TLR9 and MyD88 Are Crucial for the Development of Protective Immunity to Malaria

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Malaria caused by infection with *Plasmodium* species of protozoan parasites contributes substantially to the health crisis and death tolls around the world (1). Malaria infection is characterized by dominant proinflammatory responses with Th1 cell development during early stages of infection that decrease as infection progresses with parallel increase in production of anti-inflammatory responses (2–4). A robust production of proinflammatory cytokine responses at initial stages of infection is necessary for the efficient development of protective cell-mediated and humoral immune responses (2–4). In contrast, excessive and/or prolonged proinflammatory responses lead to the development of severe malaria clinical conditions and fatal outcomes (2, 3, 5). To overcome the detrimental effects of inflammation, as infection progresses, the proinflammatory responses are downregulated (2, 3, 5).

TLRs, a family of pathogen recognition molecules that sense certain conserved structures of pathogens, play important roles in initiating innate inflammatory responses to various pathogenic infections, including malaria (6–8). In humans and mice together, 13 TLRs (TLR1 to TLR13) have been identified, and their ligand recognition specificities have been studied extensively (8, 9). The signal initiated upon TLRs sensing microbial components is transmitted through their highly conserved cytoplasmic Toll/IL-1R domains, which in most TLRs recruit a common adaptor protein initiating innate inflammatory responses to various pathogenic infections, including malaria (6–8). In humans and mice together, 13 TLRs (TLR1 to TLR13) have been identified, and their ligand recognition specificities have been studied extensively (8, 9). The signal initiated upon TLRs sensing microbial components is transmitted through their highly conserved cytoplasmic Toll/IL-1R domains, which in most TLRs recruit a common adaptor protein

Several TLRs have been reported to recognize different components of malaria parasites. TLR2 and TLR4 mediate the activation of macrophages by *Plasmodium falciparum* GPs (10), TLR4 recognizes heme and microparticles released from parasite-infected erythrocytes (11, 12), and TLR9 is a receptor for the activation of dendritic cells (DCs) by parasite DNA (13–15). Additionally, in human and mouse malaria parasites, profilin has been reported to activate DCs through TLR11 (16, 17). In mouse malaria parasites, *Plasmodium berghei, Plasmodium chabaudi chabaudi* AS, and *Plasmodium yoelii*, TLR2 and TLR9 have been reported to be involved in the activation of innate immune system (18–20). However, not much is known about the role of TLRs in the regulation of innate and adaptive cellular and humoral immunity to malaria infection.

In addition to TLRs, a family of intracellular receptors called nucleotide-binding oligomerization domain-like receptors senses microbial components or the endogenous danger signals and forms multiprotein complexes known as inflammasomes (21). The inflammasome is involved in the proteolytic cleavage of cell-associated pro–IL-1β and pro–IL-18 into secreted IL-1β and IL-18. The production of active IL-1β and IL-18 requires two signal components, as follows: 1) TLR-dependent activation of cells that leads to gene transcription and synthesis of pro–IL-1β and pro–IL-
18, and 2) activation of caspase-1 of the inflammasome complex, most likely by a second microbial stimuli, resulting in the cleavage of pro–IL-1β and pro–IL-18 into active cytokines. In malaria, NALP3-mediated inflammasome has been reported to be involved in hemozoin- and uric acid-induced maturation of pro–IL-1β and pro–IL-18 (22, 23).

Among cells of the innate immune system, DCs play crucial roles in TLR- and other pathogen-specific receptor-mediated recognition, and initiation of innate immune responses and development of adaptive immunity (24–26). At early stages of malaria infection, DCs efficiently produce proinflammatory cytokines, and, as the infection progresses, their ability to produce proinflammatory responses becomes low, but acquires increased capacity to produce anti-inflammatory responses (27). Additionally, DCs activate NK cells, instruct T cells to induce programmed Th1/Th2 responses, and initiate the development of cell-mediated and humoral adaptive immunity (24, 25, 28, 29). Thus, DCs provide a critical link between the innate and adaptive immune responses and help shape the pathogen-specific adaptive immune responses. TLRs and MyD88 are also expressed by T and B cells and play important roles in the function of these cells (30–32). For example, MyD88-mediated signaling is essential for T cell-mediated resistance to Toxoplasma gondii (33), and TLR2-MyD88-mediated signaling is necessary for CD8+ T cell clonal expansion and memory cell formation (34, 35). MyD88 has also been shown to regulate virus-specific CD4+ T cell responses (36), virus-induced B cell activation, and Ab production and Ig class switching to IgG2c (37, 38).

Of different immunostimulatory components of malaria parasites that activate TLRs (10–17), parasite protein-DNA complex/nucleosome is the major factor that activates DCs through TLR9/MyD88-mediated signaling and induces the production of proinflammatory responses (15, 39). Given that the cytokine milieu of the initial immune responses determines the effectiveness of adaptive immune responses, we hypothesized that TLR9 and MyD88 play crucial roles in the regulation of Th1/Th2 development and cellular and humoral adaptive immunity to malaria. In this study, we tested this hypothesis by studying innate and adaptive immune responses to the blood stage P. yoelii mouse malaria infection. The results show that TLR9 and MyD88 are critical for the robust proinflammatory responses, Th1 development, and efficient cell-mediated and humoral immunity to malaria infection. Hence, the deficiency in TLR9 and MyD88 resulted in the decreased capacity of DCs to produce proinflammatory cytokines with the increased ability to elicit anti-inflammatory cytokine responses, impaired NK and CD8+ T cell cytotoxic activity, and increased Th2-type Ab responses. Consequently, TLR9−/− and MyD88−/− mice harbored significantly higher parasitemia and exhibit lower survival rates than wild-type (WT) mice.

