MyD88-dependent activation of dendritic cells and CD4⁺ T lymphocytes mediates symptoms, but is not required for the immunological control of parasites during rodent malaria


Abstract

We investigated the role of different TLRs and MyD88 in host resistance to infection and malaria pathogenesis. TLR2⁻/⁻, TLR4⁻/⁻, TLR6⁻/⁻, TLR9⁻/⁻ or CD14⁻/⁻ mice showed no change in phenotypes (parasitemia, body weight and temperature) when infected with Plasmodium chabaudi (AS). MyD88⁻/⁻ mice displayed comparable ability to wild type animals in controlling and clearing parasitemia. Importantly, MyD88⁻/⁻ mice exhibited impaired production of TNF-α and IFN-γ as well as attenuated symptoms, as indicated by changes in body weight and temperature during parasitemia. Consistently, CD11b⁺ monocytes and CD11c⁺ dendritic cells from infected MyD88⁻/⁻ mice were shown impaired for production of pro-inflammatory cytokines, and in initiating CD4⁺ T cell responses. Importantly, the inhibition of T cell activation with anti-CD134L, mostly inhibited IFN-γ, partially inhibited TNF-α production, and protected the animals from malaria symptoms. Our findings suggest that MyD88 and possibly its associated TLRs expressed by dendritic cells play an important role in pro-inflammatory responses, T cell activation, and pathogenesis of malaria, but are not critical for the immunological control of the erythrocytic stage of P. chabaudi.

Keywords: Toll-like receptors; Innate immunity; Cytokines; Pathogenesis; Malaria and Plasmodium chabaudi

1. Introduction

Toll-like receptors (TLRs) are activated by diverse microbrial molecular structures and share a cytoplasmic domain with high homology to the IL-1 receptor (IL-1R) known as the Toll-like receptor/IL-1 resistance (TIR) domain. The TIR domains of TLRs interact with cytoplasmic adapter molecules that also contain TIR domains. The best-studied example of a TIR domain containing adapter protein is the myeloid differentiation primary-response gene 88 (MyD88), which transduces signals for all of the known TLRs (except for TLR3) and the IL-1/IL-18 Receptors [1]. Another key element, shared by TLR2 and TLR4 receptors is CD14, which is a co-receptor with lipid transferase activities that potentiates the activation of TLR2 and TLR4 by lipid containing microbial molecules [2].

To date, TLRs have been implicated in every known category of microorganism that causes human disease, including...
protozoan parasites [3]. The early elimination of invasive microorganisms is the primary goal of the innate immune system. The TLRs are critical for all aspects of this process, including the recruitment of phagocytes to infected tissue, activation of effector mechanisms and subsequent microbial killing [1]. Further, dendritic cells (DCs) activated by TLR agonists are key elements for initiating and shaping acquired immunity and development of memory lymphocytes and long term immune responses [4].

Acute infection with Plasmodium results in the production of high levels of pro-inflammatory cytokines, that orchestrate host immunity to infection, but at the same cause many of the observed symptoms [5,6]. We hypothesize that TLRs are central mediators in the pathogenesis of malaria. Evidence exists that TLRs are critical for eliciting the synthesis of pro-inflammatory cytokines during infection with protozoan parasites. For example glycosylphosphatidylinositol (GPI) anchors derived from protozoan parasites [3,7], including Plasmodium falciparum [8] have been shown to trigger the synthesis of pro-inflammatory cytokines through TLR2 and TLR4. In addition, hemozoin carrying parasite DNA have been shown to activate human and mouse DCs via TLR9 [9,10]. Consistently, microarray analysis of RNA derived from peripheral blood mononuclear cells from individuals infected with P. falciparum, indicate that gene activation by TLR signaling through NF-κB is significantly upregulated [11]. Altered frequency of functional single nucleotide polymorphisms (SNPs) in TLR2, TLR4, and TLR9 genes has been shown in individuals from endemic regions P. falciparum infection and associated with severe malaria in pregnancy [12,13]. Importantly, MyD88Δ/Δ, TLR2Δ/Δ and TLR9Δ/Δ mice have attenuated symptoms when infected with Plasmodium berghei [14,15].

