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Leishmania braziliensis Infection Induces Dendritic Cell Activation, ISG15 Transcription, and the Generation of Protective Immune Responses

Diego A. Vargas-Inchaustegui,* Lijun Xin,* and Lynn Soong2*†

Leishmania (Viannaia) braziliensis is the causative agent of cutaneous and mucosal leishmaniasis in South America, and the latter is a severe and disfiguring form of the disease. Our understanding of how L. braziliensis parasites interact with dendritic cells (DCs) is limited, partially due to the difficulty in generating axenic amastigotes. In this study, we successfully generated axenic amastigotes of L. braziliensis and used them to test the hypothesis that L. braziliensis infection efficiently triggers innate responses in DCs and the subsequent adaptive immune responses for parasite clearance. This study has revealed unique immunological features of L. braziliensis infection. Firstly, axenic amastigotes showed higher infectivity and the potential to stimulate C57BL/6 (B6) bone marrow-derived dendritic cells to produce IL-12p40 when compared with their promastigote counterparts. Both parasite-carrying and bystander DCs displayed an activated (CD11chighCD45RBlow) phenotype. Secondly, L. braziliensis infection triggered transcription and phosphorylation of STAT molecules and IFN-stimulated gene 15 (ISG15). Finally, the self-healing of the infection in mice was correlated to the expansion of IFN-γ- and IL-17-producing CD4+ cells, suggesting the existence of active mechanisms to regulate local inflammation. Collectively, this study supports the view that innate responses at the DC level determine parasite-specific T cell responses and disease outcomes.

Modulation of DC activation by Leishmania parasites appears to be species-specific (13). For example, some species (L. major and L. donovani) are capable of activating DCs to express costimulatory molecules on the surface and to produce IL-12, in the presence of IFN-γ and anti-CD40 mAb (14, 15). By contrast, New World species such as L. amazonensis and L. mexicana either fail to activate DCs or cause an impaired DC response to secondary stimuli, such as LPS and IFN-γ (16, 17). DCs are potent APCs that can stimulate both the innate and adaptive immune systems, either by producing cytokines or by directly activating specific subsets of effector T cells (16, 18). The role of DCs in the immune response against Leishmania sp. infection has been characterized in several healing and nonhealing models, and the importance of these specialized cells in adaptive immune responses is evident (15, 16, 18). Intracellular signaling events involved in Leishmania infection have been described in infected Mφs, where several Leishmania species can repress the JAK/STAT and MAPK signaling pathways (19) and alter Ag-presenting capabilities and cytokine production in the infected cells (20). At present, there is no report describing the interactions between L. braziliensis parasites and DCs (21).

In the present study, we generated axenic amastigotes of L. braziliensis and evaluated the activation/maturation events triggered by infection in DCs, and its consequences on T cell responses. The use of L. amazonensis, another member of the New World species, as a control allowed us to define molecules and T cell populations that were selectively up-regulated during L. braziliensis infection in vitro and in vivo. Using fluorescence-labeled pro- and amastigotes, we demonstrated that L. braziliensis infection induced DC activation/maturation and IL-12p40 production in both infected and bystander cells, and that DC activation was accompanied by up-regulation of the JAK/STAT signaling pathway, especially in regard to the expression of p-STAT1, p-STAT3, IFN regulatory factor 1 (IRF-1), and ISG15. Moreover, L. braziliensis-infected DCs were highly efficient in priming naive CD4+ T cells to proliferate and to produce IFN-γ and IL-17. We, therefore, propose that a rapid induction and fine regulation of IFN-γ-producing
CD4+ T cells can be attributed to the control of *L. braziliensis* infection.

**Materials and Methods**

**Mice**

Female B6 and BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were used in this study. Mice were maintained under specific pathogen-free conditions and used for experimentation at 6 to 8 wk of age, according to protocols approved by the University of Texas Medical Branch (UTMB) Institutional Animal Care and Use Committees.

**Parasite culture and Ag preparation**

Infectivity of *L. braziliensis* (strains LT1B111 and LC1418) was maintained by regular passage through Syrian golden hamsters (Harlan Sprague Dawley, Indiana). Infectivity of *L. amazonensis* (strain LV78, clone 12-1; a gift from Kwang-Poo Chang, Departments of Microbiology and Immunology, Chicago Medical School) was maintained by regular passage through BALB/c mice. All promastigotes were cultured at 23°C in Schneider’s Drosophila medium (Invitrogen) (pH 7.0), supplemented with 20% FBS (Sigma-Aldrich), 2 mM t-glutamine, and 50 μM/ml gentamicin. Stationary promastigotes of less than five passages were used for DC or animal infection. Axenic amastigotes of *L. braziliensis* (LC1418) and *L. amazonensis* (LV78) were cultured at 33°C in complete Grace’s insect cell culture medium (Invitrogen) (pH 5.0), supplemented with 20% FBS. *L. braziliensis* axenic amastigotes required 5% CO2 for their growth. To prepare promastigote lysates, parasites (2 × 10^7/ml) were subjected to six freeze-and-thaw cycles in liquid nitrogen and a 15-min sonication. Ag preparations were stored in aliquots until use.

