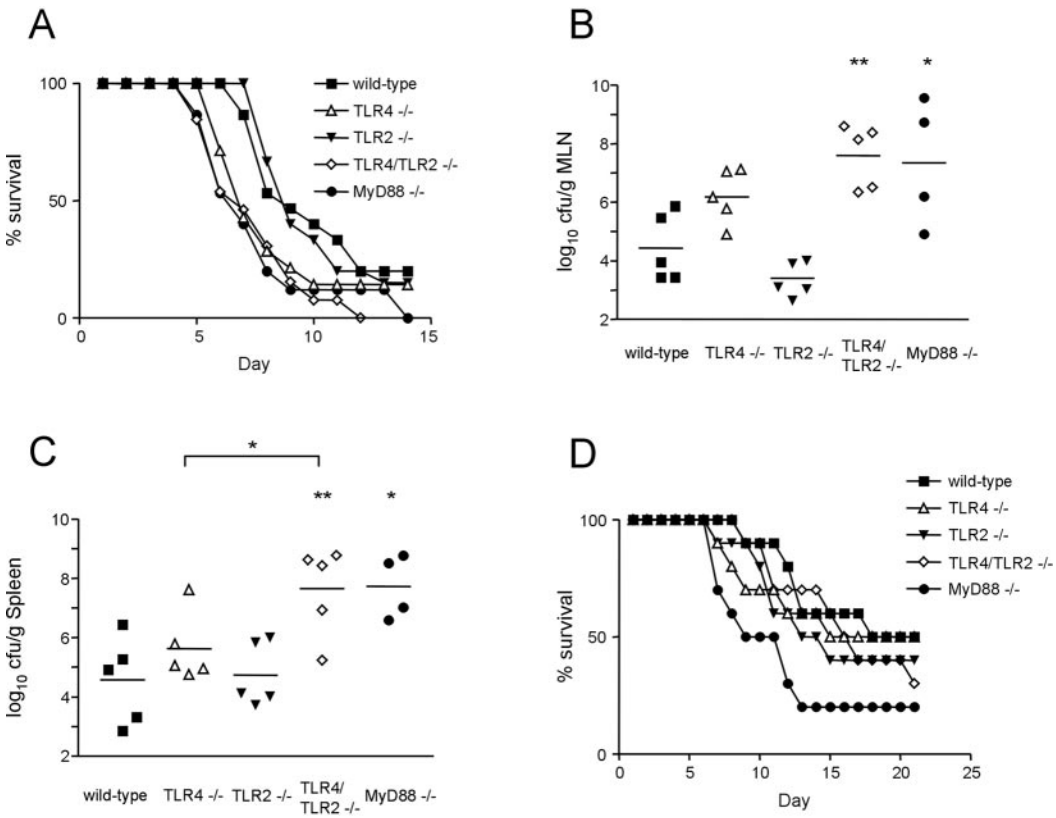

FIGURE 5. TLRs are required to control *Salmonella* oral infection. Mice were orally infected with 10^5 CFU of *Salmonella* and assayed for survival (*A*) or bacterial colonization (*B* and *C*). *A*, Statistical significance as compared with wild-type mice: *, TLR4/TLR2$^{-/-}$; *, MyD88$^{-/-}$. *B*, Mesenteric lymph nodes (MLN) and spleens (*C*) were collected on day 5 postinfection, bacteria were quantified, and the number of bacteria per g of tissue was calculated. One MyD88$^{-/-}$ mouse died before organ collection on day 5. *, $p < 0.05$; **, $p < 0.005$ as compared with wild-type. *D*, Mice were orally infected with 10^3 CFU *Salmonella* and assayed for survival. Statistical significance of MyD88$^{-/-}$ compared with wild-type mice. Data in all panels are representative of three independent experiments.