Here, we investigated the role of various TLRs into two critical immunological aspects of malaria: (i) cytokine-mediated clinical symptoms; and (ii) control of parasitemia. We observed that TLR2Δ/Δ, TLR4Δ/Δ, TLR6Δ/Δ, TLR9Δ/Δ and CD14Δ/Δ mice showed similar parasitemia, production of pro-inflammatory cytokines and pathological parameters, when compared to wild type (WT) mice. The MyD88Δ/Δ mice also showed comparable parasitemia to WT mice, which was nevertheless associated with an impaired production of TNF-α and IFN-γ and attenuated symptoms. Importantly, DCs from MyD88Δ/Δ showed impaired production of pro-inflammatory cytokines, and were less able to initiate T cell responses in infected mice. Consistently, blockage of T cell activation during primary Plasmodium chabaudi infection, greatly inhibited IFN-γ and TNF-α production, protecting animals against the malaria symptoms. In conclusion, our findings suggest that MyD88, in coordination with a combination of TLRs expressed by dendritic cells, plays a role in pro-inflammatory responses, T cell activation and pathogenesis of malaria, but are not critical for the immunological control of P. chabaudi infection.

2. Material and methods

2.1. Rodent model of malaria and knockout mice

The P. chabaudi AS strain was used in our experimental infections [16]. Mice were infected with 10^6 infected erythrocytes, and checked daily for survival; moribund animals were scored as dead, and euthanized. Laboratory values that reflect malaria-associated pathology were determined every 2–3 days throughout the 30 day study period. This evaluation included percent parasitemia by Giemsa stained blood smears (Merk, Darmstadt, Germany), determination of temperature by rectal thermocouple, and measurement of body weight [16,17]. MyD88Δ/Δ, TLR2Δ/Δ, TLR4Δ/Δ, TLR6Δ/Δ, CD14Δ/Δ and IFN-γΔ/Δ all backcrossed at least eight generations into the C57BL/6 background were used in the experiments described above.

2.2. Mouse treatment with monoclonal antibodies

Isotype matched monoclonal antibodies anti-b-galactosidase (GL113 – control), anti-CD4 (GK 1.5) and anti-CD134L (OX89) were purified from ascites, and given intraperitoneally, at a dose of 0.5 mg/mouse diluted in 200 μl of PBS, at days 7 and 1 one day prior infection and once a week thereafter.

2.3. Cytokine measurements

Supernatant of splenocyte cultures and sera were collected from each mouse, for measuring the levels of cytokines (i.e., IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12p70). Splenocytes were obtained by macerating spleens through a nylon mesh, erythrocytes lysed with ice-cold isotonic solution (155 mM NH₄Cl, 10 mM KHCO₃, 100 mM EDTA; pH 7.4). Splenocytes were then suspended in RPMI, 5% fetal calf serum (Cultilab, Campinas Brazil), 1% gentamicyn (Schering Plough, RJ, Brazil) at concentration of 5 x 10⁶ cells/ml and cultured in 24-well plate for 48 h without stimulus. Cytokine levels were measured in sera and culture supernatants by using Cytokine bead array kit (CBA) (BD Biosciences), according to the manufacture’s protocol.

2.4. Staining to determine splenocytes profile and single-cell cytoplasmic cytokine staining

Five hundred thousand cells were analyzed after 4 h cultured in 96-well plates in 200 μl medium with brefeldin-A (1 μg/ml). After that, the cells were washed and incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)- or phycoerythrin-cy-chrome (PE-CY) labeled antibody solutions for 20 min at 4 °C. After washed twice, the preparations were fixed with 200 μl of 2% formaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS. The fixed cells were permeabilized with a solution of saponin, stained, using anti-cytokine monoclonal antibodies, fixed and analyzed using FACS. At least 35,000-gated events were acquired for later analysis. The antibodies used for the staining were immunoglobulin FITC and PE controls, anti-TCR-FITC, anti-CD4-FITC, anti-CD8-FITC, anti-NK-FITC, anti-CD69-FITC, anti-CD25-FITC, anti-CD25-FITC, anti-11b-FITC, anti-CD4-PE, anti-CD8-PE, anti-IL-6-PE, anti-IL-12-PE, anti-IL-1α-PE, anti-TNF-ζ-PE, anti-IFN-γ-PE, anti-CD4-PE-Cy, anti-CD8-PE-Cy (PharMingen, San Diego, CA).
Fig. 1. C57BL/6 (WT), TLR2−/−, TLR4−/−, TLR6−/−, TLR9−/−, CD14−/−, MyD88−/− and IFN-γ−/− mice were challenged with 1 × 10^E5 infected erythrocytes and followed every three days for parasitemia (top panels), body weight (middle panel), and body temperature (bottom panel). The results are averages of eight animals from a representative of one out of three experiments that yielded similar results. Asterisk indicates that difference is statistically significant (p < 0.05) when comparing results from a specific knockout lineage (i.e. MyD88−/− or IFN-γ−/−) with the wild type (C57BL/6) mice.