**DC generation and infection**

Bone marrow-derived DCs were generated from B6 mice in complete IMDM (Invitrogen) containing 10% FBS, supplemented with 20 ng/ml recombinant GM-CSF (eBioscience), and harvested at day 8. Parasites (5 × 10^7/ml in PBS) were labeled with CFSE (5 μM, Sigma-Aldrich) at room temperature for 5 min. The efficiency of parasite labeling was >99%, as judged by FACS and microscopic examination. Labeled parasites were washed four times and incubated with DCs (8:1 parasite-to-cell ratio) at 33°C for 12 h, and then at 37°C for another 12 h. LPS (100 ng/ml) from *Salmonella enterica* serovar Typhimurium (Sigma-Aldrich) plus IFN-γ (100 ng/ml, Leinco Technologies) was used as a positive control in all of the experiments. At 24 h postinfection, cells were collected for FACS analysis and extracted for RNA or proteins, and culture supernatants were harvested for cytokine detection.

**DC sorting and immunofluorescence**

A total of 4 × 10^6 DCs infected with CFSE-labeled *L. braziliensis* parasites were sorted based on their FL1 fluorescence with a FACSAria Cell Sorter (BD Biosciences). CFSE+ and CFSE− DCs were then cytospun onto glass slides, counterstained with mounting medium containing 4',6'-diamidino-2-phenylindole (Vector Laboratories), and assessed for infection status using an Olympus BX51/52 fluorescence microscope (Olympus America). The percentage of infected cells in each sorted subpopulation was determined by counting infected and uninfected cells using a total of 10 pictures obtained from random visual fields. The percentage of infection was calculated as the number of infected cells/total number of cells × 100.

**T cell priming in vitro**

Naïve CD4+ T cells were purified from the spleen of B6 mice by negative selection using magnetic beads (Miltenyi Biotec), and their purity was routinely around 95%, as judged by CD4 staining and FACS analysis. Purified CD4+ T cells (2 × 10^4) were cocultured with parasite-infected, mitomycin C-pretreated DCs at a 10:1 ratio in 96-well plates for 3 days. Supernatants were harvested for cytokine detection. To assess T cell proliferation, 1 μCi of [3H]thymidine was added at 18 h before harvest, and incorporated radioactivity was determined on a microplate scintillation and luminescence counter (Packard Instrument Company). Naïve CD4+ T cells were also cocultured with sorted CFSE+ and CFSE− DCs using the same protocols.

**Intracellular staining and FACS analysis**

The following specific mAbs and their corresponding isotype controls were purchased from eBiosciences: FITC-conjugated anti-IFN-γ (XMG1.2), anti-CD45RB (C363.16A), and rat IgG2a; PE-conjugated anti-IL-17A (eBio17B7), anti-IL-10 (JES5-16E3), anti-IL-12/IL-23p40 (C17.8), anti-IL-17 (eBio21B10), anti-IL-12p70 (eBio405.4), PE-Cy5-conjugated anti-CD4 (GK1.5), anti-CD11c (N418), and hamster IgG. In brief, cells were washed, blocked with 1 μg/ml FcγR blocker (CD16/32, eBioscience), stained for specific surface molecules, fixed and/or permeabilized with a Cytofix/Cytoperm Kit (BD Biosciences), and then stained for specific intracellular molecules. To detect intracellular cytokines, 1 μg/ml of Golgi Stop (BD Biosciences) was added for the last 6 h of cultivation. Cells were read on a FACScan (BD Biosciences) and analyzed using FlowJo V8.5 Software (TreeStar).