Materials and Methods

Reagents

DMEM and RPMI 1640 medium were purchased from Mediatech (Manassas, VA). Penicillin/Streptomycin solution was from Invitrogen (Carlsbad, CA). FBS was purchased from Atlanta Biologicals (Lawrenceville, GA). Collagenase D was from Roche Applied Science (Mannheim, Germany). Coating and biotin-labeled detecting Abs against mouse against mouse IgG1, IgG2a, and rat IgG2a isotype control; PerCP-Cy5.5–conjugated anti-CD8a mAb (53-6.7); allopurinol-conjugated-anti CD8a mAb (clone 145-2C11), mouse CD40 (1C10), and mouse IFN-γ (XM12); and rat IgG1 isotype control were from eBioscience (San Diego, CA). PerCP-Cy5.5–conjugated anti-mouse IL-4 (11B11) and PE-conjugated anti-mouse IL-12 Ab (clone 16.5) were from BD Biosciences (San Jose, CA).

Ethics statement

The Institutional Animal Care and Use Committee of the Pennsylvania State University College of Medicine, Hershey, has reviewed and approved the protocols for use of animals in this study. The animal care was according to the institutional guidelines of the Pennsylvania State University College of Medicine.

Mice

WT, TLR2−/−, TLR4−/−, and MyD88 knockout mice, and OT-II transgenic mice expressing TCR for OVA323–339 peptide on CD4 T cells were housed in a pathogen-free environment. All mice used in this study were in C57BL/6j background.

Parasite infection, parasitism, and survival rate measurements

WT C57BL/6 mice were infected with cryopreserved nonlethal P. yoelii (Py17XLN strain) parasites, and blood from these mice was used for infecting experimental mice. Experimental mice were infected by i.p. injection of 1 × 106 infected erythrocytes from infected donor mice in 100 μl saline. Parasitemia was monitored on alternative days postinfection by examining Giemsa-stained thin smears of tail blood on glass slides, and results were expressed as percentage of parasite-infected erythrocytes. Mice were monitored for survival twice per day. Blood was collected, and sera were prepared and stored at −80°C until used for cytokine and Ab analyses by ELISA (15). Mice were also infected as above with P. berghei NK65 strain. Infected RBCs from P. yoelii- and P. berghei NK65-infected mice were isolated and used for testing the Ag specificity of cytotoxic CD8+ T cells.

Preparation of Fms-like tyrosine kinase 3 ligand- and GM-CSF–differentiated DCs

Fms-like tyrosine kinase 3 (FLT3) ligand-differentiated DCs (FL-DCs) were prepared by culturing mouse bone marrow cells in complete DMEM supplemented with 15% of FLT3 ligand-containing conditioned medium, which is obtained by culturing B16 cells expressing retrovirus-coded FLT3 ligand (15, 40).

GM-CSF–differentiated DCs (GM-DCs) were obtained by culturing bone marrow cells from WT mice for 7 or 8 d in complete DMEM containing 10% conditioned DMEM from the cultured GM-CSF–producing cells (41).
Isolation of spleen and liver cells

Single-cell suspensions of mouse spleens were prepared, as described previously (15), and used for the isolation of total T cells and CD8+ T cells by magnetic column separation (MACS). Total T cells from mouse spleens were isolated using anti-mouse CD90.2 Ab-conjugated magnetic beads; the purity of cells as assessed by flow cytometry after staining with anti-CD3ε Ab was ~90%. CD8+ T cells were isolated using CD8αε T cell isolation kit; the purity of cells by flow cytometry analysis after staining with anti-CD3ε and anti-CD8αε Abs was ~92%.

DCs were isolated from the single-cell suspensions of mouse spleens, prepared by digesting with 1 mg/ml collagenase D (15), by MACS after staining with anti-mouse NK1.1 Ab was NK cell isolation kit. The purity of cells by flow cytometry analysis after collected and washed; and NK cells were isolated by MACS using the mouse NK cell isolation kit. The purity of cells by flow cytometry after staining with anti-CD11c Ab was ~90%.

For NK cell isolation, livers were flushed with 10 ml PBS (pH 7.2), crushed, and filtered through a 70-mm strainer to obtain single-cell suspensions. The cell suspensions were centrifuged on Isolymph cushions at 1200 × g for 15 min; buffy coat on the top of Isolymph was collected and washed; and NK cells were isolated by MACS using the mouse NK cell isolation kit. The purity of cells as assessed by flow cytometry after staining with anti-mouse NK1.1 Ab was ~60%.

Isolation of P. yoelii- and P. berghei-infected RBCs and preparation of cell lysates

Erythrocyte pellets from blood samples of parasite-infected mice were suspended in 2 vol of PBS (pH 7.2) and centrifuged on Isolymph cushions at 1200 × g at 4°C for 15 min, and the buffy coat was removed. The erythrocyte pellets were resuspended in 2 vol of PBS and centrifuged on 75% Percoll cushions at 1200 × g at 4°C for 15 min. The IRBCs on the top of Percoll were collected and washed.

Lysates of P. yoelii IRBCs were prepared by alternative freezing and thawing of IRBC suspensions three times, followed by sonication in water-bath sonicator for 5 min. After centrifugation at 13,000 rpm in microcentrifuge for 10 min, the protein contents in the clear supernatants were measured using Pierce micro bichinonic acid protein estimation kit (Thermo Scientific, Rockford, IL), and used for Ab analysis in mice sera.

Stimulation of DCs and cocultures of DCs plus OT-II T cells

Mouse spleen DCs (1 × 10^7/well) from infected mice were seeded into 96-well plates. Cells in 200 μl complete DMEM were stimulated with standard TLR ligands, as follows: Pam3CysK (TLR2 ligand, 10 ng/ml), polyinosinic-polycytidylic acid (TLR3 ligand, 2 μg/ml), LPS (TLR4 ligand, 100 ng/ml), or CpG oligodeoxynucleotide (TLR9 ligand, 2 μg/ml). After 24 h, the culture supernatants were collected and cytokines were measured by ELISA (15). In coculturing experiments, spleen DCs (1 × 10^5/well) from infected mice were either untreated or treated with 2 μg/ml OVA25-33 peptide and cocultured with spleen T cells (0.5 × 10^6/well) from the naive OT-II transgenic mice that express OVA-specific TCR in 96-well U-bottom plates containing 200 μl complete medium. OT-II T cells alone were similarly stimulated with OVA25-33 peptide as controls. After 72 h, the culture supernatants were collected, and cytokines were analyzed by ELISA (15).

