Genetically Resistant Mice Lacking MyD88-Adapter Protein Display a High Susceptibility to *Leishmania major* Infection Associated with a Polarized Th2 Response[1]

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Host resistance to the intracellular protozoan *Leishmania major* is highly dependent on IL-12 production by APCs. Genetically resistant C57BL/6 mice develop IL-12-mediated Th1 immune response dominated by IFN-γ and exhibit only small cutaneous lesions that resolve spontaneously. In contrast, because of several genetic differences, BALB/c mice develop an IL-4-mediated Th2 immune response and a chronic mutilating disease. Myeloid differentiation marker 88 (MyD88) is an adaptator protein that links the IL-1/Toll-like receptor family to IL-1R-associated protein kinase. Toll-like receptors recognize pathogen associated molecular patterns and are crucially implicated in the induction of IL-12 secretion by APC. The role of MyD88 protein in the development of protective immune response against parasites is largely unknown. Following inoculation of *L. major*, MyD88$^{-/-}$ C57BL/6 mice presented large footpad lesions containing numerous infected cells and frequent mutilations. In response to soluble *Leishmania* Ag, cells from lesion-draining lymph node showed a typical Th2 profile, similar to infected BALB/c mice. IL-12p40 plasma level collapses in infected MyD88$^{-/-}$ mice compared with infected wild-type C57BL/6 mice. Importantly, administration of exogenous IL-12 rescues *L. major*-infected MyD88$^{-/-}$ mice, demonstrating that the susceptibility of these mice is a direct consequence of IL-12 deficiency. In conclusion, MyD88-dependent pathways appear essential for the development of the protective IL-12-mediated Th1 response against the *Leishmania major* parasite. In absence of MyD88 protein, infected mice develop a nonprotective Th2 response. ***The Journal of Immunology*, 2003, 170: 4237–4241.**

Leishmania are protozoan parasites belonging to the *Trypanosomatidae* family. They are transmitted by phlebotomine sand flies to several mammals, including humans (reviewed in Refs. 1 and 2). *Leishmania* parasites induce a large spectrum of diseases in humans, from cutaneous lesions to progressive fatal visceralizing diseases. Clinical manifestations depend on the parasite species, immune response, and genetic of the host. A lot of information about these factors has been drawn from the murine models of *Leishmania major* infection. Clearance of *L. major* parasites in infected cells implicate effector mechanisms, such as TNF-α, NO, and Fas-mediated apoptosis (3, 4), positively regulated by IFN-γ-producing CD4$^+$ T cells (Th1 cells) and down-regulated by IL-4/IL-10-producing CD4$^+$ T cells (Th2 cells) (reviewed in Refs. 2 and 5). Most inbred mouse strains (including C3H, C57BL/6) develop a protective Th1 immune response and are able to control infection. In contrast, BALB/c mice develop an IL-4-mediated Th2 response and a progressive fatal disease (6). The key role played by IL-12 in Th1 differentiation has been extensively documented in *Leishmania* infection (7, 8).

Dendritic cell, a lineage of professional APCs (reviewed in Ref. 9), appear to be the main source of IL-12 in response to *Leishmania* parasites (10). Numerous studies have indicated that the capacity of dendritic cells to secrete IL-12 is directly conditioned by the recognition of microbial products. The best-characterized molecules involved in the recognition of these molecules are Toll-like receptors (TLRs).[4] The TLRs recognize microbial products, termed pathogen associated molecular patterns, shared by large groups of pathogens but not present in the host, suggesting that the TLRs are critical to sensing invading microorganisms (reviewed in Refs. 11 and 12). Similar cytoplasmic domains allow TLRs to use the same signaling molecules used by the IL-1Rs (13), including the adapter molecule called myeloid differentiation marker 88 (MyD88), the IL-1R-associated protein kinase, and the TNF receptor-activated factor 6 (14). Particularly, analysis of genetically deficient mice has revealed a pivotal role for MyD88 in the activation of innate immunity (reviewed in Ref. 15). Dendritic cells (16) and macrophages (17, 18) purified from MyD88$^{-/-}$ mice loose the ability to produce proinflammatory cytokines, such as IL-12, in response to a large number of pathogen associated molecular patterns. The present work was undertaken to analyze the role of MyD88 protein in the development of a protective response to *L. major* parasites. In this order, we compared BALB/c mice (developing a nonprotective Th2 response), C57BL/6 (B6.WT) mice (developing a protective Th1 response), and MyD88$^{-/-}$ mice

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[4] Abbreviations used in this paper: TLR, Toll-like receptor; MyD88, myeloid differentiation marker 88; SLA, soluble *Leishmania* Ag.