**Cytokine ELISA**

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

**JAK/STAT microarray and IL-12p40 real-time RT-PCR**

Total RNA was extracted from 1–2 × 10^6 DCs using the RNeasy system (Qiagen). Genomic DNA was digested with the on-column RNA-free DNase (Qiagen). Total RNA (10 μg) was subjected to Oligo GEArray analyses (SuperArray Bioscience Corporation) for the JAK/STAT signaling pathway. For detecting IL-12 transcripts, cDNA was synthesized from 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) primed with oligo(dt). Real-time RT-PCR was performed at UTMB’s institutional Real-Time PCR core facility, and all reagents were purchased from Applied Biosystems: 20× assay mixtures of primers, TaqMan MGB probes (FAM dye-labeled) for mouse IL-12p35 and IL-12p40 (P/N 4331182), predeveloped 18s RNA (VIC dye-labeled, as an endogenous control), and TaqMan assay reagent (P/N 4319143E). Separate-tube (singleplex) real-time RT-PCR was performed with 40 ng cDNA for both target genes and the endogenous control, using a Taqman gene expression master mix (P/N 4370074). The PCR cycling parameters were: uracil-N-glycosylase activation at 2 min 50°C, AmpliTaq activation 95°C for 10 min, denaturation 95°C for 15 s, and annealing and/or extension 60°C for 1 min (repeat 40 times) on ABI7000 (Applied Biosystems). Duplicate CT values were analyzed in Microsoft Excel using the comparative CT (ΔΔCT) method, as described by the manufacturer (Applied Biosystems).

The amount of target (2−ΔΔCT) was obtained by normalized-to-endogenous reference (18s) and relative to uninfected control samples.

**Western blot**

A total of 4 × 10^6 DCs were infected with parasites at a 10:1 parasite-to-cell ratio. At 6 h p.i., some groups were stimulated with either LPS (100 ng/ml) plus IFN-γ (100 ng/ml) or LPS (100 ng/ml) plus IFN-α (100 U/ml, Cell Sciences) for 30 min before protein extraction. Cells were lysed in ice-cold RIPA lysis buffer containing complete EDTA-free protease and phosphatase inhibitors (Pierce Biotechnology). Protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology). Equal amounts of proteins were loaded onto SDS-polyacrylamide gels, which were either a 10% (to detect STATs and IRFs) or 15% (to detect ISG15) gel, and then transferred to polyvinylidene difluoride membranes (Millipore). Rabbit anti-mouse STAT1, p-STAT1, STAT2, STAT3, p-STAT3, IRF-1, ISG15 molecules, as well as HRP-conjugated goat anti-rabbit IgG and HRP-conjugated anti-mouse IgG were purchased from Santa Cruz Biotechnology. Rabbit anti-mouse IRF-3 and p-STAT2 were purchased from Cell Signaling Technology and Upstate Biotechnology, respectively. Mouse anti-actin mAb (Sigma-Aldrich) was obtained from Dr. Jaeren Sun (Department of Microbiology and Immunology, UTMB, TX). Membranes were incubated with primary Abs (diluted 1:1,000 except for anti-ISG15 [1/500] in PBS buffer containing 5% nonfat milk and 0.05% Tween 20) at 4°C overnight, washed, and incubated with HRP-conjugated secondary Abs (1/2,000) for 1 h. Blots developed with the enzyme chemiluminescence kit ECL (Amersham Biosciences). Densitometry analysis of blots was performed using AlphaEase FC Stand-Alone Software (version 4.0, Alpha Innotech).

**Evaluation of infection and T cell activation**

Metacyclic promastigotes of *L. braziliensis* and *L. amazonensis* were purified according to a previously described protocol (22) and injected i.c. in the right hind foot of B6 mice (2 × 10^4 parasites/mouse, five mice/group). Lesion sizes were monitored weekly with a digital caliper (Control Company), and tissue parasite burdens were measured via a limiting dilution assay using LDA software (Oxford University Press) (23). At 4 and 8 wk p.i., mice were euthanized, and the popliteal LN cells were collected and stained immediately for the expression of activation markers on CD4+ T cells. Some LN cells (3 × 10^3/well/ml) were stimulated with PMA/ionomycin/Golgi Plug in the absence of parasite Ag for 6 h (ex vivo) before examination of intracellular IFN-γ, IL-17, and IL-10 expression.
L. braziliensis axenic amastigotes are highly efficient in activating DCs. Bone marrow-derived DCs (BMDCs) derived from B6 mice were infected with promastigotes (Pm) and amastigotes (Am) of L. braziliensis or L. amazonensis at the indicated parasite-to-cell ratios for 24 h. A, CFSE-labeled parasites were used to infect DCs and measure the percentage of infected cells (gated on CD11c and CFSE). B, IL-12p40 production in the culture supernatants of Pm- or Am-infected DC was assayed by ELISA. Dotted line represents baseline production by uninfected DCs.