Flow cytometry analysis of cytokine expression and costimulatory molecules

To determine the cell types that produce cytokines in coculture experiments, cells were stimulated and treated with GolgiPlug (BD Biosciences) for 6 h. Cells were harvested, treated with Fc block (anti-mouse CD16/32 Ab), and stained for surface markers followed by intracellular staining with anti-cytokine Abs (15). The survival of DCs was assessed by flow cytometry using FACSCalibur (BD Biosciences) after staining with annexin V.

For the analysis of costimulatory molecules, total spleen cells from WT, TLR9−/− and MyD88−/− mice at 3 d postinfection were stained with dye-conjugated Abs against costimulatory molecules. After washing, cells were analyzed by flow cytometry, and the results were analyzed using CellQuest software (BD Biosciences).

Restimulation of T cells from P. yoelii-infected mice

Total spleen T cells isolated from the parasite-infected mice were allowed to rest overnight in complete DMEM and plated (1 × 10^5 cells/well) into 96-well U-bottom plates. To each well was added FL-DCs (5 × 10^5/well) and P. yoelii IRBCs (1.5 × 10^6/well) in 200 μl complete DMEM. After 72 h, the culture supernatants were collected and cytokines were measured by ELISA. Supernatants of T cells cocultured with WT FL-DCs were analyzed by flow cytometry, and the results were analyzed using CellQuest software (BD Biosciences).

Statistical analysis

The data were plotted as mean values ± SD or SEM. Statistical analysis of data was performed by one-way ANOVA, followed by the Newman–Keuls test. GraphPad Prism software version 3.0 was used for the analysis. A p value <0.05 was considered statistically significant.

Results

TLR9 and MyD88 regulate malaria-induced DC functions

Malaria infection is characterized by a robust proinflammatory cytokine production during the early stages of infection that declines as infection progresses with parallel gradual increase in the production of anti-inflammatory cytokines (2–4). The initial cytokine responses play important roles in the regulation and development of immunity to malaria (2–5). Although several TLRs, including TLR9, TLR2, and TLR4, have been shown to recognize various components of malaria parasites and induce proinflammatory cytokines (10–17), which of these TLRs play key roles in the generation of protective immunity to malaria remains unknown. Because DCs are central to the development of innate and adaptive immunity to malaria (2–4), we first studied the roles of TLRs and their key shared adaptor protein MyD88 in the regulation of pro- and anti-inflammatory cytokine responses by DCs isolated from the spleen of WT, TLR2−/−, TLR4−/−, TLR9−/−, or MyD88−/− mice infected with P. yoelii. At the early stages of infection (5 d postinfection), when the parasite is establishing a stable infection (see later), DCs obtained from WT, TLR2−/−, and TLR4−/− mice, but not those from TLR9−/− and
MyD88−/− mice, efficiently produced TNF-α and IL-12 (Fig. 1). At the later stages (10 and 17 d) of infection, that is, when parasites grew exponentially, the levels of these cytokines produced by DCs from the WT, TLR2−/−, and TLR4−/− mice were markedly declined. In parallel, however, DCs from these mice at 10 and 17 d postinfection produced increased levels of IL-4 as compared with DCs from the infected mice at 5 d postinfection. The expression of IL-10 was also modestly increased in DCs from mice at 10 d postinfection compared with cells from mice at 5 d postinfection, but decreased considerably in DCs from mice at 17 d postinfection when parasitemia was high (Fig. 1). Furthermore, DCs produced high levels of cytokines in response to standard immunostimulatory ligands in TLR-specific manner (Supplemental Fig. 1). Together these results indicated that at the early stages of infection, DCs produce mainly proinflammatory responses, which are known to instruct the immune system to develop effective Th1 adaptive immunity for parasite clearance, whereas at later stages of infection these cells produce primarily anti-inflammatory cytokines, presumably leading to Th2 responses.

In contrast to infected WT, TLR2−/−, and TLR4−/− mice, DCs from infected TLR9−/− and MyD88−/− mice produced low levels of TNF-α and IL-12, and higher levels of IL-10 and IL-4, at all stages of infection (Fig. 1). Notably, at early stages of infection, TNF-α and IL-12 levels produced by DCs from infected TLR9−/− or MyD88−/− mice were comparable to those produced by DCs from the infected WT, TLR2−/−, and TLR4−/− mice at later stages (10 and 17 d) of infection. Collectively, these results revealed that TLR9- and MyD88-mediated signaling dominantly drives proinflammatory cytokine responses to malaria parasites during the early stages of infection, and thus, the deficiency in TLR9 or MyD88 leads to type 2 cytokine/anti-inflammatory responses by DCs. The results are consistent with our recent report that TLR9 is the major sensor of P. falciparum that mediates proinflammatory responses (15).

Given that cytokine responses by DCs from infected TLR9−/− and MyD88−/− mice were markedly different from those by DCs from infected WT mice, it was of interest to determine whether DCs from these mice differ in their activation status. Therefore, we evaluated maturation of DCs in the infected animals by analyzing surface expression of costimulatory molecules. Spleen DCs from WT, TLR9−/−, and MyD88−/− mice at 3 d postinfection exhibited significantly increased levels of CD40, CD80, and CD86 than DCs from naive WT mice (Supplemental Fig. 2). These results demonstrate that, regardless of the critical requirement of TLR9 and MyD88 for the production of proinflammatory cytokines by DCs, malaria parasites can activate and induce maturation of DCs in TLR9- and MyD88-independent manner. These results agree with the ability of DCs from infected TLR9−/− and MyD88−/− mice to efficiently produce anti-inflammatory responses (see Fig. 1).