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in the C57BL/6 background (B6.MyD). Our results show that B6.MyD mice present high susceptibility to *L. major* and develop a typical Th2 response, suggesting that MyD88-dependent pathways are essential for the development of protective IL-12-mediated Th1 response against the *L. major* parasite.

Materials and Methods

Mice, reagents, and parasites

The 6- to 8-wk-old female BALB/c and C57BL/6 mice were purchased from Harlan Nederland (Horst, The Netherlands). MyD88$^{-/-}$ mice (17) backcrossed eight times to the C57BL/6 background were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan) and bred in our own animal facility. The maintenance and care of mice complied with the guidelines of the Université Libre de Bruxelles ethics committee for the human use of laboratory animals. Mitogenic anti-CD3e (hamster Ig, 145-2C11; American Type Culture Collection, Manassas, VA) was produced and purified in our laboratory according to standard procedure. Bioactive recombinant murine IL-12 was provided by PeproTech (London, U.K.). Promastigotes of *L. major* (MHOM/IR/–/173 strain) were obtained after passage in BALB/c mice and propagated in vitro as previously described (19). Parasites harvested in stationary phase after 8–10 days of culture were centrifuged (2500 × *g*, 10 min, 4°C) and washed three times in RPMI 1640 (Seromed, Berlin, Germany) before being used for inoculation to animals. Soluble *Leishmania* Ag (SLA) was produced as described (20).

Leishmania *infection, lesion monitoring, and tissue processing*

Mice were infected s.c. in the left hind footpad with 5×10^6 stationary phase promastigotes of *L. major* in a final volume of 25 μl (in RPMI 1640 medium). The contralateral right footpad received an identical volume of RPMI medium without parasites as internal control. The thickness of infected and uninfected footpads was regularly measured with a metric caliper, and the difference between both measurements corresponded to the size of lesions as previously described (19). At selected time points, some mice were killed by cervical dislocation. Footpad lesions (or normal tissue in controls) were cut tangentially to the bone ground and collected for immunohistochemical studies (see below). The distribution and enumeration of infected cells were determined in organ sections stained with H&E.

Immunohistochemistry for granulocyte detection

In this study we used Immunohistowax processing, a new fixation and embedding method for light microscopy that preserves Ag immunoreactivity and morphological structures (21). Briefly, primary lesions and draining lymph nodes were fixed for 3 days in Immunohistofix (Aphase, Gosselies, Belgium), followed by dehydration in graded series of ethanol solution (30, 50, 70, 90, and 100%) for 30 min each at room temperature. Tissues were embedded in Immunohistowax (Aphase) to perform sections of 3–6 μm, de-embedded by washing in acetone for 10 min, and transferred to PBS. The tissue sections were treated for 30 min with blocking reagent (1% in PBS; Boehringer Mannheim, Mannheim, Germany) to saturate the sites of nonspecific reactions. The endogenous peroxidase activity was neutralized by 3% H_2O_2 in PBS for 30 min. The slides were then incubated for 60 min with the RB6-8C5 anti-GR1 mAb (BD PharMingen, San Diego, CA). They were further incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and stained with a solution of diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO), giving brown precipitates. Digitized images were captured using a CCD color camera (Ikegami Tsushinki, Tokyo, Japan) and analyzed using the CorelDraw 7 software (Corel, Ottawa, Ontario, Canada).

In vitro cell stimulation

The complete medium used in all experiments was RPMI 1640 supplemented with 2% HY ULTROSER (a serum-free medium purchased from Life Technologies, Merelbeke, Belgium), penicillin, streptomycin, nonessential amino acids, sodium pyruvate, 2-ME, and L-glutamine (Flow ICN Biomedicals, Bucks, U.K.). Lymph node cells (3×10^5) from control and infected mice were cultivated with either RPMI 1640 alone as control, mitogenic anti-CD3 (0.5 μg/ml), or SLA (50 μg/ml) in a total volume of 0.2 ml in 96-well U-bottom plates. Supernatants were collected after 48 h of culture, frozen, and assayed for IL-4 and IFN-γ content by ELISA (see below).