FIGURE 1. L. braziliensis axenic amastigotes are highly efficient in activating DCs. Bone marrow-derived DCs (BMDCs) derived from B6 mice were infected with promastigotes (Pm) and amastigotes (Am) of L. braziliensis or L. amazonensis at the indicated parasite-to-cell ratios for 24 h. A, CFSE-labeled parasites were used to infect DCs and measure the percentage of infected cells (gated on CD11c and CFSE). B, IL-12p40 production in the culture supernatants of Pm- or Am-infected DC was assayed by ELISA. Dotted line represents baseline production by uninfected DCs.

Statistical analysis

The difference between two different groups was determined by the Student’s t test. One- or two-way ANOVA was used for multiple group comparisons (GraphPad Software v4.0). Statistically significant values are referred to as follows: *, p < 0.05; ***, p < 0.01.

Results

L. braziliensis axenic amastigotes have enhanced infectivity and induce high levels of IL-12p40 secretion in DCs

Although there is increasing evidence for a marked suppression of DC function by L. amazonensis and L. mexicana amastigotes (16–18), we find no reports in the literature as to how DCs respond to L. braziliensis amastigotes. To address this issue, we generated short-term axenic amastigotes for L. braziliensis and first examined their infectivity in DCs. After exposing DCs to two different doses (4:1 and 8:1 parasite-to-cell ratios) of CFSE-labeled L. braziliensis promastigotes and amastigotes for 24 h, we consistently observed significantly higher percentages of CD11c⁺ DCs that were infected with amastigotes when compared with those infected with promastigotes (Fig. 1A). To assess the function of these infected DCs, we included cells infected with L. amazonensis as a control and tested several infection doses (2/1, 4/1, and 8/1). As shown in Fig. 1B, while both promastigotes were capable of inducing IL-12p40 secretion in DCs, L. braziliensis promastigotes were more potent in doing so than their L. amazonensis counterparts (p < 0.05 in all three tested doses). In sharp contrast to the general failure of L. amazonensis amastigotes in activating DCs (16, 18), L. braziliensis amastigotes were highly competent in activating DCs and tended to stimulate higher (6 ng/ml) levels of IL-12p40 than did their promastigote counterparts (4 ng/ml) at an 8/1 infection dose (p < 0.05). These results suggested remarkable differential responses of murine DCs to two New World Leishmania species, and prompted us to examine the molecular basis underlying these differences.

DC maturation is induced after L. braziliensis infection

To examine the status of DC maturation and/or activation, we measured the expression of surface markers (CD40, CD80, and CD83) and intracellular molecules (IL-12p40) at the single-cell level at 24 h p.i. with L. braziliensis parasites. The distribution of IL-12p40 and CD40 among CD11c⁺ DCs is shown as an example of FACS plots in Fig. 2A, and the results from the pooled data from four independent repeats are given in Fig. 2B. DCs stimulated with
LPS/IFN-γ were included in all tests as positive controls for general quality/responsiveness of DCs. As compared with nonstimulated DCs, it was evident that infection with *L. braziliensis* promastigotes stimulated DCs to express significantly higher levels of IL-12p40, CD40, and CD83 (p < 0.01 and p < 0.05), whereas significant increases were detected for IL-12p40 and CD40 following infection with *L. braziliensis* amastigotes (p < 0.05). Consistent with the data shown in Fig. 1, both developmental forms of *L. braziliensis* parasites induced higher levels of DC activation than did their *L. amazonensis* counterparts (data not shown). Together, these FACS studies confirmed and extended the ELISA data, indicating the ability of *L. braziliensis* to activate DCs.

**DC activation during L. braziliensis infection occurs in both parasite-carrying and bystander cells**

Given that ~55–70% CD11c+ DCs carried CFSE+ parasites (Fig. 1A), we wondered whether the observed DC responses were due to infection or to bystander activation. To address this issue, we infected DCs with CFSE-labeled *L. braziliensis* parasites and analyzed the activation status of DCs between the parasite-carrying (CD11c+CFSE+) and bystander (CD11c+CFSE−) cells.

As shown in Fig. 3A, comparable percentages (3.6 vs 4.6%) of IL-12p40+ cells were detected in CD11c+CFSE+ and CD11c+CFSE− subsets of DCs at 24 h of infection with promastigotes; however, it appeared that higher percentages (8.1%) of IL-12p40+ cells were detected in CD11c+CFSE+ DCs following infection with amastigotes than in CD11c+CFSE− DCs (2.5%). Similar trends were observed when DCs were infected with parasites for 6 h and then treated with LPS/IFN-γ for an additional 18 h (Fig. 3A). To ensure that the CFSE labeling accurately reflected parasite infection, we sorted the CFSE+ and CFSE− subsets by flow cytometry (Fig. 3B) and assessed the percentages of infected cells under a fluorescence microscope (Fig. 3C). Having confirmed the CFSE-labeling protocol, we then quantified the percentages of CFSE+ and CFSE− subsets among IL-12p40+ and CD40+DC11c+ cells following infection with amastigotes than in CD11c+CFSE− DCs (2.5%).
Transient IL-12p40 gene expression and the activation of the JAK/STAT signaling pathway in L. braziliensis-infected DCs

