Distinct Roles for MyD88 and Toll-Like Receptor 2 during *Leishmania braziliensis* Infection in Mice[▽]

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Received 9 February 2009/Returned for modification 20 March 2009/Accepted 6 April 2009

We have previously reported that *Leishmania braziliensis* infection can activate murine dendritic cells (DCs) and upregulate signaling pathways that are essential for the initiation of innate immunity. However, it remains unclear whether Toll-like receptors (TLRs) are involved in *L. braziliensis*-mediated DC activation. To address this issue, we generated bone marrow-derived DCs from MyD88^{-/-} and TLR2^{-/-} mice and examined their responsiveness to parasite infection. While wild-type DCs were efficiently activated to produce cytokines and prime naïve CD4⁺ T cells, *L. braziliensis*-infected MyD88^{-/-} DCs exhibited less activation and decreased production of interleukin-12 (IL-12) p40. Furthermore, MyD88^{-/-} mice were more susceptible to infection in that they developed larger and prolonged lesions compared to those in control mice. In sharp contrast, the lack of TLR2 resulted in an enhanced DC activation and increased IL-12 p40 production after infection. As such, *L. braziliensis*-infected TLR2^{-/-} DCs were more competent in priming naïve CD4⁺ T cells in vitro than were their controls, findings which correlated with an increased gamma interferon production in vivo and enhanced resistance to infection. Our results suggest that while MyD88 is indispensable for the generation of protective immunity to *L. braziliensis*, TLR2 seems to have a regulatory role during infection.

Leishmaniasis is a vector-borne disease that has a great socioeconomic impact in many tropical and neotropical countries (40). *Leishmania* parasites multiply as flagellated promastigotes in the midguts of sand flies and are transmitted to the vertebrate host via the bites of parasite-carrying female flies (3, 22). The insult at the bite site initiates a strong neutrophil influx and parasite capture by these cells (38). Interestingly, some of the captured parasites remain viable, and these infected neutrophils actually facilitate the silent entry of parasites into macrophages (Mφs) (29), where parasites survive and replicate as intracellular amastigotes (3). The magnitude and nature of inflammatory responses at the infection site and the profile of subsequent T-cell responses determine the outcome of the infection. In South America, *Leishmania braziliensis* infection causes cutaneous leishmaniasis in most cases and mucocutaneous leishmaniasis in some individuals. The latter is a severe and disfiguring form of the disease. At present, it remains unclear why the infection is controlled in some individuals but progressive in others (40).

Dendritic cell (DC)-pathogen interactions are initiated by interaction between receptors on DCs and pathogen-associated molecular patterns, including lipopolysaccharide (LPS), glycolipids, and nucleic acids. Signals through Toll-like receptors (TLRs) can induce DC maturation and the production of proinflammatory cytokines (20, 39), thereby bridging the innate and adaptive immune responses (9). Upon ligand binding,

downstream signaling of all TLRs (with the exception of TLR3) uses the adaptor protein MyD88 (32). Gene knockout studies in mice have suggested that TLR signaling is essential for the immune responses against *Leishmania* parasites (52). For example, MyD88 and TLR4 contribute to the control of *Leishmania major* infection in C57BL/6 mice (27, 33). TLR9 is involved in NK cell activation in animal models of visceral (*Leishmania donovani*) and cutaneous (*L. major* and *L. braziliensis*) leishmaniasis (30, 45), while TLR2 and TLR3 are required for the intracellular killing of *L. donovani* in gamma interferon (IFN-γ)-primed Mφs (15). *Leishmania* lipophosphoglycan (LPG), an abundant molecule in the surfaces of promastigotes, not only is a virulence factor for some *Leishmania* species (e.g., *L. major* and *L. donovani*) (49) but also acts as a ligand for TLR2-mediated signaling (5). However, different species of *Leishmania* display relatively high variations (biochemical modifications) in LPG molecules (7). In the case of *L. braziliensis*, the procyclic form of the parasite lacks side chain sugar substitutions on its LPG, whereas the metacyclic form appears to contain decreased amounts of LPG compared to other *Leishmania* species (47). On the DC surface, TLR2 is present as preexisting heterodimers of TLR2/1 and/or TLR2/6, recognizing triacylated and diacylated lipoproteins, respectively (51). TLR2 has been shown to be important for NK cell activation in vitro by purified *L. major* LPG (5); however, the functional roles of TLR2 remain largely unclear during both parasite-DC interactions and the course of *Leishmania* infection in vivo.

Most inbred strains of mice are genetically resistant to *L. braziliensis* infection, due to the capacity of mice to establish a strong Th1 response (43). This self control of infection is accompanied by the selective expansion of IFN-γ-producing

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[▽] Published ahead of print on 13 April 2009.

CD4⁺ T cells, which induce nitric oxide production in infected Mφs to promote parasite killing (3, 12). We have previously revealed that several key molecules in the innate immunity pathways (e.g., STAT1, STAT3, and ISG15) were upregulated in *L. braziliensis*-infected DCs and that such DCs were highly efficient in priming CD4⁺ T cells in vitro and in vivo (53). However, it remains unclear whether DC-*Leishmania* cell interactions in the absence of MyD88 and TLR2 impact T-cell functions and in vivo containment of infection. In the present study, we generated bone marrow-derived DCs (BMDCs) from MyD88^{-/-} and TLR2^{-/-} mice and examined their responsiveness to *L. braziliensis* infection. We found that infected MyD88^{-/-} DCs showed low levels of cell activation and interleukin-12 (IL-12) p40 production, which correlated with increased susceptibilities of these mice to *L. braziliensis* infection and decreased expansion of IFN-γ-producing and IL-17-producing CD4⁺ T cells during the course of infection. Given that most TLR pathways share MyD88 and that TLR2 is involved in LPG recognition, we then examined the role of TLR2 in *L. braziliensis* recognition. Contrary to MyD88^{-/-} DCs, the lack of TLR2 enhanced DC activation, IL-12 p40 production, and T-cell priming in vitro. Consequently, TLR2^{-/-} mice were more resistant to infection than were the control mice, a finding that was associated with enhanced IFN-γ production in the draining lymph nodes (dLN). Collectively, our results show that while MyD88 is critical for *L. braziliensis* recognition in vitro and in vivo, TLR2 appears to have a regulatory role in modulating immune responses to the parasite.