Fig. 2. (A) C57BL/6 (WT), TLR2−/−, and MyD88−/− mice were challenged with 1 × 10^E5 infected erythrocytes and followed every three days for parasitemia; (B) C57BL/6 (WT), TLR2−/−, and MyD88−/− mice challenged with 1 × 10^E5 infected erythrocytes were re-challenged with 1 × 10^E8 infected erythrocytes at 90 days after initial infection, and naive C57BL/6 control mice receiving the same inoculums were use as controls; (C) the levels of anti-P. chabaudi specific IgG1 and IgG2a antibodies were measured in the sera of C57BL/6, TLR2−/− and MyD88−/− at 90 days post-infection, as well as in the sera of uninfected control mice. The results are averages of eight animals from a representative of two experiments that yielded similar results. Asterisk indicates that difference in parasite specific IgG1 level is statistically significant (p < 0.05) when comparing results from a MyD88−/− and the wild type (C57BL/6) mice.

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Fig. 3. Splenocytes from C57BL/6 (WT), MyD88−/−, and TLR2−/− mice were harvested before (day 0), during (day 8) and after (day 28) the peak of parasitemia and cultured in absence of any exogenous stimulation. After 48 h of culture supernatants were frozen at −70 °C until use. Cytokine levels in the supernatant were
2.5. Analysis of FACS data

Lymphocytes, NK cells, and DCs were analyzed, making use of known positioning of mononuclear cells based on size and granularity profiles, for their intracellular cytokine expression patterns and for surface markers in a number of ways using the Cell Quest (Becton & Dickinson, San José, CA). Limits for the quadrant markers were always set based on negative populations and isotype controls, and cellular staining analyzed in a FACScan flow cytometer and analyzed using Cell Quest software (Becton Dickinson, San José, CA).

2.6. Purification of CD11c\(^+\) splenic DCs

Spleens from naïve or infected mice were treated for 30 min at 37 °C with 1 mg/ml collagenase IV (Sigma Aldrich). CD11c\(^+\) splenic DCs were isolated using CD11c microbeads (Miltenyi Biotec) accordingly to manufactures instructions. Cells (3 \times 10^5) were Fc blocked with 2.4G2 mAb (BD Pharmingen San Diego, CA) and labeled with FITC-conjugated anti-CD11c, PE-Cy-conjugated anti-CD40 and CD80 mAbs (BD Pharmingen San Diego, CA). A non-related IgG mAb was used as a control for staining specificity. For intracellular cells were process as described above.

2.7. Statistical analysis

Each experiment was performed using eight individual C57BL/6 mice and correspondent group of knockout animals infected with P. chabaudi. Each experiment was repeated two or three times. Each animal was analyzed individually. The numbers of animals used for cytokine measurements and FACS analysis are indicated in the figure legends. Arithmetic means (parasitemia, temperature, body weight, cytokines, antibody levels and cell numbers/percentage obtained in FACS analysis) and standard errors of the means were calculated. T student’s t-test was used to determine the statistical significance of the observed differences. Differences were considered significant when \( p < 0.05 \).

3. Results

3.1. Role of Toll-Like Receptors (TLR2, TLR4, TLR6 or TLR9), related co-receptor (CD14) and MyD88 in host resistance and malaria pathogenesis