DC responsiveness to different pathogens varies greatly in intensity and duration (24, 25). Because biologically functional IL-12p70 is composed of p40 and p35 heterodimers, we evaluated the kinetics of IL-12 gene expression during L. braziliensis infection via real-time RT-PCR. As shown in Fig. 4A, there were 43- and 12-fold increases in IL-12p40 expression at 8 and 24 h, respectively. RNA extracted at 24 h was used for microarray analysis of the JAK/STAT signaling pathway. RNA extracted from L. amazonensis-infected BMDCs under similar conditions was used for establishing controls. Shown are representative results from two independent experiments.

FIGURE 4. DC activation following L. braziliensis infection is transient and accompanied by up-regulation of STAT molecules. BMDCs were infected with L. braziliensis (Pm and Am, 8:1 parasite-to-cell ratio) for 8 or 24 h before RNA extraction. A, IL-12p40 expression was quantified by real-time RT-PCR. B, RNA extracted at 24 h was used for microarray analysis of the JAK/STAT signaling pathway. RNA extracted from L. amazonensis-infected BMDCs under similar conditions was used for establishing controls. Shown are representative results from two independent experiments.

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FIGURE 5. L. braziliensis infection up-regulates phosphorylated STAT molecules and ISG15. BMDCs were infected with L. braziliensis or L. amazonensis promastigotes (Pm) or amastigotes (Am) at 10:1 parasite-to-cell ratio for 6 h. Cells were then treated with LPS/IFN-γ and LPS/IFN-α (A), or LPS/IFN-α (C) for 30 min. Expression levels of indicated total or phosphorylated proteins were evaluated using cell lysates on Western blots. Fold of increase for p-STAT1, p-STAT3, and ISG15, IRF-1 was calculated by densitometry analysis. Shown are representative images of three independent experiments with similar results. *p < 0.05; **p < 0.01; and ***p < 0.001 following one-way ANOVA analysis, indicate statistically significant differences between the control and infected groups.

FIGURE 6. L. braziliensis-infected DCs are highly potent in priming naive CD4+ T cells in vitro and promoting the production of IFN-γ. BMDCs were infected with L. braziliensis and L. amazonensis promastigotes (A and B) and CFSE-labeled L. braziliensis promastigotes (C) at a parasite-to-cell ratio of 8:1 for 24 h. DCs were treated with mitomycin C (50 μg/ml) and cocultured with spleen-derived naive B6 CD4+ T cells (2 × 10^6/ml) at a 1:10 DC-to-T ratio. LPS-treated DCs were used as a control. A, T cell proliferation was measured by [3H]-thymidine uptake after 4 days of coculture. B, The presence of IFN-γ, IL-10, and IL-17 in culture supernatants was assayed by ELISA. C, Enrichment of CFSE+ (parasite-carrying) cells was performed by passing the cells through a FACSaria cell sorter. CFSE+ and CFSE- DCs were then treated with mitomycin C before coculture with naive CD4+ T cells. Shown are pooled data from three independent experiments. Bars represent statistically significant differences between compared groups. *, p < 0.05; **, p < 0.01; and ***, p < 0.001 indicate statistically significant differences between the control and infected groups by one-way ANOVA analysis.
differences between the compared groups. Bars represent statistically significant differences between the naive and infected groups by one-way ANOVA analysis.

To understand the intracellular events triggered at the early stage of infection, we examined the JAK/STAT signaling pathway by an Oligo GE Array analysis. Side-by-side comparison of the gene expression profiles revealed that nearly 55 out of 120 tested genes were up-regulated in DCs at 24 h p.i with *L. braziliensis* and *L. amazonensis* promastigotes, respectively (data not shown). In comparison to the *L. amazonensis*-infected and uninfected controls, there was an increased transcription of STAT1, STAT2, and NO synthase 2 (Nos2) in *L. braziliensis*-infected DCs (Fig. 4B). Notably, *L. braziliensis* infection triggered a 23-fold increase in the transcription of ISG15 (Fig. 4B), a molecule known to be important in viral infection (26, 27). Semiquantitative RT-PCR analyses confirmed the up-regulation of STAT1, STAT2, STAT3, and ISG15 genes (data not shown).