MATERIALS AND METHODS

Mice. Female TLR2^{-/-} mice (nine generations back-crossed to C57BL/6J [B6]) and wild-type (WT) B6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). MyD88^{-/-} mice (10 generations back-crossed to B6) were bred at the Baylor College of Medicine, Houston, TX, as described previously (24). Mice were maintained under specific-pathogen-free conditions and used at 6 to 8 weeks of age, according to protocols approved by the Institutional Animal Care and Use Committees.

Parasite culture and antigen preparation. Infectivity of *L. braziliensis* (MHOM-BR-79-LTB111) was maintained by regular passage through golden Syrian hamsters (Harlan Sprague Dawley, Indianapolis, IN). Infectivity of *Leishmania amazonensis* (MHOM/BR/73/LV78) was maintained by regular passage through BALB/c mice (Harlan Sprague Dawley). Promastigotes were cultured at 23°C in Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA), pH 7.0, supplemented with 20% fetal bovine serum (Sigma, St. Louis, MO), 2 mM L-glutamine, and 50 µg/ml gentamicin. Stationary promastigote cultures of less than five passages were used for DC or animal infection. To prepare soluble leishmanial antigens (SLA), *L. braziliensis* promastigotes (2 × 10⁸/ml) were subjected to six freeze-and-thaw cycles in liquid nitrogen and a 15-min sonication. SLA preparations were divided into aliquots and stored at -20°C until use.

DC generation and infection. BMDCs were generated from B6, MyD88^{-/-}, and TLR2^{-/-} mice in complete Iscove's modified Dulbecco's medium (Invitrogen) containing 10% fetal bovine serum, supplemented with 20 ng/ml recombinant granulocyte-macrophage colony-stimulating factor (eBioscience, San Diego, CA), and harvested on day 8. Parasites (5 × 10⁷/ml in phosphate-buffered saline) were washed twice and incubated with DCs (an 8:1 or 10:1 parasite-to-cell ratio) at 33°C for 8 h and then at 37°C for another 16 h. LPS (100 ng/ml) of *Salmonella enterica* serovar Typhimurium (Sigma) and IFN-γ (100 ng/ml; Leinco Technologies, St. Louis, MO) were used as positive controls in all of the experiments. Pam₃CSK₄ (100 ng/ml; InvivoGen, San Diego, CA), a synthetic agonist of TLR2, was used as a means of quality control in the TLR2^{-/-} in vitro experiments. At 24 h postinfection (p.i.), culture supernatants were harvested for cytokine detection and cells were collected for fluorescence-activated cell sorter (FACS) analysis.

T-cell priming in vitro. Naïve CD4⁺ T cells were purified from the spleens of B6 mice by negative selection using magnetic beads (Miltenyi Biotec, Auburn, CA), and their purity was routinely around 95%, as judged by CD4 staining and

FACS analysis. Purified CD4⁺ T cells (2 × 10⁵) were cocultured with parasite-infected, mitomycin C-pretreated DCs at a 10:1 ratio in 96-well plates for 4 days. Supernatants were harvested for cytokine detection. To assess T-cell proliferation, we added 1 µCi of [³H]thymidine 18 h before harvest and determined incorporated radioactivity on a microplate scintillation and luminescence counter (Packard Instrument Company, Downers Grove, IL). Stimulation indexes were calculated by normalizing the proliferation of CD4⁺ T cells induced by parasite-infected DCs to the proliferation induced by uninfected DCs.

Intracellular staining and FACS analysis. The following specific monoclonal antibodies and their corresponding isotype controls were purchased from eBiosciences: fluorescein isothiocyanate-conjugated anti-IFN-γ (XMG1.2) and rat immunoglobulin G2a (IgG2a); phycoerythrin (PE)-conjugated anti-IL-10 (JESS-16E3); anti-IL-12/IL-23 p40 (C17.8); anti-CD40 (1C10); anti-CD80 (16-10A1); and anti-CD83 (Michel-17); as well as rat IgG1, IgG2a, and IgG2b; PE-Cy5-conjugated anti-CD11c (N418); hamster IgG; PerCP-Cy5.5 anti-IL-17A (eBio17B7) and rat IgG2a; antigen-presenting cell (APC)-conjugated anti-CD4 (GK1.5) and rat IgG2b; and PE-Cy7-conjugated anti-CD8a (53-6.7) and rat IgG2a. APC-Cy7-conjugated anti-CD3e (145-2C11) and Armenian hamster IgG1 were purchased from BD Biosciences (Franklin Lakes, NJ). Briefly, cells were washed, blocked with 1 µg/ml FcRγ blocker (CD16/32; eBioscience), stained for specific surface molecules, fixed/permeabilized with a Cytofix/Cytoperm kit (BD Biosciences), and then stained for specific intracellular molecules. To detect intracellular cytokines, we added 1 µl/ml of Golgi Stop (BD Biosciences) for the last 6 h of cultivation. Cells were read on a FACSCanto flow cytometer (BD Biosciences) and analyzed using FlowJo v8.5 software (TreeStar, Ashland, OR).

Cytokine ELISA. The levels of cytokines in culture supernatants were measured by using enzyme-linked immunosorbent assay (ELISA) kits purchased from BD Biosciences (IL-12 p40 and IL-10) or eBioscience (IFN-γ and IL-17A). Detection limits were 15 pg/ml for IFN-γ, 4 pg/ml for IL-10, 10 pg/ml for IL-12 p40, and 8 pg/ml for IL-17, respectively.

SOCS1 and SOCS3 real-time RT-PCR. At 4 h p.i., the total RNA was extracted from ~1 × 10⁶ to ~2 × 10⁶ *Leishmania* parasite-infected DCs (a 10:1 parasite-to-cell ratio) using the RNeasy system (Qiagen, Valencia, CA). Genomic DNA was digested with the on-column RNase-free DNase (Qiagen). For detecting suppressor or cytokine signaling 1 (SOCS1) and SOCS3 transcripts, cDNA was synthesized from 2 µg of total RNA by using the SuperScript III first-strand system (Invitrogen) primed with random hexamers. Real-time reverse transcription-PCR (RT-PCR) was performed at the institutional real-time PCR core facility, and all reagents were purchased from Applied Biosystems (Foster City, CA) and consisted of the following: a 20× assay mixture of primers, TaqMan minor groove binder probes (6-carboxyfluorescein dye labeled) for mouse SOCS1 and SOCS3 (P/N 4331182), predeveloped 18S rRNA (VIC dye labeled, as an endogenous control), and TaqMan assay reagent (P/N 4319413E). Separate-tube (singleplex), real-time PCR analysis was performed with 40 ng cDNA for both target genes and the endogenous control by using TaqMan gene expression master mix (P/N 4370074). The PCR cycling parameters were as follows: uracil-N-glycosylase activation at 50°C for 2 min, AmpliTaq activation at 95°C for 10 min, denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min (repeated 40 times) on ABI Prism 7000 (Applied Biosystems). Duplicate threshold cycle (C_T) values were analyzed in Microsoft Excel by using the comparative C_T(ΔΔC_T) method, as described by the manufacturer (Applied Biosystems). The amount of target (2^{-ΔΔC_T}) was normalized to the endogenous reference (18s), and fold induction was calculated relative to uninfected control samples.