Infection with P. chabaudi, a rodent of malaria, induces high serum levels of IFN-γ, MCP-1, TNF-α, IL-6 and IL-10 that coincides with parasitemia, weight loss and drop in temperature in the wild type C57BL/6 (WT) mice (data not shown). Here, we evaluated the role of different TLRs and associated molecules in host resistance and pathogenesis of infection with P. chabaudi. As controls, we used the WT and IFN-γ\(^-/-\) mice that are resistant and susceptible to P. chabaudi infection, respectively, as indicated by their ability to clear or not parasitemia after 15–20 days of infection. The results presented in Fig. 1 show that parasitemia was different, when comparing MyD88\(^-/-\), TLR2\(^-/-\), TLR4\(^-/-\), TLR6\(^-/-\), TLR9\(^-/-\) and CD14\(^-/-\) to WT mice. Except for the IFN-γ\(^-/-\) mice, all the knockout mice were capable of clearing parasitemia, at the same time of the WT mice. Consistently, by end of the experiment, at 28 days post-infection, we observed significantly increased lethality (>50%) only in the IFN-γ\(^-/-\) mice infected with P. chabaudi. In the other knockout mouse strains the lethality varied from 0% to 20% and was not statistically different from WT mice (data not shown).

Similar to the WT mice, the TLR2\(^-/-\), TLR4\(^-/-\), TLR6\(^-/-\), TLR9\(^-/-\) and CD14\(^-/-\) mice had a clear drop in the temperature and weight loss (\( p < 0.05 \)) that corresponded to the peak of parasitemia (Fig. 1). Interestingly, the MyD88\(^-/-\) infected with P. chabaudi, showed no significant change in temperature and body weight. In contrast, parasitemia was similar to the WT mice. To further confirm that MyD88 was not required for development of protective acquired immunity, we infected WT, MyD88\(^-/-\) or TLR2\(^-/-\) mice with 10E5 infected erythrocytes and then gave a secondary challenge using high P. chabaudi (i.e. 10E8 infected erythrocytes) dose at 90 days after the initial infection (Fig. 2A and B). The MyD88\(^-/-\) infected with P. chabaudi showed immune response skewed towards a Th2 phenotype, as indicated by the enhancement of parasite specific IgG1 antibody isotypes (Fig. 2C) and lower IL-12 (Figs. 3–5) production. Nevertheless, MyD88\(^-/-\) mice were completely resistant to the secondary challenge, as indicated by parasitemia, weight loss and change in temperature.

3.2. Cytokemia during infection with P. chabaudi in mice deficient in specific TLRs (TLR2, TLR4, TLR6 or TLR9), the TLR co-receptor (CD14) or the adapter molecule, MyD88

We next evaluated the cytokine responses of knockout mice at 8 and 28 days post-infection by the Cytomation Bead Arrays. Fig. 3A (left panel) shows the size (SSC) and forward (FSC) scattering and indicate in R1 the population of beads selected for analysis. Fig. 3A (right panel) shows the FL3 measured by using the pro-inflammatory cytokines – Cytomix Bead Array (CBA). (A) Gated population of beads analyzed, and the correspondence between the bead stain intensity (FL3) and cytokine (FL2) analyzed, respectively. (B) Analysis of splenocyte supernatants from representative individual mice from each lineage analyzed at 0, 8 and 28 days post-infection. (C) Cytokine levels in the supernatant of splenocyte cultures obtained from C57BL/6, TLR2\(^-/-\), TLR9\(^-/-\) and MyD88\(^-/-\) mice. The results represent an average of eight mice per group pooled from two different experiments. (D) Sera from C57BL/6 (WT), TLR2\(^-/-\), TLR4\(^-/-\), TLR6\(^-/-\), TLR9\(^-/-\) and MyD88\(^-/-\) mice were obtained before (day 0), during (day 8) and after (day 28) the peak of parasitemia and frozen at -70 °C until use. Cytokine levels in the sera were measured by using the pro-inflammatory cytokines Cytomix Bead Array (CBA). Each panel shows the analysis of a representative individual mouse from different mouse lineage analyzed at 0, 8 and 28 days post-infection. The results represent an average of eight mice per group pooled from two different experiments. One (\( p < 0.05 \)) and two (\( p < 0.01 \)) asterisks indicate that difference is statistically significant when comparing results from a specific knockout lineage (i.e. MyD88\(^-/-\), TLR2\(^-/-\) or TLR9\(^-/-\)) with the wild type (C57BL/6) mice.