Cytokine assays

IFN-γ levels were determined by ELISA using anti-IFN-γ mAb F1 and Db1 as previously described (22). IL-4 levels were determined by ELISA using anti-Il-4 mAb 11B11 and biotinylated anti-IL-4 from BD PharMingen. Serum was assayed for IL-12 p40 by ELISA using Ab specific for p40 (clone 5D9 and SC3) as previously described (22). Standard curves were generated using recombinant murine cytokines. Results are expressed as nanograms per milliliter.

Results

MyD88$^{-/-}$ C57BL/6 mice, like BALB/c mice, present a high susceptibility to L. major *infection*

B6.WT, B6.MyD, and BALB/c were infected with 5×10^6 *L. major* promastigotes, and the course of infection was monitored. Resistant B6.WT mice displayed a small skin lesion at the site of inoculation, but they were able to resolve it and control the infection. Susceptible BALB/c mice, in contrast, developed ulcerating skin lesions that progressed without healing (Fig. 1). B6.MyD presented skin lesions similar in kinetic and size to those observed in BALB/c mice (Fig. 1). Immunohistochemical analyses were performed in footpad lesions to determine the number of infected cells. On day 28 postinfection, the number of infected cells in both BALB/c and B6.MyD mice was 10 times more than in B6.WT mice (Fig. 2, *A* and *B*). At 9 wk we observed that only infected BALB/c and B6.MyD mice presented footpad mutilation (80 and 60%, respectively). Globally, these results show that, like BALB/c mice, B6.MyD mice are highly susceptible to *L. major* infection.

Lack of resistance of MyD88$^{-/-}$ C57BL/6 mice to L. major *is associated with Th2 polarization of immune response*

To determine whether the susceptibility of B6.MyD mice was the consequence of a reduced Th1 response or a nonprotective Th2 response (like in BALB/c mice), lymph node cells from mice infected since 4 wk were isolated and stimulated by either control medium, mitogenic anti-CD3, or SLA. Supernatants were collected 48 h later and analyzed for their IL-4 and IFN-γ contents (Fig. 3). As classically described, stimulated lymph node cells from infected BALB/c mice produced high levels of IL-4 and low levels of IFN-γ in regard to B6.WT. B6.MyD displayed a clear Th2 profile similar to that observed in BALB/c mice. Note that lymph node cells from noninfected B6.MyD produced IFN-γ at a level similar to B6.WT in response to mitogenic anti-CD3, demonstrating the capacity of these mice to produce IFN-γ.

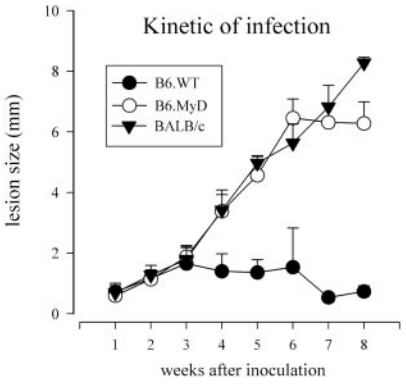

FIGURE 1. Size of primary footpad lesions during the course of *L. major* infection in B6.WT, B6.MyD, and BALB/c mice. Results illustrate one representative experiment performed with 10 animals of each strain and expressed as means ± SD. Two independent experiments have been performed.