In vivo evaluation of infection and T-cell activation. *L. braziliensis* stationary promastigotes were injected subcutaneously in the right hind foot (2 × 10⁶ parasites/mouse; four mice/group). Lesion sizes were monitored weekly with a digital caliper (Control Company, Friendswood, TX), and tissue parasite burdens were measured via a limiting dilution assay by using LDA software (Oxford University Press, New York, NY) (19). At 4 and 8 weeks p.i., mice were euthanized, and the popliteal LN cells (1 × 10⁶/well/ml) were collected and stimulated with phorbol myristate acetate (PMA)-ionomycin-GolgiPlug for 6 h (ex vivo) prior to examination of intracellular IFN-γ, IL-17, and IL-10 expression. Similarly, LN cells were also cultured in the presence of SLA (a 1:1 parasite-to-cell ratio) for 72 h (recall response), and cytokine concentrations were assayed in culture supernatants by ELISA.

Statistical analysis. One-way analysis of variance (ANOVA) was used for multiple group comparisons. Differences between individual treatment groups were determined using Student's *t* test. A *P* value of ≤0.05 was considered statistically significant (GraphPad Software v4.0, San Diego, CA).

RESULTS

MyD88^{-/-} DCs have impaired activation and cytokine production after *L. braziliensis* infection. MyD88 is essential for DC activation/maturation after *L. donovani* and *L. major* infection (11, 14, 33). Given that *L. braziliensis* infection in B6 mice is self-healing due to a strong Th1 immune response (53), we investigated whether MyD88 is required for parasite control. We generated BMDCs from WT and MyD88^{-/-} mice and infected cells with *L. braziliensis* promastigotes at an 8:1 parasite-to-cell ratio. We consistently observed significantly lower percentages of activated DCs (CD11c⁺ CD40⁺) compared to those from the WT controls (Fig. 1A). Since marked differences are reported for DC responsiveness to *Leishmania* species (53, 57), we also infected MyD88^{-/-} DCs with *L. amazonensis*, a closely related New World species known for its selective impairment in DC activation, and used them as controls.

Upon pathogen capture, cytokines produced by DCs can shape both innate and adaptive immune responses, and the production of IL-12 p40 greatly promotes Th1 cell differentiation (55). As shown in Fig. 1B, *L. braziliensis*-infected MyD88^{-/-} DCs produced significantly smaller amounts of IL-12 p40 than their WT counterparts ($P < 0.01$). This impairment was not due to an intrinsic failure in the knockout cells, since WT and MyD88^{-/-} DCs produced similar levels of IL-12 p40 following stimulation with LPS and IFN- γ (Fig. 1B). Compared to the WT counterparts, MyD88^{-/-} DCs produced significantly larger amounts of IL-10 after infection with either *L. amazonensis* or *L. braziliensis* in the presence of LPS/IFN- γ (Fig. 1C). Thus, in the absence of MyD88, DC activation and IL-12 p40 production after *L. braziliensis* infection is not only hampered but also skewed toward enhanced IL-10 production.

Enhanced susceptibility of MyD88^{-/-} mice to *L. braziliensis* infection. It has been shown that MyD88^{-/-} B6 mice are highly susceptible to *L. major* infection due to the skew from protective Th1 responses to a detrimental Th2-type immune response (11, 14, 33). To examine the role of MyD88 in the control of *L. braziliensis*, we infected MyD88^{-/-} mice with 2×10^6 stationary promastigotes and monitored lesion development weekly. At 4 weeks p.i., lesion sizes were comparable in both groups of mice; however, at 5 to 8 weeks, a significant increase in lesion sizes was observed in *L. braziliensis*-infected MyD88^{-/-} mice ($P < 0.05$) (Fig. 2A). Consistent with increased lesion sizes, infected MyD88^{-/-} mice also contained significantly higher numbers of parasites in their footpads at 8 weeks than the WT controls ($-\log_{10} 2.82 \pm 0.35$ versus nondetectable; $P < 0.001$). To understand the mechanisms underlying the increased susceptibility in MyD88^{-/-} mice, we examined the cytokine profiles of in vivo-primed CD4⁺ T cells in the dLN. At 4 weeks p.i., when comparable lesion sizes (Fig. 2A) and parasite burdens (data not shown) were detected in both groups, MyD88^{-/-} mice had significantly lower numbers of IFN- γ - and IL-17-producing CD4⁺ T cells (Fig. 2B). The frequencies of cytokine-producing CD4⁺ T cells remained significantly low in infected MyD88^{-/-} mice even at 8 weeks, by which time the WT mice had already cleared the parasites (Fig. 2C). We observed similar trends when dLN cells were restimulated with parasite antigens for 3 days (data not shown). Our results suggest that MyD88 is necessary for the generation of immune

FIG. 1. The absence of MyD88 impairs IL-12 p40 production and DC activation after *L. braziliensis* infection. BMDCs were derived from WT (open bars) and MyD88^{-/-} (closed bars) mice and infected with *L. braziliensis* (Lb) and *L. amazonensis* (La) promastigotes at an 8:1 parasite-to-cell ratio for 24 h. (A) Expression of CD40 on DC surfaces was measured by FACS analysis. (B) IL-12 p40 production in the culture supernatants was assayed by ELISA. (C) IL-10 production in the culture supernatants in response to parasite infection plus LPS (100 ng/ml) and IFN- γ (100 ng/ml) was assayed by ELISA. Results from three independent repeats were pooled. Statistically significant differences between the groups determined by one-way ANOVA and Student's *t* test are as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. n.d., not detectable.

responses against *L. braziliensis* infection in vivo, especially for the priming of IFN- γ - and IL-17-producing CD4⁺ T cells.