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Fig. 4. (A) Splenocytes from C57BL/6 and MyD88−/− mice were harvested on day 0 (Control) and day 8 post-infection (peak of parasitemia and cytokinemia) and cytokine production evaluated by intracellular staining and FACS analysis employing anti-CD11b-FITC to label monocytes and anti-IL-1-α-PE, TNF-α-PE and IL-12(p70)-PE for intracellular staining of cytokine producing cells. CD11c+ splenic DCs (3 × 10^5) from naïve and P. chabaudi-infected (9 days post-infection) mice were purified using CD11c microbeads (Miltenyi Biotec), Fc blocked and stained for the surface expression of CD11c-FITC, CD40, CD86-Cy5 (B) or intracellular expression of IL-1, IL-6, IL-12 and TNF-α (C). (B) Histograms show the CD40 and CD80 expression on splenic CD11C+ DCs of naïve (shaded background) and 9 days infected (continuous line) mice. A non-related PE-conjugated IgG mAb was used as a control for staining specificity (dotted line). (C) For intracellular staining of cytokines, 3 × 10^5 DCs were treated with brefeldin-A, Fc blocked, stained for surface expression of CD11c and fixed. Cells from naïve mice (shaded background) and infected mice (continuous line) were then permeabilized with saponin, and stained for intracellular cytokines IL-1, IL-6, IL-12 and TNF-α using specific PE-conjugated mAbs. The grafts were designed based on the reason of percentages obtained by FACs analysis from cells of infected/control mice. Data are representative of two independent experiments. Asterisk indicates that difference in parasite specific IgG1 level is statistically significant (p < 0.05) when comparing results from a MyD88−/− and the wild type (C57BL/6) mice.
indicating the peak of intensity for fluorescence of beads coated with specific cytokine antibody (lower intensity for IL-12 and higher intensity IL-6). Fig. 3B shows a representative of a single wild type, TLR2\(^{-/-}\) and MyD88\(^{-/-}\) prior infection (uninfected controls), at 8 and 28 days post-infection. FL3 shows the differential staining of beads coated with specific anti-cytokine antibodies, the level of FL2 fluorescence staining is determined by the levels and of cytokine present in the splenocyte culture supernatants and their interaction with each of the beads. The results show no detection of cytokinemia in days 0 and 28 post-infection, whereas cytokines, mainly IFN-\(\gamma\), TNF-\(\alpha\) and MCP-1 were augmented on day 8 post-infection. The results presented in bars represent pooled data from three different experiments and are shown in Fig. 3C (culture supernatants) and 3D (sera). We observed an impaired synthesis of TNF-\(\alpha\) and IFN-\(\gamma\), but not MCP-1, by splenocytes from MyD88\(^{-/-}\) mice at day 8 post-infection (\(p<0.01\)) (Fig. 3C). We also observed a partial impairment in the synthesis of TNF-\(\alpha\) and IFN-\(\gamma\) by splenocytes from TLR2\(^{-/-}\) and TLR9\(^{-/-}\) mice, respectively (\(p<0.05\)). No impairment of cytokine production, was observed in spleen cells from TLR4\(^{-/-}\), TLR6\(^{-/-}\) or CD14\(^{-/-}\) mice at 8 days post-infection (data not shown).

Serum cytokine levels (Fig. 3D) trended along the same lines as those of the splenocytes. While statistically significant (\(p<0.01\)), the impairment of TNF-\(\alpha\) and IFN-\(\gamma\) levels in MyD88\(^{-/-}\) mice infected with \(P.\ chabaudi\) was only partial. MCP-1 was produced in a MyD88-independent manner. The levels of TNF-\(\alpha\), IFN-\(\gamma\) and MCP-1 were not altered in the sera of TLR2\(^{-/-}\), TLR4\(^{-/-}\), TLR6\(^{-/-}\), TLR9\(^{-/-}\) or CD14\(^{-/-}\) mice infected with \(P.\ chabaudi\).

3.3. MyD88 is a critical element for cytokine production by monocytes, DCs and T lymphocytes during acute episodes of malaria in mice infected with \(P.\ chabaudi\)