**FIGURE 7.** Self-healing in *L. braziliensis*-infected mice is associated with the expansion of IFN-γ-producing CD4+ T cells. B6 mice (five/group) were infected in the hind foot with 2 × 10^6 metacyclic promastigotes of *L. braziliensis* or *L. amazonensis*. A, Lesion sizes were monitored weekly with a digital caliper. B, Mice were sacrificed at 4 and 8 wk postinfection, and parasite burdens in foot tissues were determined by limited dilution. Draining LN cells were collected at 4 (C) and 8 wk postinfection (D) and stimulated with PMA/ionomycin/Golgi Plug for 6 h. Intracellular staining for IFN-γ, IL-17, and IL-10 was gated on CD4+ T cells. Bars represent statistically significant differences between the compared groups. *, p < 0.05; **, p < 0.01; and ***, p < 0.001 indicate statistically significant differences between the naive and infected groups by one-way ANOVA analysis.

L. braziliensis infection up-regulates phosphorylated STAT molecules and the expression of ISG15 proteins

To investigate whether the increased gene expression correlated to total or phosphorylated protein levels, we infected DCs with *L. braziliensis* for 6 h and then stimulated cells with LPS/IFN-γ for 30 min before protein extraction and Western blot analyses. We found that infection with *L. braziliensis* promastigotes resulted in a 2-fold increase in the levels of p-STAT1, and that infection with pro- and amastigotes led to 1.5- to 2-fold increases, respectively, in p-STAT3 (Fig. 5A and B). *L. braziliensis* infection did not cause major changes in the levels of STAT1, STAT2, p-STAT2, STAT3 (Fig. 5A), STAT4, and p-STAT4 (data not shown).

Consistent with the microarray data, infection with *L. braziliensis*, but not *L. amazonensis*, pro- and amastigotes induced a 3.5-fold increase in the levels of free ISG15 (comparing lanes 5 and 6 with lanes 3 and 4, respectively, Fig. 5C). Because ISG15 expression can be modulated through type I IFN signaling (28), we also evaluated the expression levels for IRF-1 and IRF-3. Infection with *L. braziliensis* pro- and amastigotes resulted in 1.5- and 1.9-fold increases, respectively, in the expression of IRF-1 (Fig. 5D). Of note, down-regulation of IRF-1 below the baseline level was observed in *L. amazonensis* amastigote-infected DCs (Fig. 5C, lane 4). No major changes were detected for IRF-3 (Fig. 5C). Together, these results suggested that *L. braziliensis* infection selectively up-regulates DC signaling pathways known to be critical for innate immune responses and the production of proinflammatory cytokines.
vitro, and the potential of *L. braziliensis*-infected DCs in activating CD4+ T cells to produce high levels of IFN-γ, IL-10, and IL-17.

**Self-healing in *L. braziliensis*-infected mice is associated with an early induction and tight regulation of IFN-γ-producing CD4+ T cells**

To validate our in vitro T cell-priming observations, we next analyzed the CD4+ T cell phenotype during *L. braziliensis* infection in vivo. B6 mice were infected s.c. with 2 × 10^8 *L. braziliensis* or *L. amazonensis* promastigotes in the hind foot, and lesion development was monitored weekly. As expected, *L. amazonensis* and *L. braziliensis* infections led to both non- and self-healing diseases, respectively (Fig. 7A), which correlated well with tissue parasite burdens at 4 and 8 wk p.i. (Fig. 7B). To evaluate the phenotypes of in vivo-primed CD4+ T cells, we examined the percentages (data not shown) and the total number of CD4+ T cells in draining LNs. At 4 wk, when comparable lesion sizes and parasite burdens were detected in both infection groups, there was a remarkable expansion (180 × 10^3 cells/LN) of IFN-γ-producing CD4+ T cells in the LNs of *L. braziliensis*-infected mice (p < 0.001, Fig. 7C). The percentages and total numbers of IFN-γ-, IL-17-, and IL-10-producing CD4+ T cells in *L. braziliensis*-infected mice, however, decreased considerably by 8 wk (Fig. 7D), which correlated with parasite clearance (Fig. 7B). This early activation of IFN-γ-producing CD4+ T cells was blunted in *L. amazonensis*-infected mice, but these mice displayed relatively high numbers of IFN-γ- and IL-10-producing cells at 8 wk (p < 0.001 and p < 0.01). Similar statistical trends were obtained when LN cells were re-stimulated with soluble leishmanial Ags for 4 days (data not shown). These data collectively suggest that a strong induction of and fine balance among proinflammatory and regulatory cytokines is linked to the control of *L. braziliensis* infection.