***L. braziliensis*-infected TLR2^{-/-} DCs display high levels of activation and APC function.** Given that MyD88 signaling is

FIG. 2. Increased susceptibility of MyD88^{-/-} mice to *L. braziliensis* infection is associated with a decrease in IFN-γ- and IL-17-producing CD4⁺ T cells. MyD88^{-/-} and WT mice (four per group) were infected in the hind foot with 2×10^6 promastigotes of *L. braziliensis*. (A) Lesion sizes were monitored weekly with a digital caliper. Parasite loads at 8 weeks p.i. are indicated inside the graph ($-\log_{10} \pm$ the standard deviation). n.d., not detectable. At 4 (B) and 8 (C) weeks, dLN cells were collected and stimulated with PMA-ionomycin-GolgiPlug for 6 h. Intracellular staining for IFN-γ, IL-17, and IL-10 was gated on CD4⁺ T cells. Results shown are from one experiment and representative of two independent experiments. # ($P < 0.05$) and ## ($P < 0.01$) represent statistically significant differences between WT and MyD88^{-/-} mice. Statistically significant differences between the naive and infected groups determined by one-way ANOVA and Student's *t* test are as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Lb, *L. braziliensis*; La, *L. amazonensis*.

shared by almost all TLRs and that TLR2 is a key molecule in host-*Leishmania* species interactions (4, 15), we then investigated if TLR2 was involved in the recognition of *L. braziliensis*. We generated DCs from TLR2^{-/-} mice and infected them with *L. braziliensis* promastigotes. Unexpectedly, we found that the absence of TLR2 resulted in an increased expression of CD40 (Fig. 3A) and CD80 (Fig. 3B) in CD11c⁺ cells. Upon *L. braziliensis* infection, TLR2^{-/-} DCs produced larger amounts of IL-12 p40 (Fig. 3C) but smaller amounts of IL-10 (Fig. 3D) than did WT DCs. It is known that TLR engagement can induce regulatory pathways, including the expression of SOCS molecules (28), and that *Leishmania* infection can activate the SOCS1 and SOCS3 genes in human Mφs (6). Therefore, we investigated whether changes in the transcription of the SOCS genes could explain the enhanced activation in infected TLR2^{-/-} DCs. To our surprise, *L. braziliensis*-infected TLR2^{-/-} DCs had significantly higher levels of SOCS1 (Fig. 3E) and SOCS3 (Fig. 3F) transcription compared to infected WT DCs, suggesting a TLR2-independent upregulation of SOCS1 and SOCS3 transcription in *L. braziliensis*-infected DCs.

The finding of enhanced DC activation following *L. braziliensis* infection in the absence of TLR2 prompted us to examine DC-T-cell interactions. Using an in vitro T-cell priming assay, we found that a coculture of *L. braziliensis*-infected or *L. amazonensis*-infected TLR2^{-/-} DCs with purified naive CD4⁺ T cells resulted in a significant increase in both T-cell proliferation (Fig. 4A) and IFN-γ production (Fig. 4B). Collectively, our in vitro infection studies suggested to us that TLR2 deficiency promotes DC activation and IL-12 p40 production, in addition to CD4⁺ T-cell activation and IFN-γ production.

Enhanced resistance of TLR2^{-/-} mice to *L. braziliensis* infection in vivo. To further examine the biological functions of TLR2, we infected WT and TLR2^{-/-} mice with 2×10^6 promastigotes in the hind foot. We consistently observed that TLR2^{-/-} mice developed significantly smaller lesions than those found in infected WT mice from 2 to 5 weeks p.i. (Fig. 5A), even though lesions from both groups contained comparable parasite loads at 4 and 8 weeks (Fig. 5B). To determine if this enhanced protection in infected TLR2^{-/-} mice was associated with changes in the T-cell cytokine profile, we assessed in vivo-primed CD4⁺ T cells via FACS analysis. Both infection groups contained comparable numbers of IFN-γ-, IL-17-, and IL-10-producing CD4⁺ T cells at 4 weeks (Fig. 5C); however, the numbers of IFN-γ-producing CD4⁺ T cells in TLR2^{-/-} mice were significantly higher than those in WT mice at 8 weeks ($P < 0.01$) (Fig. 5D). By comparison, the numbers of IL-17-producing and IL-10-producing CD4⁺ T cells were comparable in TLR2^{-/-} and WT mice at 8 weeks (Fig. 5D). To confirm the sustained production of IFN-γ in infected TLR2^{-/-} mice, we restimulated dLN cells in vitro with *L. braziliensis* antigens for 3 days and measured IFN-γ and IL-10 levels in culture supernatants by using ELISA. As shown in Fig. 6A, while antigen-specific IFN-γ production decreased markedly from 4 to 8 weeks p.i. in WT mice ($P < 0.05$), IFN-γ production was maintained at a relatively high level during this period in TLR2^{-/-} mice. This trend was unique for IFN-γ since IL-10 production by dLN cells in response to antigen

FIG. 3. TLR2^{-/-} DCs show enhanced activation after *L. braziliensis* infection. WT and TLR2^{-/-} BMDCs were infected with *L. braziliensis* (Lb) and *L. amazonensis* (La) promastigotes at an 8:1 parasite-to-cell ratio for 24 h. Expression of DC surface maturation markers CD40 (A) and CD80 (B) was measured by FACS analysis. The levels of IL-12 p40 (C) and IL-10 (D) in the culture supernatants were assayed by ELISA. (E and F) DCs were infected at a 10:1 parasite-to-cell ratio for 4 h before RNA extraction. Total RNA was extracted and used for real-time RT-PCR measurement of SOCS1 (E) and SOCS3 (F) levels. Data are expressed as a fold change of expression relative to uninfected samples and normalized to the 18s ribosomal control. P₃CSK₄ (Pam₃CSK₄) was used as a quality control for TLR2. Data were pooled from three independent repeats and are shown in the plots. Statistically significant differences between the groups determined by one-way ANOVA and Student's *t* test are as follows: *, *P* < 0.05; **, *P* < 0.01; and ***, *P* < 0.001. KO, knockout.

restimulation was reduced in both TLR2^{-/-} and WT mice by 8 weeks (Fig. 6B).

