Published in final edited form as:

Mol Immunol. 2008 July ; 45(12): 3371–3382. doi:10.1016/j.molimm.2008.04.018.

Down-regulation of dendritic cell signaling pathways by *Leishmania amazonensis* amastigotes

Lijun Xin^a, Kui Li^{a,b}, and Lynn Soong^{a,c*}

^aDepartment of Microbiology, University of Texas Medical Branch Galveston, TX, USA

^bDepartment of Pathology, University of Texas Medical Branch Galveston, TX, USA

^cCenter for Biodefense and Emerging Infectious Diseases, Sealy Center for Vaccine Development, Institute for Human Infections and Immunity, Sealy Center for Cancer Cell Biology, University of Texas Medical Branch Galveston, TX, USA

Abstract

We have previously reported a link between a deficient Th1 response to *Leishmania amazonensis* (*La*) parasites and profound impairments in the cytokine/chemokine network at early stages of the infection. To define the molecular basis of these deficiencies, we focused on early and intracellular events in *La*-infected dendritic cells (DCs) in this study. Compared with *La* promastigote-infected counterparts, amastigote-infected DCs were less mature and less potent as antigen-presenting cells (APC) as evidenced by the lower expression of CD40 and CD83, suppressed cytokine expression (IL-12p40 and IL-10), reduced effectiveness for priming CD4⁺ T cells from naïve or infected mice. Infection with *La* promastigotes, but not amastigotes, triggered transient expression of IL-12p40 by DC. Both forms of parasites markedly suppressed IL-12p40, IL-12p70, and IL-6 production and increased IL-10 production when DCs were treated with LPS, IFN- γ /LPS or IFN- α /LPS as positive stimuli. Of note, pre-infection of DCs with live amastigotes resulted in multiple alterations in innate signaling pathways, including degradation of STAT2, decreased phosphorylation of STAT1, 2, 3 and ERK1/2, and markedly reduced expression of interferon regulatory factor-1 (IRF-1) and IRF-8, some of which were partially reversed by pretreatment of parasites with proteasome or protease inhibitors. The impaired IL-12 production in infected DCs was not attributed to increased IL-10 production. Together, our data suggest that *La* parasites, especially in their intracellular forms, have evolved unique strategies to actively downregulate early innate signaling events, resulting in impaired DC function and Th1 activation.

Keywords

Leishmania; protozoan parasites; DC; innate immune response; JAK/STAT

*Address correspondence and reprint response to Dr. Lynn Soong, Department of Microbiology and Immunology, University of Texas Medical Branch, Medical Research Building 3.132, 301 University Blvd., Galveston, TX 77555-1070, Phone (409) 772-8149; Fax: (409) 747-6869; Email: lysoong@utmb.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Extensive studies have been conducted to examine how *Leishmania* promastigotes (the insect stage of the parasite) and amastigotes (the intracellular form) infect target cells and suppress the intracellular signaling events in macrophages [reviewed by (Kima, 2007) and (Olivier et al., 2005)]. However, limited information is available regarding the molecular details as to how DCs respond to the intracellular amastigotes. As professional APCs and potential targets for *Leishmania* infection, DCs play multiple roles in leishmaniasis: updating/transporting parasites (Moll et al., 1995), initiating innate immunity (Schleicher et al., 2007), priming parasite-specific CD4⁺ and CD8⁺ T cells, and maintaining T cell memory/activity (Baldwin et al., 2004; Belkaid et al., 2002; Bertholet et al., 2005; Zaph et al., 2004). Recently, DC-based vaccination for the control of *L. major*-induced cutaneous leishmaniasis has gained special attention (Ramirez-Pineda et al., 2004; Remer et al., 2007). However, studies from our and other groups have revealed marked differences in DC responsiveness to Old World versus New World *Leishmania* parasites. For example, while appreciable levels of IL-12 are produced in murine DCs infected with *L. major* or *L. donovani* promastigotes (Gorak et al., 1998; von Stebut et al., 1998), C57BL/6 (B6) DCs infected with *L. mexicana* amastigotes show no sign of activation and no detectable levels of IL-12 and other proinflammatory cytokines (Bennett et al., 2001). Likewise, *Leishmania amazonensis* (*La*) amastigotes failed to induce CD40-dependent IL-12 production from both BALB/c and C3H DCs (Qi et al., 2001). Notably, DCs infected with *La* promastigotes displayed a “semi-activation” phenotype, produced relatively low levels of IL-12, and preferentially induced pathogenic CD4⁺ T cells (Xin et al., 2007).

IL-12-producing DCs and MΦs are essential for host defense against intracellular pathogens. It has been well-established in the *L. major* infection model that the outcome of cutaneous leishmaniasis is determined by the paradigm of Th1/Th2 responses. Resolution of *L. major* in genetically resistant mice is ascribed to a polarized Th1 immune response, while the susceptibility in BALB/c mice is attributable to selective expansion of Th2 cells (Sacks and Noben-Trauth, 2002). However, we and others have found that *La* infection results in non-healing disease in most inbred mouse strains due to the induction of a weak, Th1/Th2 mixed response (Ji et al., 2002; Jones et al., 2000). The deficient Th1 response seen in *La*-infected mice is due largely to profound, multiple impairments in numerous cytokines, chemokines, and their corresponding receptors at an early stage of the infection (Ji et al., 2003). Our recent evidence of intrinsic defects in *La*-infected DCs support the concept that *Leishmania* parasites can manipulate the immune response at the APC level (Xin et al., 2007).