**Discussion**

Recent studies from our laboratory, as well as that of others, have described how *L. amazonensis* infection in DCs and Mφs leads to a profound impairment in the IL-12 signaling pathway and alters the Ag-presenting capacity of these cells (13, 18, 23, 29, 30). However, little is known about how other New World *Leishmania* species interact with DCs at early stages of infection and how these interactions influence disease outcome. In this study, we examined in detail whether DCs responded to *L. braziliensis* pro- and amastigotes differently, as well as the downstream events triggered by these infections. We have provided evidence that, in sharp contrast to *L. amazonensis*, *L. braziliensis* (especially its amastigote form) can efficiently activate DCs to produce proinflammatory cytokines. To the best of our knowledge, this is the first report to describe DC-*L. braziliensis* interactions at the cellular and molecular levels. More importantly, this study provides new insight into early and differential DC responses to different *Leishmania* species and developmental stages and the impact of these responses on T cell activation and disease outcomes.

DC surface activation markers have been widely used to evaluate DC responsiveness to different pathogens. In this study, we used the surface expression of CD40, CD80, and CD83 molecules, as well as the production of IL-12p40, as the readouts for DC activation because of their known sensitivity to *Leishmania* infection in vivo (16, 18). In addition, we used CFSE-labeled pro- and amastigotes to define the status of DC activation at the single-cell level. Our in vitro DC infection and T cell-priming studies have revealed several interesting findings. First, in contrast to *L. amazonensis* infection, which induces relatively low levels of DC maturation/activation (16), *L. braziliensis* infection induced high levels of activation in both parasite-carrying (CD11c+ CFSE+) and bystander (CD11c+ CFSE−) DCs (Fig. 3). Although the “bystander activation” would be caused by a small number (<2%) of unlabeled parasites, it was more likely that it was due to cytokine/chemokine release from parasite-infected DCs. Second, the presence of *L. braziliensis* parasites did not alter DC responsiveness to secondary stimuli, because parasite-carrying DCs responded to LPS/IFN-γ by up-regulating surface activation markers and intracellular IL-12p40. Third, although both parasite-carrying and bystander DCs were capable of priming naive CD4+ T cells in vitro, the former were highly competent in doing so (Fig. 6). Therefore, strong DC activation is a hallmark for *L. braziliensis* infection.

In our in vitro studies, secreted and intracellular IL-12p40 proteins were readily detected in DCs infected with *L. braziliensis* pro- or amastigotes in the absence of other exogenous stimuli (Figs. 1–3). Similar to other reports (14, 31) (L. Xin, K. Li, and L. Soong, submitted for publication), infection with parasites alone failed to stimulate IL-12p70 production, although *L. braziliensis*-infected LPS/IFN-γ-stimulated DCs were capable of producing IL-12p70 (data not shown). Given that the expression of the IL-12p40 gene can precede the formation and release of bioactive IL-12p70, serving as an early event in host-pathogen interactions (32), we examined the expression of IL-12p40 and IL-12p35 genes via quantitative real-time RT-PCR. We found that infection with *L. braziliensis* pro- or amastigotes alone triggered a 45- to 55-fold increase of IL-12p40 mRNA at 8 h, which dropped dramatically by 24 h (Fig. 4), suggesting a transient induction feature of the IL-12p40 gene during *Leishmania* infection (L. Xin, K. Li, and L. Soong, submitted for publication). In our hands, *L. braziliensis* infection alone was insufficient to stimulate IL-12p35 gene expression in DCs (data not shown), which may explain the lack of detectable IL-12p70 in our in vitro studies. Given that production of IL-12p70 by APCs requires T cell-dependent signals, such as CD40-CD40L interactions (33), it will be interesting to further examine whether *L. braziliensis*-infected DCs are capable of producing IL-12p70 in vivo or in the presence of T cells in vitro.