DISCUSSION

Since their discovery, TLRs have been extensively studied for their role in the recognition of pathogen components and the initiation of innate immune responses (17, 32). We have

recently described that *L. braziliensis* infection triggers DC activation and cytokine production, partially via the activation of the JAK/STAT signaling pathway (53). However, little is known about how innate immunity contributes to *L. braziliensis* recognition by DCs and parasite containment in vivo. In the present study, we used both an in vitro DC infection system and an in vivo infection model to analyze the roles of MyD88

FIG. 4. *Leishmania* parasite-infected TLR2^{-/-} DCs induce stronger in vitro T-cell responses than WT DCs. WT and TLR2^{-/-} BMDCs were infected with *L. braziliensis* and *L. amazonensis* promastigotes at an 8:1 parasite-to-cell ratio for 24 h. DCs were then treated with mitomycin C (50 mg/ml) and cocultured with spleen-derived, naïve syngeneic CD4⁺ T cells (2×10^6 /ml) at a 1:10 DC-to-T-cell ratio. (A) T-cell proliferation was measured by [³H]thymidine uptake after 4 days of coculture. (B) The presence of IFN-γ in culture supernatants was assayed by ELISA. Shown are pooled data from three independent experiments. Statistically significant differences between the groups determined by one-way ANOVA and Student's *t* test are shown as follows: **, *P* < 0.01; and ***, *P* < 0.001.

and TLR2 in *L. braziliensis*-DC interactions and in the generation of antigen-specific CD4⁺ T-cell responses. Our data indicate that MyD88 was important for DC recognition of *L. braziliensis* promastigotes in vitro and for the control of infection in vivo because of significant impairments in DC and T-cell responses and protective immunity in MyD88^{-/-} mice. Unexpectedly, we found that DC and T-cell activation in response to *L. braziliensis* infection in vitro was markedly enhanced in the absence of the TLR2 gene. As such, TLR2^{-/-} mice were more resistant to *L. braziliensis* infection than were WT controls, partially due to the sustained production of IFN-γ in infected knockouts. To the best of our knowledge, this is the first report to describe the differential roles of MyD88 and TLR2 in *L. braziliensis*-DC interactions and the generation of protective immunity to this parasite species.

Our studies involving MyD88^{-/-} DCs are in line with previous reports confirming that in the absence of this adaptor protein, DC maturation and pathogen recognition are affected (13, 31, 33, 46). The first control point for the establishment of a protective Th1 immune response is at the level of IL-12 production by DCs, a process that can be regulated by MyD88-dependent and MyD88-independent mechanisms (2). In *Toxo-*

plasma gondii infection models, it has been shown that the production of both IL-12 p40 and IL-12 p70 in splenic DCs is mediated via MyD88-dependent events (44). In our hands, *L. braziliensis*-infected, but not *L. amazonensis*-infected, MyD88^{-/-} DCs expressed smaller amounts of IL-12 p40 and costimulatory molecule CD40 than did WT cells (Fig. 1). It is documented that *L. amazonensis* parasites can directly affect antigen presentation by targeting the JAK/STAT signaling pathway in Mφs (35) and DCs (56), therefore downregulating IL-12 production and Th1 responses. Thus, our comparative studies involving two distinct New World *Leishmania* species clearly indicate that successful DC activation by *L. braziliensis* parasites is partially dependent on the TLR-MyD88 signaling pathway. A second mechanism to regulate Th1 cell development by APCs is the induction of IL-10 production (57). Although infection of DCs with promastigotes alone did not trigger IL-10 production (53), we observed that infection with both parasite species, in the presence of LPS/IFN-γ, caused significantly higher levels of IL-10 secretion in MyD88^{-/-} DCs than it did in their WT controls (Fig. 1C). Collectively, our in vitro results suggest that the absence of MyD88 differentially affects IL-12 p40 and IL-10 production by *Leishmania* parasite-infected DCs.

MyD88^{-/-} mice have been reported to be more susceptible to infection with a wide variety of viral, bacterial, and protozoan pathogens, such as respiratory syncytial virus (10), *Burkholderia pseudomallei* (54), *T. gondii* (44), *Plasmodium berghei* (1), and *L. major* (11, 14, 33). Our in vivo studies with *L. braziliensis* also indicate that MyD88 is required for effective parasite clearance and lesion resolution in B6 mice, a mouse strain that spontaneously heals the lesions at around 8 weeks (41). We have previously reported that *L. braziliensis* infection leads to a strong induction of IL-17-producing CD4⁺ T cells (Th17 cells) both in vitro and in vivo (53). Interestingly, *L. braziliensis* infection in MyD88^{-/-} mice failed to trigger Th17 cell differentiation throughout the observation period (Fig. 2B and C). It is known that Th17 immune responses are crucial during infections that cause tissue inflammation and cell recruitment (37), especially because IL-17 can induce the production of IL-1β, tumor necrosis factor alpha, and IL-6 in different cell types (26). Studies in a murine model of inflammatory bowel disease showed that in the absence of MyD88, Th17 cell differentiation was strongly and particularly affected (16), a finding that was similar to those in our studies. Even though Th17 cells seem to play an important role during infection, Th1 activation is a prerequisite for the development of a protective immune response against *Leishmania* parasites (50). In agreement with this view, we observed that the lack of MyD88 caused a decrease in, but not the complete abolishment of, the intensity of Th1 responses. Our results add up to previous reports showing a crucial role for MyD88 in the establishment of an IL-12-mediated Th1 immune response against *L. major* (11, 14, 33). MyD88-dependent resistance to *L. braziliensis* infection can also be attributed to TLR-independent pathways, because certain inflammatory responses (e.g., the IL-1 and IL-8 signaling pathways) also use MyD88 as an adaptor protein (8). Since *L. braziliensis* is not extremely pathogenic in mice, it is not surprising that the infected MyD88^{-/-} mice displayed increased susceptibilities with delayed parasite clearance rather than uncontrolled parasite rep-

FIG. 5. TLR2^{-/-} mice have enhanced resistance to *L. braziliensis* infection. TLR2^{-/-} and WT mice (five per group) were infected in the hind foot with 2×10^6 promastigotes of *L. braziliensis*. (A) Lesion sizes were monitored weekly with a digital caliper. (B) Mice were sacrificed at 4 and 8 weeks, and parasite burdens in foot tissues were determined by limited dilution. Draining LN cells were collected at 4 (C) and 8 (D) weeks p.i. and stimulated with PMA-ionomycin-GolgiPlug for 6 h. Intracellular staining for IFN- γ , IL-17, and IL-10 was gated on CD3⁺CD4⁺ T cells. ## ($P < 0.01$) represents statistically significant differences between WT and TLR2^{-/-} mice. Statistically significant differences between the naive and infected groups determined by one-way ANOVA and Student's *t* test are shown as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

lication as observed in *L. major*-infected MyD88^{-/-} mice (11, 33). Furthermore, we did not observe significant production of IL-4 by dLN cells of WT and MyD88^{-/-} mice at 8 and 12 weeks p.i. (data not shown), a finding that was in contrast to *L. major*-infected MyD88^{-/-} mice (11, 33). Since the IL-17-producing CD4⁺ T subset appeared to be affected mostly by the absence of MyD88, we are currently investigating the role of Th17 cells in *Leishmania* infection.