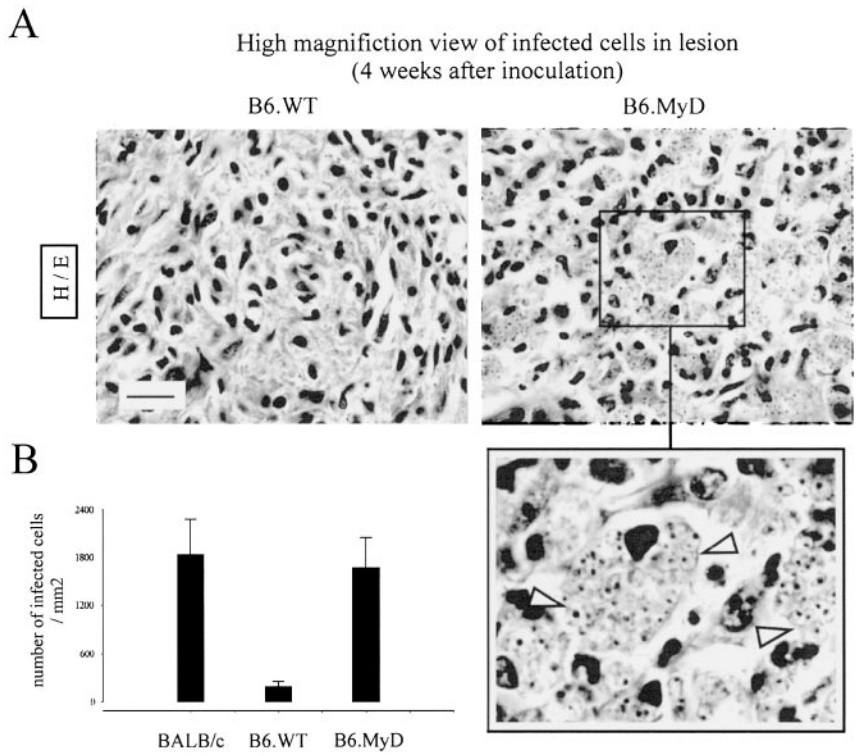

FIGURE 2. *A*, High magnification view of amastigotes stained by H&E in section of footpad lesion. Scale bar = 10 μm. *B*, Numbers of infected cells present in footpad at 4 wk of infection. Each value represents the mean ± SD of cell counts per mm^2 of tissue (10 sections from five mice). These results are representative of two independent experiments.

Rescue of L. major*-infected MyD88$^{-/-}$ C57BL/6 mice by administration of exogenous IL-12*

Our results strongly suggest that a dysregulation of Th1/Th2 differentiation of T cells is involved in the B6.MyD susceptibility to *L. major* parasite. Thus, as IL-12 is a key factor promoting the development of Th1 response in *L. major*-resistant mice (7, 8), we analyzed the IL-12 level in the plasma of B6.WT, B6.MyD, and BALB/c mice 4 wk after inoculation (Fig. 4*A*). Although, IL-12 p40 was detected in serum of infected B6.WT, it remained undetectable in infected B6.MyD and BALB/c. Note that we are not able to detect circulating levels of IL-12p70 in infected B6.WT, suggesting that it is under the detection limit of our ELISA.

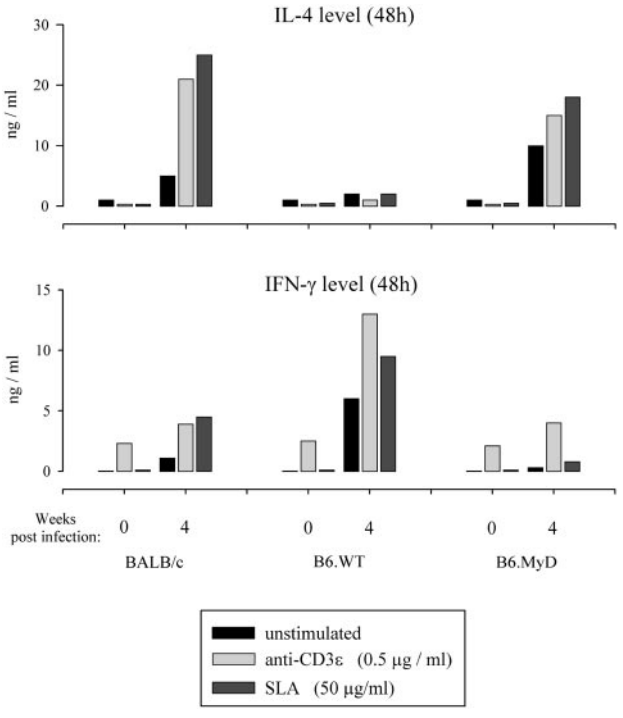

FIGURE 3. Production of IFN-γ and IL-4 by cells from lymph node draining the lesions of noninfected and infected B6.WT, B6.MyD, and BALB/c mice. Pooled lymph node cells obtained from five mice infected since 4 wk with *L. major* were cultivated with either culture medium alone as control, mitogenic anti-CD3, or SLA as described in *Materials and Methods*. After 48 h, supernatants were collected and tested by ELISA for their IFN-γ and IL-4 contents. These results are representative of two independent experiments

The reduced levels of IL-12p40 in infected B6.MyD suggests that susceptibility of B6.MyD could be dependent on MyD88-mediated IL-12 deficiency. To test this hypothesis, BALB/C and B6.MyD mice were treated daily for 7 days after *L. major* infection by i.p. administration of 1 μg of bioactive IL-12. The lesion size was monitored during 6 wk. Results (Fig. 4*B*) clearly show that IL-12-treated B6.MyD displayed reduced lesions compared with nontreated B6.MyD, demonstrating that the susceptibility of B6.MyD to *L. major* is a direct consequence of IL-12 deficiency.