To further understand the role of TLR9 and MyD88 in malaria parasite-induced function of DCs, we assessed the ability of ex vivo DCs to induce cytokine responses in T cells. DCs from the spleens of mice at 5, 10, and 17 d postinfection were cocultured with OT-II T cells and treated with OVA peptide, and the levels of TNF-α, IL-12, IFN-γ, IL-4, and IL-10 in the culture supernatants were analyzed by ELISA. The cytokine levels produced by the cocultures were significantly higher than those produced by the control DCs (p < 0.05 to < 0.001) (Fig. 2A–E). Intracellular staining with anti-cytokine Abs and flow cytometry analysis showed that DCs from mice at both 5 and 10 d postinfection can efficiently induce the production of TNF-α and IFN-γ by OT-II T cells (Fig. 2F); cells were gated as indicated in Supplemental Fig. 3A. DCs from mice at 5 d postinfection induced substantially higher levels of TNF-α and IFN-γ production by OT-II T cells than DCs from mice at 10 d postinfection. These results are consistent with a previous report, based on ELISA analysis of cytokines produced by cocultures, that the ability of DCs from malaria-infected mice to induce T cells to produce proinflammatory responses decreases as the infection progresses (27). However, in contrast (27), DCs from mice at both 5 and 10 d postinfection were unable to induce detectable levels of IL-4 and IL-10 by OT-II T cells (Supplemental Fig. 3B and data not shown), even though the cocultures produced substantially higher levels of these cytokines than DCs from control mice. Therefore, it appears that, in vivo, besides DCs, cytokine milieu and other APCs such as macrophages and mast cells influence T cells to produce IL-4 and IL-10. Macrophages uptake IRBCs efficiently and produce IL-10 and IL-4 (44) (X. Wu, N.M. Gowda, and D.C. Gowda, unpublished results). Consistent with the fact that T cells produce little or no IL-12 (45), the expression of this cytokine by OT-II T cells was not evident. However, interestingly, the cocultures produced significantly higher levels of IL-12 than control DCs (Fig. 2C). Therefore, the observed increased production of IL-4, IL-10, and IL-12 by cocultures, even though OT-II T cells were unable to produce

**FIGURE 1.** TLR9 and MyD88 regulate pro- and anti-inflammatory cytokine production by DCs in malaria parasite-infected mice. Spleen DCs, isolated from P. yoelii-infected WT, TLR2−/−, TLR4−/−, TLR9−/−, and MyD88−/− mice at 5, 10, and 17 d postinfection were cultured in 96-well plates. After 24 h, TNF-α (A), IL-12 (B), IL-10 (C), and IL-4 (D) released into the culture media were analyzed by ELISA. Spleen DCs from naive WT mice were analyzed as controls. Experiments were performed three times, and, each time, ELISA was performed in duplicates. The results of a representative experiment are shown. Error bars represent mean values ± SD. The letters a, b, and c denote the statistical significance between the levels of cytokines produced by DCs from the indicated gene knockout mice and those produced by DCs from the corresponding infected WT mice. 

*p < 0.001, †p < 0.01, ‡p < 0.05.

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these cytokines, suggested that OT-II T cells in turn influenced DCs to induce increased expression of cytokines and/or enabled DCs to survive longer, leading to increased production of cytokines. The influence of T cells in inducing DCs to produce higher levels of cytokines is also evident from enhanced production of TNF-α and IL-4 by DCs cocultured with OT-II T cells as compared with DCs cultured alone (Supplemental Fig. 3C). To determine the possibility that ligation of OT-II T cells leads to increased life span of DCs in coculture, we assessed the duration of survival of cocultured DCs by measuring the levels of cell death at different time points by flow cytometry after staining with annexin V.

It is known that annexin V binds phosphatidylserine expressed on the surface of apoptotic cells and the annexin V-binding assay is used for measuring the extent of cell death (46, 47). The results showed that the levels of annexin V-positive DCs were substantially higher in control DC culture than that in coculture (Fig. 2G). Together the above data clearly demonstrated that ligation of OT-II T cells leads to increased life span of DCs, and hence, increased production of cytokines by DCs. These results are consistent with the previous report that ligation of CD40 and CD40L leads to increased production of IL-12 by DCs (48, 49).

Furthermore, in three independent experiments, we consistently found that OT-II T cells cocultured with spleen DCs from infected TLR9−/− and MyD88−/− mice produced noticeably lower levels of IFN-γ than OT-II cells cocultured with DCs from infected WT mice, although there was no difference in the level of TNF-α production (Fig. 2F). However, in all three groups, OT-II T cells cocultured with DCs from mice at 5 d postinfection produced markedly higher levels of TNF-α and IFN-γ than OT-II T cells cocultured with DCs from mice at 10 d postinfection. Thus, these
results demonstrated that DCs are programmed to induce a strong Th1 development at early stages of infection in a TLR9- and MyD88-dependent manner, and that the ability of DCs to induce Th1 polarization decreases as the infection progresses.

**TLR9 and MyD88 differentially regulate cytokine production in response to malaria infection**

In addition to DCs, various other cell types, including T and B cells, produce cytokines, contributing to serum cytokine profiles of malaria-infected mice. This is especially the case at later stages of infection. To determine the role of TLR9 and MyD88 on cytokine production in vivo in response to malaria infection, we analyzed cytokine responses in *P. yoelii*-infected WT, TLR2−/−, TLR4−/−, TLR9−/−, and MyD88−/− mice. The serum TNF-α, IL-12, IL-10, and IL-4 profile in WT, TLR2−/−, TLR4−/−, and TLR9−/− mice at the early stages of infection was similar to those produced by DC ex vivo (Fig. 3), that is, higher levels of proinflammatory cytokines, including IFN-γ, and lower Th2-type/anti-inflammatory cytokines by WT, TLR2−/−, and TLR4−/− mice, and vice versa by TLR9−/− mice. However, at the later stages of infection, whereas the profiles of TNF-α and IL-10 in the sera of infected WT, TLR2−/−, and TLR4−/− mice were comparable to those produced by ex vivo DCs, the serum levels of IL-12 and IL-4 were substantially increased in all these mice.