TLR2 can bind to a wide variety of pathogenic molecules, including lipoteichoic acid, peptidoglycan, atypical LPS, and other lipoproteins from both gram-positive and gram-negative bacteria (18, 23). Importantly, the surfaces of *Leishmania* promastigotes are intensively covered by glycosylphosphatidylinositol-anchored and glycosylphosphatidylinositol-related proteins (such as gp63 and LPG) (34, 47). de Veer et al. have described that purified *L. major* LPG can activate mitogen-activated protein kinase signaling and induce the production of IL-12 p40 and tumor necrosis factor α in bone marrow-derived M ϕ s in a TLR2- and MyD88-dependent manner (14). Activation of TLR2 signaling, however, can also induce regulatory pathways in a wide variety of cell types (36). For example, findings from studies of human peripheral blood DCs stimulated with peptidoglycan and *Porphyromonas gingivalis* fimbriae showed a decreased capacity of the DCs to stimulate allogeneic T-cell proliferation compared to that for LPS-stimulated DCs (21). Similarly, Ropert et al. showed that spleno-

cytes of TLR2^{-/-} mice produce increased levels of IFN- γ and nitric oxide after an in vivo challenge with *Trypanosoma cruzi*, compared to WT controls (42). Our in vitro studies with DCs derived from TLR2^{-/-} and WT mice showed that *L. braziliensis* infection induced significantly high levels of DC activation and IL-12 p40 production (Fig. 3A to C), as well as decreased levels of IL-10 production (Fig. 3D). These results suggest a negative/regulatory role for TLR2 in DC activation. This conclusion was supported by our findings that, in the absence of TLR2, *Leishmania* parasite-infected DCs were highly potent APCs for activating syngeneic CD4⁺ T cells to proliferate and produce IFN- γ (Fig. 4).

It has been shown that *L. donovani* is capable of inducing expression of SOCS1 and SOCS3 in human M ϕ s as a potent inhibitory strategy to prevent cell activation (6). Our real-time RT-PCR studies for the expression levels of SOCS1 and SOCS3 suggest that the upregulation of these proteins in *L. braziliensis*-infected DCs (Fig. 3) is TLR2 independent. At this stage, the intracellular events that lead to the enhanced DC and T-cell activation in *Leishmania* parasite-infected TLR2^{-/-} mice remain unclear.

Several infection models have indicated that TLR2 deficiency enhances the susceptibility of mice to bacterial and viral pathogens such as *Streptococcus pneumoniae* (25) and herpes simplex virus (48). Regardless of the mechanisms underlying enhanced DC activation during *L. braziliensis* infection in the

FIG. 6. Decreased susceptibility of TLR2^{-/-} mice to *L. braziliensis* infection is associated with a sustained production of IFN- γ in the dLN. TLR2^{-/-} and B6 WT mice (four per group) were infected in the hind foot with 2×10^6 promastigotes of *L. braziliensis*. At 4 and 8 weeks, dLN cells were collected and restimulated with parasite antigens for 3 days. Supernatants were collected and assayed by ELISA to determine the concentrations of IFN- γ (A) and IL-10 (B). Shown are representative results from one of two independent experiments with similar trends. Statistically significant differences between the groups determined by one-way ANOVA and Student's *t* test are shown as follows: *, $P < 0.05$; and **, $P < 0.01$. n.d., not detectable; n.s., not significant.

absence of TLR2, our in vivo studies indicate that TLR2^{-/-} mice were capable of controlling *L. braziliensis* and developed smaller lesions than did the WT controls (Fig. 5A). Despite this, *L. braziliensis*-infected TLR2^{-/-} and WT mice showed comparable parasite loads at 4 weeks p.i., suggesting that the reduction in lesion sizes in TLR2^{-/-} mice was not linked to increased parasite killing in this model (Fig. 5B). The decreased lesion sizes in TLR2^{-/-} mice correlated with an enhancement in IFN- γ production in LN cells in response to restimulation with *L. braziliensis* SLA, especially at late stages of the infection (Fig. 5 and 6). These in vivo and ex vivo findings clearly support our in vitro data, which suggested to us that enhanced DC activation and T-cell-priming capacity had a positive impact on protective immunity to *Leishmania* infection in vivo.

In summary, this study addresses the role of MyD88 during the immune response to *L. braziliensis* infection and how the absence of this molecule greatly impairs protective immunity. Additionally, we show that not all signaling pathways that use MyD88 act alike, since the absence of TLR2 had a positive

impact on the disease outcome. Findings from our in vitro studies using MyD88^{-/-} and TLR2^{-/-} DCs, which were similar to those in our previous reports (48), have revealed a direct correlation between DC activation status in vitro and the outcome of infection in the host. Furthermore, unraveling the role played by key components of the innate immune system would provide novel strategies for the control of leishmaniasis.

ACKNOWLEDGMENTS

This study was supported in part by NIH grants AI043003 (to L.S.) and AI057696 (to D.B.C.) and by the James W. McLaughlin Fellowship Fund (to D.A.V.-I. and A.E.H.).

We thank Janice J. Endsley for helpful discussions and critical review of the manuscript and Mardelle Susman for assisting in manuscript preparation.