Discussion

Previous studies have documented a dual role for MyD88 protein during bacterial and parasite infection. B6.MyD mice show enhanced susceptibility to *Staphylococcus aureus* (23) and *Toxoplasma gondii* (16). In contrast, B6.MyD mice have been reported to present improved resistance to lethal shock caused by administration of high-dose LPS (17), peritonitis induced by bacterial polymicrobial infection (24), and liver injury induced by *Plasmodium berghei* (25). These results suggest that the role played by MyD88 protein was strictly dependent on the nature of the inflammatory stimuli and the type of pathogen. This study reveals new insights into the role of MyD88 adaptator protein in the mechanism of resistance vs susceptibility to experimental cutaneous Leishmaniasis.

Studies of infection with *L. major* have established a paradigm for the role of different Th subsets during infection. Early production of IFN-γ and IL-12 in genetically resistant B6.WT mice is

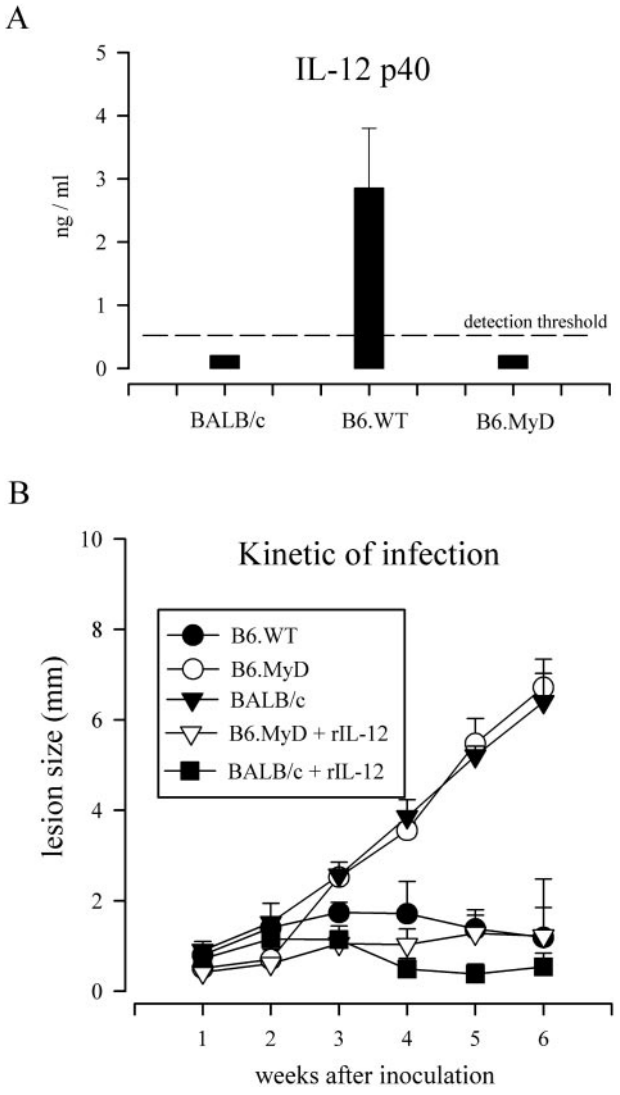

FIGURE 4. *A*, Production of IL-12 p40 by *L. major* infected B6.WT, B6.MyD, and BALB/c. Mice were infected, and serum was collected 4 wk after parasite inoculation. Data are expressed as mean ± SD, $n = 6$. These results are representative of two independent experiments. *B*, Size of primary footpad lesions during the course of *L. major* infection in B6.WT, B6.MyD, BALB/c, Il-12-treated B6.MyD, and IL-12-treated BALB/c mice ($n = 12$ for each group of mice). IL-12-treated mice were treated daily for 7 days after *L. major* infection by i.p. administration of 1 μg of bioactive IL-12. Results are expressed as means ± SD.