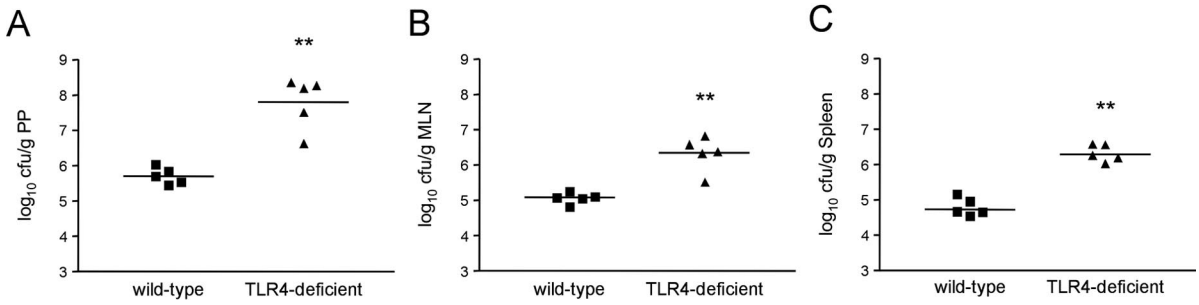

FIGURE 6. TLR4 contributes to host defense against *Salmonella* oral infection in C3H/HeN mice. Mice were orally infected with 10^9 CFU *Salmonella* and assayed for bacterial colonization in the Peyer's patches (PP; *A*), mesenteric lymph nodes (MLN; *B*) and spleen on day 10 postinfection (*C*). ■, Wild-type C3H/HeN mice; ▲, TLR4-deficient C3H/HeJ mice. **, $p < 0.005$ as compared with wild type. Data in each panel are representative of three independent experiments.

TLR4 contributes to host defense against Salmonella *oral infection in C3H/HeN mice*

The genetic background of mice can have a profound impact on their phenotype (24). To verify the requirement of TLR4 in host defense against *Salmonella* oral infection, we inoculated C3H/HeJ mice, which carry a dominant-negative mutation in TLR4 (25, 26). We used a high dose of bacteria because C3H/HeJ mice, and the control wild-type C3H/HeN mice, encode the wild-type phagosomal membrane protein, NRAMP1, that is important in host defense (27). This makes them more resistant to *Salmonella* infection than C57BL/6 mice that encode a mutant NRAMP1. In bacterial colonization experiments, TLR4-defective C3H/HeJ mice harbored 10- to 100-fold higher bacterial loads than control C3H/HeN mice in all organs tested (Fig. 6). These data confirm that TLR4 is required for host defense against *Salmonella* oral infection and show that this phenotype is independent of NRAMP1.

Discussion

We show that TLR4 and TLR2 are sequentially involved in macrophage responses to a bacterial pathogen. This correlates with the timing of their expression because TLR4 is expressed on resting mouse macrophages, whereas TLR2 is induced upon activation (Fig. 2*A*) (9). We show that macrophages produce TNF-α in response to LPS, the TLR4 agonist, at 6 h, but to BLP, the TLR2 agonist, only at 24 h (Fig. 2, *B* and *C*). Correspondingly, in *Salmonella*-infected cells, TLR4 is required for early TNF-α production, and TLR2 is involved in the late response (Fig. 3). MyD88, an adaptor protein that is critical for TLR-mediated cytokine production, is, as expected, required for both early and late responses. TLR4 and TLR2 show the same sequential involvement in macrophage killing of *Salmonella* (Fig. 1, *B* and *C*). Interestingly, TNF receptor p55 knockout macrophages also have a defect in killing *Salmonella*, suggesting that TLR-mediated TNF-α production may be required for efficient macrophage killing of intracellular *Salmonella* (22).

The role of TLR2 in host defense against *Salmonella* can be detected only by comparing TLR4$^{-/-}$ and TLR4/TLR2$^{-/-}$ cells. TLR2$^{-/-}$ macrophages are not deficient in host responses as compared with wild-type cells. These data suggest that the TLR4-dependent early response is sufficient for host defense independently of TLR2. In addition to TLR2, another late acting MyD88-dependent receptor may signal for host defense because, at 24 h postinfection, MyD88$^{-/-}$ macrophages produce less TNF-α than do TLR4/TLR2$^{-/-}$ cells (Fig. 3).