REFERENCES

- Adachi, K., H. Tsutsui, S. Kashiwamura, E. Seki, H. Nakano, O. Takeuchi, K. Takeda, K. Okumura, L. Van Kaer, H. Okamura, S. Akira, and K. Nakanishi. 2001. *Plasmodium berghei* infection in mice induces liver injury by an IL-12- and toll-like receptor/myeloid differentiation factor 88-dependent mechanism. *J. Immunol.* 167:5928–5934.
- Alexander, J., and K. Bryson. 2005. T helper (h)1/Th2 and *Leishmania*: paradox rather than paradigm. *Immunol. Lett.* 99:17–23.
- Alexander, J., A. R. Satoskar, and D. G. Russell. 1999. *Leishmania* species: models of intracellular parasitism. *J. Cell Sci.* 112:2993–3002.
- Banerjee, A., and S. Gerondakis. 2007. Coordinating TLR-activated signaling pathways in cells of the immune system. *Immunol. Cell Biol.* 85:420–424.
- Becker, I., N. Salaiza, M. Aguirre, J. Delgado, N. Carrillo-Carrasco, L. G. Koheb, A. Ruiz, R. Caervantes, A. P. Torres, N. Cabrera, A. Gonzalez, C. Maldonado, and A. Isibasi. 2003. *Leishmania* lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. *Mol. Biochem. Parasitol.* 130:65–74.
- Bertholet, S., H. L. Dickensheets, F. Sheikh, A. A. Gam, R. P. Donnelly, and R. T. Kenney. 2003. *Leishmania donovani*-induced expression of suppressor of cytokine signaling 3 in human macrophages: a novel mechanism for intracellular parasite suppression of activation. *Infect. Immun.* 71:2095–2101.
- Beverley, S. M., and S. J. Turco. 1998. Lipophosphoglycan (LPG) and the identification of virulence genes in the protozoan parasite *Leishmania*. *Trends Microbiol.* 6:35–40.
- Braddock, M., and A. Quinn. 2004. Targeting IL-1 in inflammatory disease: new opportunities for therapeutic intervention. *Nat. Rev. Drug. Discov.* 3:330–339.
- Chandra, D., and S. Naik. 2008. *Leishmania donovani* infection down-regulates TLR2-stimulated IL-12 p40 and activates IL-10 in cells of macrophage/monocytic lineage by modulating MAPK pathways through a contact-dependent mechanism. *Clin. Exp. Immunol.* 154:224–234.
- Cyr, S. L., I. Angers, L. Guillot, I. Stoica-Popescu, M. Lussier, S. Qureshi, D. S. Burt, and B. J. Ward. 2009. TLR4 and MyD88 control protection and pulmonary granulocytic recruitment in a murine intranasal RSV immunization and challenge model. *Vaccine* 27:421–430.
- Debus, A., J. Glasner, M. Rollinghoff, and A. Gessner. 2003. High levels of susceptibility and T helper 2 response in MyD88-deficient mice infected with *Leishmania major* are interleukin-4 dependent. *Infect. Immun.* 71:7215–7218.
- DeKrey, G. K., H. C. Lima, and R. G. Titus. 1998. Analysis of the immune responses of mice to infection with *Leishmania braziliensis*. *Infect. Immun.* 66:827–829.
- De Trez, C., M. Brait, O. Leo, T. Aebischer, F. A. Torrentera, Y. Carlier, and E. Muraille. 2004. MyD88-dependent in vivo maturation of splenic dendritic cells induced by *Leishmania donovani* and other *Leishmania* species. *Infect. Immun.* 72:824–832.
- de Veer, M. J., J. M. Curtis, T. M. Baldwin, J. A. DiDonato, A. Sexton, M. J. McConville, E. Handman, and L. Schofield. 2003. MyD88 is essential for clearance of *Leishmania major*: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. *Eur. J. Immunol.* 33:2822–2831.
- Flandin, J. F., F. Chano, and A. Descoteaux. 2006. RNA interference reveals a role for TLR2 and TLR3 in the recognition of *Leishmania donovani* promastigotes by interferon-gamma-primed macrophages. *Eur. J. Immunol.* 36:411–420.
- Fukata, M., K. Breglio, A. Chen, A. S. Vamadevan, T. Goo, D. Hsu, D. Conduah, R. Xu, and M. T. Abreu. 2008. The myeloid differentiation factor 88 (MyD88) is required for CD4⁺ T cell effector function in a murine model of inflammatory bowel disease. *J. Immunol.* 180:1886–1894.
- Gazzinelli, R. T., and E. Y. Denkers. 2006. Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. *Nat. Rev. Immunol.* 6:895–906.