responsible for Th1 development and disease resistance. In genetically susceptible BALB/c mice, an early IL-4 response induces the down-regulation of IL-12 receptor β2 chain expression on $CD4^+$ T cells, resulting in a state of unresponsiveness to IL-12 and consequently, a reduction of IFN-γ production (26). Collectively, our findings indicate that genetically resistant mice lacking MyD88 protein fail to develop a protective response against *L. major* parasite. The phenotype of B6.MyD mice is strikingly reminiscent of that observed typically in susceptible BALB/c mice, because they displayed large footpad lesions containing numerous infected cells and frequent mutilations. Interestingly, immunohistochemical analysis of footpad lesions showed that 4 wk after inoculation, B6.MyD presented a granulocyte recruitment intermediate between infected B6.WT and BALB/c mice (data not shown). Some works (6, 27) have related that Th2 response in infected BALB/c mice is instructed by an early burst of IL-4. It has been demonstrated (27) by in vivo depletion that granulocytes are necessary to promote IL-4 production by T cells.

The results of this study also reveal new information about the role of MyD88 for the production of inflammatory mediators during infectious process. The immune response of infected B6.MyD mice harbors a typical Th2 profile with high IL-4 and low IFN-γ/IL-12 levels. Importantly, we have observed that administration of exogenous IL-12 during the first week of infection rescues *L. major*-infected B6.MyD mice, demonstrating that the susceptibility of these mice is a direct consequence of impaired IL-12 production during infection. Susceptibility to *L. major* infection has been observed in several models of knockout C57BL/6 mice such as $CD40^{-/-}$ (28), $Fas^{-/-}$ (29), $IL\text{-}12^{-/-}$ (30), IFN-γ $receptor^{-/-}$ (31), migration-inhibitory $factor^{-/-}$ (32), NO synthase (33), STAT-4 (34), and $TNF^{-/-}$ (35). However, it is important to note that analysis of the cytokine profile produced during the course of infection revealed that susceptibility in these models rarely takes its origin in Th1/Th2 dysregulation. With the exception of $IL\text{-}12^{-/-}$ mice, these knockout mice present impaired (reduced, delayed, or nonefficient) Th1 responses, and their lesions rarely reach the large sizes observed in infected BALB/c mice. Our results suggest that MyD88 protein constitutes a key element in the pathways regulating the choice between Th1 and Th2 immune response during *L. major* infection. In accord, it has been demonstrated that dendritic cells from B6.MyD present an enhanced ability to induce IL-4 production by T cells in allogenic MLR, suggesting that the MyD88 deficiency can confer to dendritic cells the ability to support Th2 immune responses (36).

MyD88 is an adapter protein common to signaling pathways of IL-1R, IL-18R, and TLRs to IL-1R-associated protein kinase. It has been demonstrated that C57BL/6 mice lacking the IL-1 type 1 receptor gene (37) or IL-18 gene (38) infected with *L. major* are still able to develop a protective immune response and present small lesions that resolve spontaneously. These data suggest that the Th2 shift observed in infected B6.MyD do not derive from the blockage of IL-1- and/or IL-18-signaling in $MyD88^{-/-}$ mice. Thus, based on our present knowledge of MyD88 interacting receptors, we hypothesize that susceptibility of B6.MyD mice to *L. major* could be a consequence of impaired TLR signaling. The TLR family consists of 10 members recognizing microbial products shared by large groups of pathogens. At present, ligands for TLR2, TLR3, TLR4, TLR5, TLR6, and TLR9 have been identified, whereas those of the other TLRs remain unknown. Pathogen recognition by TLRs triggers a rapid activation of innate immunity by inducing the production of proinflammatory cytokines (16, 17, 18), such as IL-12, by macrophages and dendritic cells. Thus, the low level of IL-12 observed in infected B6.MyD favors our hypothesis linking susceptibility and impaired TLR signaling. The role of the TLR family in the recognition of *Leishmania* parasites presently remains unknown. However, it is possible that members of the TLR family are involved in the recognition of protozoa, and evidence supporting this idea is provided by studies that implicated TLR2 in the recognition of *Trypanosoma cruzi* GPI anchors (39). Taken together, our study and these data suggest that TLR interacting with MyD88 pathways might be implicated in the detection of the *L. major* parasite. Presently, there is no efficient vaccine available against Leishmaniasis. Eucaryotic parasites are complex organisms constituted with a large number of Ags rendering selection of new immunogenic Ags by systematic or random research very hazardous. Identification of parasite Ags selected by TLR might constitute an alternative and interesting way for vaccination research. Thus, our observations suggest that TLRs might provide a new and original system to identify a novel set of *Leishmania* Ags with strong immunostimulatory properties.

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