Notably, in contrast to cytokine profiles observed by DC ex vivo (see Fig. 1), the infected TLR9−/− mice presented a distinct cytokine profile at the later stages of infection, that is, significantly higher levels of IL-12 and IL-4 compared with WT, TLR2−/−, and TLR4−/− mice (Fig. 3). Furthermore, interestingly, in the case of infected MyD88−/− mice, the levels of both pro- and anti-inflammatory cytokines were markedly low at all stages of infection, despite the fact that ex vivo DCs produced significantly higher levels of IL-10 and IL-4 than DCs from the infected WT, TLR2−/−, and TLR4−/− mice (compare Figs. 1 and 3). Because cytokine profiles of DCs from WT, TLR2−/−, and TLR4−/− mice were more or less comparable to those observed in vivo in these mice at early stages of infection (compare Figs. 1 and 3), it appears that, during early stages of infection, DCs are the major contributors to the cytokine profiles observed in infected mice. However, at later stages of infection, it appears that other cell types, such as T and B cells, contribute to the production of cytokines in a TLR9-independent and MyD88-dependent manner. Thus, the results demonstrate that MyD88−/− mice, but not TLR9−/− mice, have intrinsic defect in producing pro- and anti-inflammatory cytokines in response to malaria infection (Fig. 3), although DCs deficient in TLR9 and MyD88 produce similar cytokine profiles (see Fig. 1).

**TLR9 and MyD88 contribute to cytokine production by T and B cells in response to malaria infection**

To test the prediction that, at late infection stages, T and B cells significantly contribute to the observed serum cytokine profiles (see above), we first analyzed cytokines produced by T cells. Intracellular staining showed that T cells from WT, TLR9−/−, and MyD88−/− mice at 10 and 17 d postinfection produced substantial levels of IFN-γ and IL-4 (Fig. 4A, 4B). The proinflammatory cytokine expression by T cells from the infected TLR9−/− and MyD88−/− mice was appreciably lower than that by T cells from WT mice. We next analyzed cytokine responses by T cells isolated from mice at 10 d postinfection after restimulation with *P. yoelii* IRBC Ags presented in vitro generated DCs. Previously, we showed that IRBCs can induce efficient production of cytokines by FL-DCs and spleen DCs, and that the cytokine profiles produced by these cells are similar (15). In this study, we used FL-DCs from WT mice as APCs for the restimulation of T cells from infected mice. Cytokine profiles produced by T cells restimulated with IRBC-treated FL-DCs were similar to those produced by unstimulated ex vivo T cells (Supplemental Fig. 4A, and compare Supplemental Fig. 4A with Fig. 4B). Together, the above results indicate that cytokine production by T cells in response to malaria...
infection is to a certain extent TLR9 and MyD88 dependent, and that the cytokines produced by T cells contribute significantly to the serum cytokine profiles of parasite-infected WT, TLR9^{-/-}, and MyD88^{-/-} mice.

We next measured cytokine levels in the cocultures of spleen T cells from WT, TLR9^{-/-}, and MyD88^{-/-} mice at 10 and 17 d postinfection and FL-DCs from WT mice, in which T cells were restimulated with *P. yoelii* IRBCs presented by FL-DCs. The profiles of TNF-α, IFN-γ, IL-12, IL-4, and IL-10 produced were nearly comparable to those of serum cytokines in the respective infected mice (Fig. 4C, 4E, see Fig. 3). The levels of cytokines produced by the cocultures were significantly higher than those secreted by the IRBC-stimulated WT DCs alone (Fig. 4C, 4E). Furthermore, in the case of T cells from WT mice at 10 d postinfection, the cocultures produced significantly higher levels of TNF-α, IFN-γ, and IL-12 than the case of TLR9^{-/-}
TLR9 and MyD88 regulate malaria immunity

We also assessed cytokine responses by B cells at 10 and 17 d postinfection by intracellular staining and flow cytometry. Although B cells are known to produce a wide range of cytokines, including TNF-α, IFN-γ, IL-12, IL-10, and IL-4, we analyzed IL-12 and IL-4 production as representatives of proinflammatory and type 2 cytokine responses, respectively. B cells from infected WT, TLR9−/− mice showed markedly low levels of IL-12 and IL-10 at 17 d postinfection, the cocultures produced appreciable levels of both IL-12 and IL-4. However, the levels of cytokines produced by B cells from MyD88−/− mice were lower than those produced by WT and TLR9−/− mice (Fig. 4G). Although detailed analysis of cytokine responses by B cells from WT and TLR knockout mice has not been done in the current study, the above results suggest that B cells produce substantial levels of cytokines in response to malaria infection, contributing to the serum cytokine profiles of parasite-infected mice.

Overall, the results of the above analyses demonstrate that the cytokines produced by T and B cells from infected WT, TLR9−/−, and MyD88−/− mice contribute substantially to the serum cytokine profiles of the respective mice. The results further demonstrate that TLR9 and MyD88 distinctively regulate cytokine responses by T and B cells in response to malaria infection.

TLR9-independent/MyD88-dependent and/or IL-1R/IL-18R-mediated signaling also contribute to cytokine responses to malaria parasites

Whereas the malaria parasite-induced function of DCs was dependent on both TLR9 and MyD88 (see Fig. 1, and also see Ref. 15), the serum cytokine responses to malaria infection were significantly independent of TLR9, but largely dependent on MyD88 (see Fig. 3). To gain insight into how TLR9 and MyD88 differentially regulate immune responses to malaria, we performed the following studies. The malaria parasite hemozoin has been reported to activate inflammasome-mediated signaling to induce the production of IL-1β (22, 23). To determine the contribution of inflammasome-mediated signaling, we measured the levels of IL-1β and IL-18 in the sera of P. yoelii-infected mice. Both IL-1β and IL-18 were produced throughout the course of infection (Fig. 5). The production of IL-1β decreased as the infection progressed, whereas that of IL-18 increased with increasing parasitemia. Furthermore, although the production of both cytokines was dependent, to some extent, on TLR9 at early stages of infection, it was mostly independent of TLR9 at later stages of infection (Fig. 5). These results suggested that inflammasome also plays an important role in immune responses to malaria infection. Furthermore, because infected MyD88−/−, but not TLR9−/− mice showed markedly low levels of IL-1β and IL-18, collectively, the above results suggested that TLR9-independent/MyD88-dependent and/or IL-1R/IL-18R-mediated signaling is also involved in the inflammatory cytokine responses to malaria parasites. Because several cell types, including monocytes and NK, T, and B cells, have IL-1R/IL-18R (50), which trigger signaling through MyD88 (51), it is likely that high levels of IL-18 produced during later stages of infection induce IL-12 and IL-4 production by these cells. This explains the increased production of IL-12 and IL-4 by infected TLR9−/− mice. Furthermore, because production of IL-1β and IL-18 by inflammasome-mediated signaling requires the pathogen recognition receptor-mediated expression of pro–IL-1β and pro–IL-18, it appears that TLRs other than TLR9 also contribute to certain extent in these cytokine responses. Because serum cytokine profiles of infected TLR2−/− and TLR4−/− mice were similar to those of the infected WT mice (see Fig. 3), despite parasites having TLR2 and TLR4 as well as TLR11 ligands (10–17), it appears that collective signaling strength of TLR2, TLR4, and TLR11 is responsible for the MyD88-dependent cytokine-inducing activity seen in TLR9-deficient mice.