Previous studies showed that activation of the JAK/STAT signaling pathway plays an important role in DC differentiation and maturation, and STAT1^{-/-} and IRF-1^{-/-} DCs are unable to undergo full maturation and secrete IL-12 (Gabriele et al., 2006; Jackson et al., 2004). Most recently, Johnson and colleagues have further interpreted the failure to control *L. major* infection in STAT1^{-/-} mice to be due to impaired up-regulation of MHC and costimulatory molecules in STAT1^{-/-} DCs (Johnson and Scott, 2007). In this study, we further explored the molecular mechanisms underlying impaired DC activation and IL-12 suppression following infection with *La* pro- and amastigotes. Our results demonstrated that although *La* promastigotes induced a weak and transient DC activation and IL-12p40 production, both developmental forms suppressed IL-12 production, and induced IL-10, in the presence of secondary stimuli (IFN-γ/LPS, IFN-α/LPS). Infection of DCs with *La* parasites resulted in multiple alterations in the JAK/STAT signaling pathway, including a protease- and proteasome-dependent down-regulation of STAT activation and degradation of STAT2 protein. Furthermore, we showed that alterations in intracellular signaling and suppression of IL-12 production were caused by direct effects of amastigotes rather than by the induction of endogenous IL-10. To the best of our knowledge, this is the first report that provides molecular

details for host-amastigote interactions and highlights the functional impairment of DC activation by direct effect of intracellular parasites.

2. Materials and methods

2.1. Mice

Female C57BL/6 (B6) and BALB/c mice were purchased from Harlan Sprague Dawley. All mice were maintained under specific pathogen-free conditions and used for experiments at 6–8 wk of age according to protocols approved by the Animal Care and Use Committee of the University of Texas Medical Branch (UTMB, Galveston, TX).

2.2. Reagents and chemicals

Recombinant mouse IFN- γ was purchased from Leinco Technologies (St. Louis, MO) and CHO-derived recombinant mouse IFN- α was purchased from Cell Sciences (Canton, MA). LPS from *Salmonella enterica* serovar Typhimurium and CFSE (5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester) were purchased from Sigma-Aldrich (St. Louis, MO). All inhibitors, including proteasome inhibitor MG132, protease inhibitor E64, protein tyrosine phosphatase inhibitor bisperoxovanadium bpV (phen), and MEK/ERK inhibitor U0126, were purchased from Calbiochem (San Diego, CA).

2.3. Antibodies

The following fluorescence-conjugated, mouse-specific mAbs were purchased from eBioscience (San Diego, CA): FITC-conjugated anti-MHC class II (I-A/I-E) (M5/114.15.2); PE-conjugated anti-CD83 (Michel-17), anti-CD80 (16-10A1), anti-CD86 (PO3.1), anti-CD40 (1C10), anti-IL-12p40 (C17.8); PE-Cy5-conjugated anti-CD11c (N418), as well as isotype control Abs, including FITC-conjugated rat IgG2a; PE-conjugated rat IgG1, IgG2a, and IgG2b; PE-Cy5-conjugated Hamster IgG. Antibodies, including rabbit anti-mouse STAT1, p-STAT1, STAT2, STAT3, p-STAT3, and IRF-1, as well as HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-mouse ERK1/2 and p-ERK1/2 mAbs were purchased from Cell Signaling Technology (Danvers, MA); rabbit anti-mouse p-STAT2 mAb was obtained from Upstate Biotechnology (Charlottesville, VA); mouse anti-IRF-8/ICSBP mAb was obtained from Invitrogen Life Technologies (San Francisco, CA) and mouse anti-actin mAb (Sigma) was obtained from Dr. Jiaren Sun (Department of Microbiology and Immunology, UTMB, TX). Purified hamster anti-mouse CD11c (N418) was purchased from eBioscience. Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated anti-hamster IgG were purchased from Molecular Probes (Eugene, OR), and mounting medium with DAPI was obtained from Vector Laboratories (Burlingame, CA).