DCs have been shown to be activated by \(Plasmodium\) products as well as play distinct roles during rodent malaria [18]. Exposure of BMDCs to erythrocytes infected with \(P.\ chabaudi\) elicited the production of IL-12. The levels were relatively low, as compared to LPS (TLR4 agonist) and Pam3Cys (TLR2 agonist). Importantly, the IL-12 response was ablated (uninfected controls), at 8 and 28 days post-infection. The results presented in bars represent pooled data from three different experiments and are shown in Fig. 3C (culture supernatants) and 3D (sera). We observed an impaired synthesis of TNF-\(\alpha\) and IFN-\(\gamma\), but not MCP-1, by splenocytes from MyD88\(^{-/-}\) mice at day 8 post-infection (\(p<0.01\)) (Fig. 3C). We also observed a partial impairment in the synthesis of TNF-\(\alpha\) and IFN-\(\gamma\) by splenocytes from TLR2\(^{-/-}\) and TLR9\(^{-/-}\) mice, respectively (\(p<0.05\)). No impairment of cytokine production, was observed in spleen cells from TLR4\(^{-/-}\), TLR6\(^{-/-}\) or CD14\(^{-/-}\) mice at 8 days post-infection (data not shown).

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On the other hand, \(\alpha\beta\)TCR\(^{+}\) lymphocytes and NK cells were shown to be the main source of IFN-\(\gamma\). The \(\alpha\beta\)TCR\(^{+}\) lymphocytes also contributed as a source of TNF-\(\alpha\) during acute infection with \(P.\ chabaudi\) (Fig. 5A). Among T lymphocytes, CD4\(^{+}\) T cells, but not CD8\(^{+}\) T cells, were shown to be the main source of cytokines. Consistently, the expression of the CD25 and CD69 activation markers was enhanced in CD4\(^{+}\) T cells, but not in CD8\(^{+}\) T cells from mice at eight days post-infection (Fig. 5B). Importantly, the cytokine production and expression of activation markers by CD4\(^{+}\) T cells was impaired in MyD88\(^{-/-}\) mice infected with \(P.\ chabaudi\) (Fig. 5A–C).

3.4. T cells are the main source of IFN-\(\gamma\) and mediate clinical symptoms during acute episodes of malaria in mice infected with \(P.\ chabaudi\)

DCs have a primary role in initiating T cell responses during infectious diseases. In order to evaluate our hypothesis that primary activation of T cells by DCs is an important step for cytokinemia and pathogenesis during acute infection with \(P.\ chabaudi\), we treated animals, with anti-CD4 (GK 1.5) or anti-CD134 (OX89) mAbs that deplete CD4\(^{+}\) T lymphocytes or block the activation of CD4\(^{+}\) T cells, respectively. The results presented in Fig. 6 show that either treatment with mAbs resulted in decrease weight loss and drop in body temperature in infected mice, as compared to mice treated with the control mAb, GL113. The attenuation of clinical symptoms in mice depleted of CD4\(^{+}\) T cells or treated with OX89 that blocks T cell activation, was associated with decreased serum levels as well as blockade of IFN-\(\gamma\) and partial inhibition of TNF-\(\alpha\) production by spleen cells from infected mice (Fig. 6). TNF-\(\alpha\) production stayed high at day 28 post-infection, in mice depleted of CD4\(^{+}\) T cells, which did not clear parasites from blood, further suggesting the activation of innate immune cells to produce TNF-\(\alpha\).

4. Discussion

The strongest data suggesting the importance of the TLR signaling pathway in host resistance and pathogenesis during parasitic diseases are those obtained from infections of MyD88\(^{-/-}\) mice with \(Toxoplasma gondii\), \(Leishmania major\), \(Trypanosoma cruzi\) or \(Trypanosoma brucei\) [19–22]. Increased susceptibility to infection with these protozoa is associated with impaired IL-12 and IFN-\(\gamma\) production [19–22]. Similarly, we found that MyD88 plays an important role in the production of pro-inflammatory cytokines, during infection with \(P.\ chabaudi\). Unexpectedly, and unlike infection with the protozoan parasites mentioned above, we show that MyD88 is not essential for control and parasite clearance in rodent malaria. Importantly, despite the skewed response towards Th2 (indicated by higher parasite specific IgG1 levels), after primary infection, the MyD88\(^{-/-}\) mice were completely protected, showing no parasites in the blood upon a secondary challenge with \(P.\ chabaudi\). This came to us as a surprise since previous studies have shown MyD88 is a critical element for the production of IL-12 and the development of Th1
lymphocytes, known as important components for host resistance to *P. chabaudi* [23]. Thus, our findings indicate that activation of TLRs and MyD88, may contribute, but is not essential for the development of protective immunity in mice challenged with *P. chabaudi*.