TLR9 and MyD88 are essential for the development of cell-mediated immunity to malaria

As shown in Fig. 1, at the early stages of infections, proinflammatory cytokine responses to malaria parasites by DCs are essentially dependent on TLR9 and MyD88, whereas, as the infection progresses, TLR9 and MyD88 differentially regulate these cytokine responses in vivo. To determine whether TLR9 and MyD88 similarly or differentially regulate cell-mediated immunity to malaria parasites, we measured cytotoxic activity of NK and CD8+ T cells from infected WT, TLR9−/−, and MyD88−/− mice at different stages of infection. It has been shown that GM-DCs efficiently internalize IRBCs by the scavenger receptor (CD36)-mediated and nonspecific phagocytosis and present Ags to T cells (52–54). Therefore, we used IRBC-pulsed GM-DCs as target cells for measuring T cell cytotoxic activity, and the cytotoxic activity of NK cells from infected mice was measured using YAC-1 cells as a target. Both NK and CD8+ T cells from infected WT mice exhibited noticeable levels of cytotoxic activity to target cells even at 5 d postinfection, and the activity was substantially increased at later stages of infection (Fig. 6). In contrast, NK and
CD8+ T cells from infected MyD88−/− mice exhibited little or no cytotoxic activity at all stages of infection, and cells from infected TLR9−/− mice exhibited low levels of cytotoxic activity. To further confirm the cytotoxic activity of CD8+ T cells, we also measured their ability to lyse anti-CD3ε−redirected EL-4 target cells. As in the case of IRBC-internalized DCs, EL-4 cells were efficiently lysed by WT CD8+ T cells, but not by CD8+ T cells from MyD88−/− mice; cells from TLR9−/− mice showed low levels of cytolytic activity (data not shown). These results demonstrate that both TLR9 and MyD88 play important roles in NK and T cells acquiring cytotoxic effector activity. These results, together with the data that proinflammatory cytokine responses by DCs at early stages of infection are critically dependent on both TLR9 and MyD88 (see Fig. 1), demonstrate that DCs contribute predominantly to the development of cytotoxic activity of NK and CD8+ T cells to malaria. Furthermore, the observed differences, albeit low, in the cytotoxic activity of TLR9−/− and MyD88−/− deficient cells suggest that TLR9-independent/MyD88-dependent and/or IL-1R/IL-18R-mediated signaling also plays a role to a certain extent in the development of cell-mediated immunity.

To determine whether the observed T cell cytotoxic activity was Ag specific, we analyzed the ability of CD8+ T cells from P. yoelii-infected WT mice to lyse GM-DCs pulsed with P. berghei IRBCs; GM-DCs pulsed with P. yoelii IRBCs were used as a control. The cytotoxic activity against P. berghei Ags was ∼20% of that against P. yoelii Ags. These results agree with the fact that many proteins of P. yoelii and P. berghei are homologous (55), and strongly suggest that the P. yoelii-induced cytotoxic activity of CD8+ T cells is largely, if not completely, Ag specific.

TLR9 and MyD88 modulate Ab responses to malaria infection

To determine whether TLR9 and MyD88 also contribute to the humoral responses to malaria infection, we analyzed total Ab responses and IgG subclasses in sera of parasite-infected mice. As shown in Fig. 7, TLR9−/− and MyD88−/− mice produced similar levels of Ab titer and similar Ab subclass profiles. Furthermore, interestingly, WT mice showed significantly lower levels of total IgG than TLR9−/− and MyD88−/− mice (Fig. 7A); this appears to be in response to the significantly lower levels of parasitemia in WT mice than in TLR9−/− and MyD88−/− mice. Ig subclass analysis showed significantly higher levels of IgG1 in the sera of parasite-infected TLR9−/− and MyD88−/− mice than in the sera of WT mice (Fig. 7B). In contrast, levels of IgG2a and IgG2b were significantly higher in infected WT mice than in TLR9−/− and MyD88−/− mice. The observed high levels of Th1-type Abs in WT mice and higher level of Th2-type Abs in TLR9−/− and MyD88−/− mice are consistent with robust Th1 responses driven by WT DCs and significantly higher type 2 cytokine responses by TLR9−/− and MyD88−/− DCs. These results are consistent with

FIGURE 6. TLR9 and MyD88 are essential for the development of NK and T cell cytotoxic activity. Liver NK cells (A–C) and spleen CD8+ T cells (D–F) from P. yoelii-infected WT, TLR9−/−, and MyD88−/− mice at 5, 10, and 17 d postinfection were cocultured, respectively, with YAC-1 or P. yoelii IRBC-pulsed GM-DCs as target cells, as described in Materials and Methods. The percentages of target cell lysed were plotted. Data shown are a representative of two independent experiments. (G) Plots of percentage of cell lysis of GM-DCs pulsed with either P. yoelii (closed circle) or P. berghei (open circle) IRBCs by spleen CD8+ T cells from WT mice at 10 d postinfection. The letters, b and c, represent the statistical significance between the cytotoxic activity of NK and T cells from the infected TLR9−/− mice and that of NK and T cells from the infected WT mice. *p < 0.01, †p < 0.05.