In *Salmonella* i.p. and oral infections in vivo, TLR4 has a predominant role over TLR2 in host survival and containment of *Salmonella* growth (Figs. 4 and 5). The reason for the greater requirement of TLR4 as compared with TLR2 may be the higher early expression of TLR4. An immediate host response is critical in acute *Salmonella* infections. A less acute, low dose infection, however, relieves the requirement for TLR4 in host defense against *Salmonella* (Fig. 5*D*). This supports a role for TLR4 during early stages of infection. The phenotype of the MyD88 knockout mice indicates that other MyD88-dependent receptors might play an essential role in vivo (Figs. 4 and 5). The hierarchy of TLRs described in vivo correlates with in vitro defects in macrophage bactericidal activity and TNF-α production. These in vivo results are in agreement with the proposed role of TLRs in host defense against other bacterial pathogens (reviewed in Ref. 28).

The protective role of TLR4 in host defense against *Salmonella* was confirmed in a different genetic background. C3H/HeJ mice are deficient in TLR4 and are more susceptible to *Salmonella* infections than the wild-type C3H/HeN mice, replicating the results obtained with knockout mice on the C57BL/6 background (Fig. 6). These oral infection results are in agreement with those of O'Brien et al. (29), who showed that C3H/HeJ mice are more susceptible to i.p. *Salmonella* infection. Surprisingly, O'Brien et al. observed a more profound difference in survival between C3H/HeN and C3H/HeJ mice than we see comparing C57BL/6 wild-type and TLR4$^{-/-}$ mice. This may be due to the differences in strains or growth conditions.

Endotoxin is thought to contribute to inflammation in *Salmonella* infections. Endotoxin is a mix of outer membrane components that includes both LPS and BLP. Here, we present results showing that TLR4$^{-/-}$ mice have a milder phenotype in *Salmonella* infections than do the TLR4/TLR2$^{-/-}$ and MyD88$^{-/-}$ mice. These results indicate that although LPS contributes to the inflammation and pathology observed in *Salmonella* infections, the combination of BLP and LPS, i.e., endotoxin, and maybe other activators, act in concert to induce inflammation.

Our finding that TLR4 is implicated early in host defense against the bacterial pathogen *Salmonella* is similar to results showing an early role for TLR4 in viral infections (30). These data suggest that TLR4 may be an early sensor of a broad range of microbes. TLR4 is involved in the host response against respiratory syncytial virus (RSV) and mouse mammary tumor virus (31–34). TLR4 activation up-regulates TLR3, which signals in response to dsRNA and also participates in antiviral defense (9). Both TLR4 and TLR3 induce the expression of a subset of antiviral genes, including IFN-β (35).

We propose a model for the cooperation of TLR4, TLR2 and TLR3 in host defense by mouse macrophages. TLR4 is an initial sentinel for both bacterial and viral infections. It is expressed on resting macrophages and is in place to signal immediately upon

infection. TLR4 activates the production of TNF-α, which plays a role in host defense against both bacterial and viral pathogens (36). TLR4 also induces the production of IFN-β which is protective against viral, but not bacterial, infections (37). Therefore, the initial TLR4-dependent macrophage response toward bacteria and viruses is similar.

Later in an infection, TLR4 is down-regulated on macrophages, whereas both TLR2 and TLR3 are up-regulated. Bacterial BLP can signal through TLR2, inducing further TNF-α production. TLR2, however, does not activate IFN-β (35). Bacteria do not contain dsRNA and cannot activate IFN-β via TLR3. In a viral infection, dsRNA activates TLR3, which signals for both TNF-α and IFN-β (35, 38). Therefore, at the late time point, the macrophage response to bacterial or viral infection is different.

This model proposes that the macrophage tailors its response depending on the type of infecting microbe and addresses the question of how TLRs can work together in host defense. It may explain the reason for the changes in TLR expression on macrophages. The sequential expression of TLRs provides initial broad protection and later customizes the immune response to different classes of microbes. Because macrophages are among the earliest cells to respond to infection, this system may represent one of the first ways in which the host tailors the immune response.

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