Nevertheless, it was clear that the MyD88$^{-/-}$ mice had less symptoms (i.e., drop in body weight and temperature) than the WT mice infected with *P. chabaudi*. Based on the results presented here, we concluded that MyD88 mediates symptoms by being essential for systemic production of pro-inflammatory cytokines (i.e., TNF-$\alpha$ and IFN-$\gamma$) observed during acute episodes of malaria. We observed that CD4$^{+}$ T lymphocytes and to a less extent NK cells were the major source of IFN-$\gamma$, whereas CD4$^{+}$ T lymphocytes, monocytes, and DCs contributed as a source of TNF-$\alpha$.

Our results also show that DCs from MyD88$^{-/-}$ mice infected with *P. chabaudi* were impaired for production of IL-1, IL-6 and IL-12 resulting and less effective in inducing IFN-$\gamma$ producing CD4$^{+}$ T cells. These findings lead us to evaluate the ability of OX89 to block excessive production of IFN-$\gamma$/TNF-$\alpha$ and cytokine-mediated symptoms observed during acute episode of rodent malaria. OX89 is an anti-CD134L monoclonal antibody that blocks the interaction of the co-stimulatory molecule CD134L (OX40L) in DCs with CD134 (OX40) in T lymphocytes, which is essential for the primary activation of T cells. Treatment with OX89 has been proposed as therapy for diseases where excessive T cell response is detrimental [24,25]. We found that treatment with anti-CD134L significantly ablated the production of IFN-$\gamma$, and to a less extent affected the TNF-$\alpha$ synthesis in infected mice. In agreement, with the critical role of these cytokines in the clinical symptoms of animals infected with *P. chabaudi*, the anti-CD134L treated animals showed no drop in body weight or temperature.

A recent study has shown that IL-18 is an important cytokine that activates and expands T cells leading to the production of IFN-$\gamma$ mediated by CD134/CD134L interaction [26]. Further, IL-18 has been shown to be involved in IFN-$\gamma$
induction and host resistance during mouse infection with P. berghei or Plasmodium yoelii [27] and in a in vitro system for induction of IFN-γ production by NK cells exposed to P. falciparum [28]. However, consistent with studies performed by Adachi et al. [14], we could not reproduce the findings obtained with P. berghei and P. yoelii infections [27], and found no changes in parasitemia, cytokinemia (IFN-γ and TNF-α) and symptoms during infection of IL-18−/− with P. chabaudi (data not shown).

Together our results show that primary T cell activation by DCs is partially dependent on MyD88, and has a critical role in the pathogenesis of malaria as early as 8 days post-infection. We also show that single deficiency of TLR2, TLR6, TLR4, CD14 (co-receptor for TLR2 and TLR4), or TLR9 had no major effect on immunological control of parasite replication, cytokinemia and cytokine-mediated symptoms associated with rodent malaria. These results, contrast with recent publication from Coban et al. [15], who demonstrated a critical role for single deficiency of either TLR2 or TLR9, making mice more resistant to cerebral malaria. The assignment of single TLR responsible for the control of protozoan infections has been a consistently difficult task [3,14,19,21,22,29]. Our main hypothesis is that protozoan parasites are recognized by several TLRs. Indeed, this seems the case with T. cruzi experimental infection in TLR2/TLR9 double knockouts that are almost as susceptible as the MyD88−/− mice infected with T. cruzi [30].

Altogether, our results show that MyD88 plays a critical role in malaria pathogenesis. We also define a main mechanism by which MyD88 is essential in mediating cytokinemia and pathogenesis during acute malaria. The role of MyD88 appears to be critical for eliciting production of pro-inflammatory cytokines, and an exuberant primary activation of CD4+ T cells, resulting in excessive production of TNF-α and IFN-γ during the peak of parasitemia. Importantly, treatment with anti-CD134L that blocks co-stimulation of T cells, had a beneficial effect blocking excessive T cell response, cytokinemia and associated symptoms during acute malaria, with no detrimental effect in terms of parasitemia and parasite clearance. Thus, primary T cell activation by DCs is a key step to interfere with malaria pathogenesis, and may be proven useful to treat patients with malaria. Further, our studies suggest that TLR antagonists maybe useful in partially blocking DC activation to prevent the excessive T cell activation and cytokine-mediated symptoms observed during acute malaria episodes.
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