The JAK/STAT signaling pathway not only plays a critical role in innate immunity by inducing expression of proinflammatory cytokines and anti-parasitic molecules (e.g., NO and oxygen radicals) (19, 34), but also affects cell fate by controlling the expression of genes involved in cell proliferation, differentiation, and apoptosis (35). Therefore, it is not surprising that infection with *L. donovani* and *L. mexicana* promastigotes in Mφs (19, 31) or *L. amazonensis* amastigotes in DCs (L. Xin, K. Li, and L. Soong, submitted for publication) can down-regulate IL-12 production by altering the JAK/STAT and MAPK signaling pathways. Interestingly, we found that infection with *L. braziliensis* pro- and amastigotes enhanced the expression of p-STAT1 and p-STAT3 (Fig. 5B). Although the role of these phosphorylated proteins in *L. braziliensis* infection remains unclear, a recent report has suggested the necessity for STAT1 signaling on APCs, but not on T cells, in generating *L. major*-specific Th1 T cells and protective immunity (36). It appears that STAT3 has complex and multiple functions, serving as a positive regulator for DC homeostasis (37, 38), but as a negative regulator (through IL-10 transcription) for DC activation (39), because inhibition of the JAK2/STAT3 signaling can increase DC activation, maturation, and the capacity to induce T cell proliferation (40). Additional studies are needed to examine whether the up-regulation of p-STAT1 and p-STAT3 in *L. braziliensis*-infected DCs is responsible for the balanced Th1 immune response observed in infected animals (Fig. 7).

One of the novel findings in this study is the marked induction of ISG15 expression during *L. braziliensis*, but not *L. amazonensis*, infection (Figs. 4B and 5C). Although ISG15 is known to be important for immune responses against influenza, herpes, and...
sindbis viruses (27), there are no reports of its involvement in parasitic infection. ISG15 is an ubiquitin-like molecule involved in a process known as ISGylation, which modifies the function of target proteins (41). Even though >100 proteins have been described as targets for ISGylation, the cellular functions of ISG15 and the modified proteins remain unknown (42). ISG15 can act within the immune system either by direct conjugation with target proteins (i.e., STAT1 (43)) or being secreted as a cytokine (27). The expression of ISG15 can be induced in different events, such as microbial infections, genotoxic stress, as well as pregnancy and retinoid-induced cellular differentiation (41). In an attempt to understand the biological relevance of ISG15 in Leishmania infection, we evaluated the levels of IRF-1 and IRF-3, two up-stream molecules for ISG15 (44). We detected an increase in IRF-1 protein after L. braziliensis infection (Fig. 5C); however, no up-regulation of IRF-3 was detected, even though induction of IRF-3 is known to be correlated with up-regulation of ISG15 (45). Given that up-regulation of ISG15 appears to be selective for L. braziliensis infection, we are currently examining the biological role of the ISG15-related pathway using targeted gene knock-out mice (28).

Regardless of the mechanisms underlying a JAK/STAT- or ISG15-mediated intracellular event, there is no doubt that DC activation status can greatly influence Ag-specific T cell responses and, therefore, disease outcomes (46). Our in vitro, T cell-priming studies using two New World Leishmania species indicate clearly a correlation between DC activation status and the magnitude of T cell activation, confirming the high potential of L. braziliensis-infected DCs in priming naive CD4+ T cells (Fig. 6) and the generation of protective immunity in mice (Fig. 7). It is evident that the hallmark of infection in L. braziliensis-infected mice is a rapid induction of IFN-γ, IL-10, and IL-17-producing CD4+ T cells at early stages of infection, but a withdrawal of T cell responses during parasite clearance; however, such regulated responses are altered during L. amazonensis infection. Delayed and insufficient host responses in L. amazonensis-infected mice failed to control this infection, or may even promote amastigote growth intracellularly (47). Although the roles of IFN-γ and IL-10 in murine models of leishmaniasis are well documented (11, 48–50), the role of IL-17 in cutaneous leishmaniasis is less clear. Our group has previously described a possible role for IL-17 during the immune response to L. amazonensis (16), especially because of the local tissue inflammation and neutrophil/Mϕ infiltration observed during Leishmania infection (51). Given that IL-17-producing CD4+ T cells are important for host defense against extracellular pathogens and organ-specific autoimmunity (52), and that IL-17 can induce the production of IL-1β, TNF, and IL-6 in a variety of cell types (53), it will be interesting to define the role of the IL-17 network in cutaneous and mucosal leishmaniasis.

In summary, this study describes a signature feature of DC responses to L. braziliensis parasites. These detailed studies of DC responses to two New World species of Leishmania and their developmental stages provide new insights into how DCs differentiate invading parasites and how the responses at the molecular level impact on the development of protective and nonprotective immune responses. Because L. amazonensis is the etiological agent for diffuse cutaneous leishmaniasis, which is a rare and immunosuppressive form of the disease (21), our comparative studies of DC infection with L. braziliensis vs L. amazonensis and L. amazonensis vs L. major (16) support the view that initial events at the DC level determine the timing and magnitude of Leishmania-specific CD4+ T cell responses and the outcome of the infection. Given the clinical importance of ML and diffuse cutaneous leishmaniasis, it will be important to extend and confirm these findings in the human system. A better understanding of molecular details in DC-parasite interactions would provide novel strategies for the control of leishmaniasis.

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