FIGURE 7. TLR9 and MyD88 play roles in Ab responses to malaria parasites. Total IgG levels (A) and Ab isotypes (B) in the pooled sera of P. yoelii-infected WT, TLR9−/−, and MyD88−/− mice at 40 d postinfection were analyzed by ELISA. Sera were 1:3 serially diluted for total IgG analysis, and Ab isotype analysis was performed using 1:800 diluted sera. Serum from naive WT mice was used as a control. The letters a, b, and c represent statistical significance between the levels of total IgG or Ab isotypes in sera of infected TLR9−/− or MyD88−/− mice and those in sera of infected WT mice. Data shown are a representative of two independent experiments. *p < 0.001, †p < 0.01, ‡p < 0.05.
TLR9 and MyD88 significantly contribute to malaria-protective immunity

Finally, we assessed the contributions of TLRs and MyD88 to the development of overall protective immunity to malaria by measuring the progression of infection in WT, TLR2−/−, TLR4−/−, TLR9−/−, and MyD88−/− mice and by assessing the survival of infected animals. In all mouse types studied, parasitemia remained low during the first week of infection and thereafter parasites grew exponentially, reaching peak parasitemia between 18 and 20 d postinfection (Fig. 8A, 8B). After 3 wk, parasitemia rapidly decreased in survived mice, and infection was completely cleared at the end of the fourth week. During the exponential growth, parasitemia was 2-fold higher in MyD88−/− than in WT, TLR2−/−, and TLR4−/− mice. TLR9−/− mice showed noticeably lower levels of parasitemia than MyD88−/− mice, but exhibited considerably higher parasitemia than WT, TLR2−/−, and TLR4−/− mice. Compared with WT mice, both MyD88−/− and TLR9−/− mice were more susceptible to infection, and 80–90% of mice eventually succumbed to death (Fig. 8C, 8D). These results are consistent with the higher cytotoxic activity and Th1-type Ab responses by WT mice than TLR9−/− and MyD88−/− mice. Thus, collectively, our results demonstrate that TLR9 and MyD88 regulate innate immune responses as well as cellular and humoral immunity to malaria infection, contributing to the development of protective immunity.

Discussion

The results presented in this work allow us to make two important conclusions that have broader implications in understanding the molecular mechanisms involved in the regulation of innate and adaptive immune responses to malaria. For one, TLR9 and MyD88 play central roles in the regulation of pro- and anti-inflammatory responses and Th1 and Th2 responses, and in the development of cytotoxic effector function and Ab responses to malaria infection. This conclusion is supported by the following findings. The deficiency in either TLR9 or MyD88 but not that in TLR2 or TLR4 leads to the following: 1) markedly decreased production of proinflammatory cytokines with concomitant increase in the production of type 2/anti-inflammatory cytokines by DCs; 2) increased commitment to Th2 development; 3) impaired NK and T cell cytolytic activity; 4) significantly lower levels of Th1-type Ab responses and increased Th2-type Abs; and 5) significantly higher levels of parasitemia and increased susceptibility to malaria. Second, the inflammasome as well as TLR9-independent/MyD88-dependent- and/or IL-1R/IL-1R–mediated signaling also contribute substantially to the development of protective immunity to malaria. This conclusion is evident from our findings that infected TLR9−/− mice produced higher levels of IL-1β and IL-18 than infected MyD88−/− mice. Furthermore, NK and CD8+ T cells from infected TLR9−/− mice exhibit cytotoxic activity, albeit at marginal levels, whereas the corresponding cells from the infected MyD88−/− mice showed little or no cytotoxic activity (see Fig. 6). Thus, our study provides valuable insights into role of TLRs in the regulation of innate and adaptive immune responses to malaria infection.

Our data also clearly demonstrate that TLR9 and MyD88 play critical roles in the development of protective immunity to malaria infection. Studies have shown that protective immunity to malaria involves strong early proinflammatory cytokine responses and Th1 polarization, leading to effective cell-mediated and humoral responses (2–4). In contrast, higher anti-inflammatory and type 2 cytokine responses at the early stages of infection result in inability to control infection, leading to pathogenesis (2–4). In agreement with this notion, in the current study, parasite-infected WT mice that were protected from infection, but not susceptible TLR9−/− and MyD88−/− mice, produced high levels of proinflammatory cytokines and low levels of type 2/anti-inflammatory cytokines, and were strongly Th1 polarized, elicited efficient NK and T cell cytotoxic activity, and produced higher levels of protective Th1-type (56), opsonizing IgG2a and IgG2b, which are likely to aid effective parasite clearance. In contrast, the deficiency in TLR9 resulted in substantially lower levels of proinflammatory cytokines and higher levels of type 2/anti-inflammatory cytokines at early stages of infection, increased production of both pro- and anti-inflammatory responses at later stages of infection, and sig-
significantly impaired NK and CD8+ T cell cytotoxic activity. The MyD88−/− deficiency, in contrast, caused low levels of both proinflammatory and anti-inflammatory cytokine responses at all stages of infection and completely impaired NK and CD8+ T cell cytotoxic activity. Furthermore, TLR9 or MyD88 deficiency significantly limited the production of Th1-type IgGs, which are known to be protective (56), while increasing the levels of Th2-type IgGs. The proinflammatory cytokines and strong Th1 polarization are known to increase macrophage phagocytic activity and proliferation of cytotoxic CD8+ T cells, and promote the production of opsonizing Abs (57, 58). All of these can contribute to efficient parasite clearance, thereby providing protection against infection in WT mice. Effective clearance of IRBC-internalized macrophages, DCs, and other phagocytic cells is also likely to be important for allowing the immune system to function efficiently against malaria infection; this function is severely compromised in TLR9−/− and MyD88−/− mice. Thus, our conclusion that TLR9 and MyD88 are crucial for the development of protective immunity to malaria is supported by the observed markedly lower ability of TLR9−/− and MyD88−/− mice to control parasitemia and substantially higher susceptibility to death compared with WT mice. Our conclusion also agrees with the results of recent studies that TLR9 provides protection against cerebral malaria in P. berghei ANKA-infected mice (59), MyD88 protects P. chabaudi chabaudi-infected mice from malaria illness (18), and MyD88 is also involved in the control of early parasitemia in nonlethal P. yoelii infection (60).