18. Hertz, C., S. Kiertscher, P. Godowski, D. Bouis, M. Norgard, M. Roth, and R. Modlin. 2001. Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. *J. Immunol.* **166**:2444–2450.
19. Ji, J., J. Sun, and L. Soong. 2003. Impaired expression of inflammatory cytokines and chemokines at early stages of infection with *Leishmania amazonensis*. *Infect. Immun.* **71**:4278–4288.
20. Kaisho, T., and S. Akira. 2001. Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice. *Trends Immunol.* **22**:78–83.
21. Kanaya, S., E. Nemoto, T. Ogawa, and H. Shimauchi. 2008. *Porphyromonas gingivalis* fimbriae induce unique dendritic cell subsets via Toll-like receptor 2. *J. Periodont. Res.* doi:10.1111/j.1600-0765.2008.01149.x.
22. Kimblin, N., N. Peters, A. Debrabant, N. Secundino, J. Egen, P. Lawyer, M. P. Fay, S. Kamhawi, and D. Sacks. 2008. Quantification of the infectious dose of *Leishmania major* transmitted to the skin by single sand flies. *Proc. Natl. Acad. Sci. USA* **105**:10125–10130.
23. Kirschning, C. J., and R. R. Schumann. 2002. TLR2: cellular sensor for microbial and endogenous molecular patterns. *Curr. Top. Microbiol. Immunol.* **270**:121–144.
24. Kiss, A., M. Montes, S. Susarla, E. A. Jaensson, S. M. Drouin, R. A. Wetsel, Z. Yao, R. Martin, N. Hamzeh, R. Adelagun, S. Amar, F. Kheradmand, and D. B. Corry. 2007. A new mechanism regulating the initiation of allergic airway inflammation. *J. Allergy Clin. Immunol.* **120**:334–342.
25. Klein, M., B. Obermaier, B. Angele, H. W. Pfister, H. Wagner, U. Koedel, and C. J. Kirschning. 2008. Innate immunity to pneumococcal infection of the central nervous system depends on toll-like receptor (TLR) 2 and TLR4. *J. Infect. Dis.* **198**:1028–1036.
26. Kolls, J. K., and A. Linden. 2004. Interleukin-17 family members and inflammation. *Immunity* **21**:467–476.
27. Kropf, P., M. A. Freudenberg, M. Modolell, H. P. Price, S. Herath, S. Antoniazzi, C. Galanos, D. F. Smith, and I. Muller. 2004. Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite *Leishmania major*. *Infect. Immun.* **72**:1920–1928.
28. Kubo, M., T. Hanada, and A. Yoshimura. 2003. Suppressors of cytokine signaling and immunity. *Nat. Immunol.* **4**:1169–1176.
29. Laskay, T., G. van Zandbergen, and W. Solbach. 2008. Neutrophil granulocytes as host cells and transport vehicles for intracellular pathogens: apoptosis as infection-promoting factor. *Immunobiology* **213**:183–191.
30. Liese, J., U. Schleicher, and C. Bogdan. 2007. TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. *Eur. J. Immunol.* **37**:3424–3434.
31. Macedo, G. C., D. M. Magnani, N. B. Carvalho, O. Bruna-Romero, R. T. Gazzinelli, and S. C. Oliveira. 2008. Central role of MyD88-dependent dendritic cell maturation and proinflammatory cytokine production to control *Brucella abortus* infection. *J. Immunol.* **180**:1080–1087.
32. Medzhitov, R. 2001. Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* **1**:135–145.
33. Muraille, E., C. De Trez, M. Brait, P. De Baetselier, O. Leo, and Y. Carlier. 2003. Genetically resistant mice lacking MyD88-adaptor protein display a high susceptibility to *Leishmania major* infection associated with a polarized Th2 response. *J. Immunol.* **170**:4237–4241.
34. Naderer, T., J. E. Vince, and M. J. McConville. 2004. Surface determinants of *Leishmania* parasites and their role in infectivity in the mammalian host. *Curr. Mol. Med.* **4**:649–665.
35. Olivier, M., D. J. Gregory, and G. Forget. 2005. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. *Clin. Microbiol. Rev.* **18**:293–305.
36. O'Neill, L. A. 2008. When signaling pathways collide: positive and negative regulation of toll-like receptor signal transduction. *Immunity* **29**:12–20.
37. Ouyang, W., J. K. Kolls, and Y. Zheng. 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* **28**:454–467.
38. Peters, N. C., J. G. Egen, N. Secundino, A. Debrabant, N. Kimblin, S. Kamhawi, P. Lawyer, M. P. Fay, R. N. Germain, and D. Sacks. 2008. *In vivo* imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science* **321**:970–974.
39. Qi, H., T. L. Denning, and L. Soong. 2003. Differential induction of interleukin-10 and interleukin-12 in dendritic cells by microbial toll-like receptor activators and skewing of T-cell cytokine profiles. *Infect. Immun.* **71**:3337–3342.
40. Reithinger, R., J.-C. Dujardin, H. Louzir, C. Pirmez, B. Alexander, and S. Brooker. 2007. Cutaneous leishmaniasis. *Lancet Infect. Dis.* **7**:581–596.
41. Rocha, F. J., U. Schleicher, J. Mattner, G. Alber, and C. Bogdan. 2007. Cytokines, signaling pathways, and effector molecules required for the control of *Leishmania (Viannia) braziliensis* in mice. *Infect. Immun.* **75**:3823–3832.
42. Ropert, C., and R. T. Gazzinelli. 2004. Regulatory role of Toll-like receptor 2 during infection with *Trypanosoma cruzi*. *J. Endotoxin Res.* **10**:425–430.
43. Samuelson, J., E. Lerner, R. Tesh, and R. Titus. 1991. A mouse model of *Leishmania braziliensis braziliensis* infection produced by coinjection with sand fly saliva. *J. Exp. Med.* **173**:49–54.
44. Scanga, C. A., J. Aliberti, D. Jankovic, F. Tilloy, S. Bennouna, E. Y. Denkers, R. Medzhitov, and A. Sher. 2002. Cutting edge: MyD88 is required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12 production by dendritic cells. *J. Immunol.* **168**:5997–6001.
45. Schleicher, U., J. Liese, I. Knippertz, C. Kurzmann, A. Hesse, A. Heit, J. A. Fischer, S. Weiss, U. Kalinke, S. Kunz, and C. Bogdan. 2007. NK cell activation in visceral leishmaniasis requires TLR9, myeloid DCs, and IL-12, but is independent of plasmacytoid DCs. *J. Exp. Med.* **204**:893–906.
46. Siegemund, S., and G. Alber. 2008. *Cryptococcus neoformans* activates bone marrow-derived conventional dendritic cells rather than plasmacytoid dendritic cells and down-regulates macrophages. *FEMS Immunol. Med. Microbiol.* **52**:417–427.
47. Soares, R. P., T. L. Cardoso, T. Barron, M. S. Araujo, P. F. Pimenta, and S. J. Turco. 2005. *Leishmania braziliensis*: a novel mechanism in the lipophosphoglycan regulation during metacyclogenesis. *Int. J. Parasitol.* **35**:245–253.
48. Sørensen, L. N., L. S. Reinert, L. Malmgaard, C. Bartholdy, A. R. Thomsen, and S. R. Paludan. 2008. TLR2 and TLR9 synergistically control herpes simplex virus infection in the brain. *J. Immunol.* **181**:8604–8612.
49. Späth, G. F., L. Epstein, B. Leader, S. M. Singer, H. A. Avila, S. J. Turco, and S. M. Beverley. 2000. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major*. *Proc. Natl. Acad. Sci. USA* **97**:9258–9263.
50. Stuart, K., R. Brun, S. Croft, A. Fairlamb, R. E. Gurtler, J. McKerrow, S. Reed, and R. Tarleton. 2008. Kinetoplastids: related protozoan pathogens, different diseases. *J. Clin. Invest.* **118**:1301–1310.
51. Triantafyllou, M., F. G. Gamper, R. M. Haston, M. A. Mouratis, S. Morath, T. Hartung, and K. Triantafyllou. 2006. Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting. *J. Biol. Chem.* **281**:31002–31011.
52. Tuon, F. F., V. S. Amato, H. A. Bacha, T. Almusawi, M. I. Duarte, and V. Amato Neto. 2008. Toll-like receptors and leishmaniasis. *Infect. Immun.* **76**:866–872.
53. Vargas-Inchaustegui, D. A., L. Xin, and L. Soong. 2008. *Leishmania braziliensis* infection induces dendritic cell activation, ISG15 transcription, and the generation of protective immune responses. *J. Immunol.* **180**:7537–7545.
54. Wiersinga, W. J., C. W. Wieland, J. J. Roelofs, and T. van der Poll. 2008. MyD88 dependent signaling contributes to protective host defense against *Burkholderia pseudomallei*. *PLoS ONE* **3**:e3494.
55. Woelbing, F., S. L. Kostka, K. Moelle, Y. Belkaid, C. Sunderkoetter, S. Verbeek, A. Waisman, A. P. Nigg, J. Knop, M. C. Udey, and E. von Stebut. 2006. Uptake of *Leishmania major* by dendritic cells is mediated by Fc-gamma receptors and facilitates acquisition of protective immunity. *J. Exp. Med.* **203**:177–188.
56. Xin, L., K. Li, and L. Soong. 2008. Down-regulation of dendritic cell signaling pathways by *Leishmania amazonensis* amastigotes. *Mol. Immunol.* **45**:3371–3382.
57. Xin, L., Y. Li, and L. Soong. 2007. Role of interleukin-1 β in activating the CD11c^{high} CD45RB⁺ dendritic cell subset and priming *Leishmania amazonensis*-specific CD4⁺ T cells in vitro and in vivo. *Infect. Immun.* **75**:5018–5026.