The results of this study further demonstrate that TLR9-independent/MyD88-dependent and/or IL-1R/IL-18R-mediated signaling were also significantly involved in the regulation of immune responses to malaria infection. This is evident from our observation that P. yoelii-infected MyD88−/− mice produced markedly lower levels of both pro- and anti-inflammatory cytokines, whereas the infected TLR9−/− mice produced substantial levels of both types of cytokines (see Fig. 3). Furthermore, in a preliminary study using FL-DCs, we noted that DCs deficient in TLR9 produced appreciable levels of TNF-α and IL-12 upon stimulation with P. yoelii-infected erythrocytes, whereas DCs deficient in MyD88 were unable to produce these cytokines (N.M. Gowda, X. Wu, and D.C. Gowda, unpublished results). Malarial GPIs, microparticles from IRBCs, and heme released from the parasite-infected erythrocytes have been shown to activate macrophages through TLR2 or TLR4 (10–12). However, the effects of TLR2 or TLR4 were not evident either from the serum cytokine profiles of the infected mice or from the cytokine pattern produced by DCs ex vivo (see Figs. 1–3). Compared with other protozoan parasites such as Trypanosoma and Leishmania, malaria parasites express relatively low levels of GPIs (61, 62), and also it is possible that levels of microparticles released by parasite-infected erythrocytes are low. Furthermore, although malaria parasite’s profilin has been reported to activate DCs via TLR11 to produce IL-12 (16, 17), its activity has been reported to be low (63). Therefore, it appears that individually these receptors are unable to activate cells to a significant extent to induce strong immune responses, but collective signaling by TLR2, TLR4, and TLR11 significantly activates the innate immune system in TLR9−/− mice in vivo to induce cytokine responses by DCs and other cells. Moreover, the production of IL-1β and IL-18 at high levels by P. yoelii-infected TLR9−/− mice, but not by MyD88−/− mice (see Fig. 5), suggests that MyD88-dependent, inflammasome- and IL-1R/IL-18R-mediated signaling also contributes substantially to the activation of cells of the immune system, especially during later stages of infection when IL-18 is produced at higher levels. Therefore, cytokines produced in response to MyD88-mediated signaling, involving TLR2, TLR4, TLR11, and/or IL-1R/IL-18R, collectively account for the observed high levels of serum cytokines in P. yoelii-infected TLR9−/− mice. This explains why parasite-infected MyD88−/− mice produce only marginal levels of cytokines.

The results presented in this work additionally demonstrate that IL-18, which has been shown to play an important role in malaria infection (60, 64–66), is produced in a TLR9-independent and MyD88-dependent manner. IL-18 is known to activate NK and T cells through its receptor-mediated signaling to produce IFN-γ, contributing to Th1 cell development (50, 67). Additionally, IL-18 enhances NK cell cytotoxicity, Fas ligand-mediated Th1 cell cytotoxicity, and proliferation of activated T cells (67, 68). In the parasite-infected mice, parallel to IL-18 induction, IL-12 is produced at increased levels as infection progresses (see Figs. 3C, 5B). IL-12 and IL-18 have shared functions and, thus, they synergistically and independently activate NK, T, and B cells through their receptors to produce cytokines (50, 69, 70). IL-18 is also known to upregulate IL-12R, and IL-12 upregulates IL-18R (70). Thus, during malaria infection, IL-18 appears to collaborate with IL-12 in the development of Th1 cells and enhancement of NK and T cell cytotoxic activity by promoting cell proliferation. This is apparent from the observed high levels of proinflammatory cytokines in mice at later stages of infection and increased cytotoxic activity of NK and T cells as infection progresses. Previous studies have shown that IL-18 and IL-12 are protective to P. yoelii and P. chabaudi malaria (71–73). IL-18 has also been shown to be protective against severe malaria in human (64–66). Based on these observations, it is expected that TLR9−/− mice should be protected from malaria infection as they produce high levels of IL-18 and IL-12. However, IL-18 is also known to induce antiinflammatory cytokines and Th2 responses depending on the cytokine milieu of the system (74). Therefore, unusually high levels of IL-4 seen in the infected TLR9−/− mice in parallel with increased production of IL-18 are most likely due to the IL-18–mediated Th2 responses. Because the deficiency in TLR9 caused Th2-biased responses, IL-18 can efficiently augment Th2 responses in TLR9−/− mice. Therefore, it appears that IL-18 by efficiently driving both Th1 and Th2 responses neutralizes the protective effect of Th1 responses by Th2 responses in TLR9−/− mice. Thus, this explains why TLR9−/− mice, similar to MyD88−/− mice, have impaired cell-mediated and protective Ab responses, and thus, are more susceptible to malaria infection than WT mice.

Overall, results of the current study demonstrate that initial robust proinflammatory cytokines are critical for the development of protective immunity to malaria. DCs are the major cells of the innate immune system, which play a crucial role in the initiation of innate immune responses and development of adaptive immunity by connecting the innate immune arm to that of adaptive immune system. This is evident from our observation that DCs from the infected WT mice produced substantially high levels of proinflammatory cytokines at early stages of infection that correlated with high levels of NK and CD8+ T cell cytotoxicity and protective Th1 Ab production. In contrast, DCs from TLR9−/− and MyD88−/− mice produced low levels of proinflammatory cytokines that resulted in complete absence or marginal levels of cytotoxic activity and decreased Th1 Ab production.

Finally, our observations have broader implication in understanding the roles of TLRs in the regulation of innate and adaptive immunity in other pathogenic infections as well. Although TLRs have been recognized to be crucial for producing proinflammatory responses during infections by diverse group of pathogens such as bacteria, fungi, and parasites, the roles of these receptors in the regulation of immune responses remain poorly understood. The results presented in this study implicate that TLRs play central roles...
in the regulation of innate and adaptive immunity to various pathogenic microorganisms. Detailed understanding of the TLR-dependent immune regulations is likely to provide strategies for the development of therapeutics or vaccines against pathogenic infections.

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Disclosures

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References