2.4. Parasites and Ag preparation

The infectivity of *L. amazonensis* (MHOM/BR/77/LTB0016 or RAT/BA/74/LV78 clone 12-1) and *L. major* (MRHO/SU/59/P/LV39) was maintained by regular passage through BALB/c mice. Promastigotes were cultured at 23°C in Schneider's *Drosophila* medium, pH 7.0 (Invitrogen Life Technologies) supplemented with 20% FBS (Sigma), 2 mM L-glutamine, and 50 μ g/ml gentamicin. Stationary promastigote cultures of less than five *in vitro* passages were used for animal or DC infection. Axenic amastigotes of LV78 *La* (obtained from Dr. K.-P. Chang, Department of Microbiology and Immunology, Chicago Medical School) were maintained in complete Grace's insect cell culture medium (Invitrogen, pH 5.0), supplemented with 20% fetal bovine serum (HyClone, Logan, UT) at 33°C (Dutta et al., 2005). To label parasites, promastigotes or amastigotes, were suspended at 5×10^7 /ml in PBS containing 5 μ M CFSE and incubated at room temperature for 5 min. Labeled parasites were washed four times

with PBS and culture medium before they were added to DCs. To prepare heat-killed parasites, promastigotes or amastigotes ($1 \times 10^8/\text{ml}$ in PBS) were incubated in a 60°C water bath for 30 min. To prepare parasite lysates, parasites ($2 \times 10^8/\text{ml}$ in PBS) were subjected to six freeze-thaw cycles and a 15-min sonication in an ice bath, and then were stored at -70°C in aliquot.

2.5. DC generation

Bone marrow-derived DCs (BMDCs) were generated from B6 mice using complete IMDM containing 10% FBS (Sigma), supplemented with 20 ng/ml rGM-CSF (eBioscience), as previously described (Qi et al., 2001). Half of the culture medium was replaced with fresh GM-CSF-containing medium at days 3 and 6. At day 8, DCs were harvested and adjusted to $1 \times 10^6/\text{ml}$ for infection. Usually, the percentage of CD11c⁺ cells at day 8 of culture was over 70%, as determined by FACS analysis.

2.6. DC/T cell cocultures and proliferation assay

Naïve CD4⁺ T cells or primed CD4⁺ T cells were respectively purified from naïve mouse spleen or draining LN of mice that were infected with 2×10^6 stationary promastigotes of *La* for 7–8 wk by negative selection using magnetic beads (Miltenyi Biotec, Auburn, CA). Purified CD4⁺ T cells (2×10^5) were cocultured with mitomycin C-pretreated, promastigote- or amastigote-infected DCs (2×10^4) in a final volume of 200 μl per well in a round-bottomed, 96-well plate for 3 days. 1 μCi of [³H] thymidine was added 18 h prior to cell harvest, and incorporated radioactivity was determined on a microplate scintillation and luminescence counter (Packard Instrument Company, Downers Grove, IL).

2.7. Cytokine ELISA

BMDCs ($1 \times 10^6/\text{ml}$) were infected with *L. major* promastigotes, or *La* pro- or amastigotes at a 10:1 parasite-to-cell ratio for 6 h and then stimulated with IFN- γ (100 ng/ml), IFN- α (100 U/ml), LPS (100 ng/ml), LPS/IFN- γ , and LPS/IFN- α for 18 h. In some experiments, a neutralizing anti-IL-10 mAb (5 $\mu\text{g}/\text{ml}$) was added at the beginning of infection. The levels of cytokines in supernatants were measured by using ELISA kits specific for IL-12p40, IL-10, IL-6 (BD Biosciences), and IL-12p70 (eBioscience). Detection limits were 7 pg/ml for IL-12p40, 4 pg/ml for IL-10 and IL-6, and 15 pg/ml for IL-12p70, respectively.

2.8. FACS and intracellular staining

At 24 h post-infection with either promastigotes or amastigotes, DCs were stained for surface markers, including CD11c, CD40, CD83, CD80, CD86 and MHC class II. For intracellular cytokine staining, DCs were infected with parasites in the presence or absence of IFN- γ or LPS/IFN- γ for 24 h, 1 μl Golgi Stop (BD Biosciences) was added 6 h before harvest. Cells were washed, blocked with 1 $\mu\text{g}/\text{ml}$ Fc γ blocker (BD Biosciences), stained with mAbs specific to surface markers, fixed/permeabilized with a Cytofix/Cytoperm Kit (BD Biosciences), and then stained with mAbs specific to cytokines, or appropriate isotype controls. Cells were washed and analyzed on a FACScan (BD Biosciences), and data analyzed with FlowJo software (TreeStar, Ashland, OR).

2.9. Western blot

A total of 4×10^6 BM-DCs were infected with *La* promastigotes or amastigotes at a 10:1 parasite-to-cell ratio. At either 6 h or 20 h post-infection, cells were treated with various stimuli for the indicated times. Where indicated, parasites were pretreated with different inhibitors for 1 h, washed three times, and then added to DC cultures. Cells were then lysed in an ice-cold lysis buffer (100 mM Tris, pH 8, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100) supplemented with complete EDTA-free protease inhibitors (Pierce Biotechnology, Rockford, IL), which include 5 mM sodium vanadate, 10 mM sodium fluoride, 10 mM β -glycerophosphate sodium,

and 5 mM sodium pyrophosphate). For *in vitro* DC-parasite lysate interaction, BMDCs and parasites were lysed in ice-cold lysis buffer without inhibitors. Protein concentrations were determined using BCA protein assay kits (Pierce Biotechnology). Equal amounts of proteins were loaded onto 10% SDS-polyacrylamide gels, and then transferred to polyvinylidene difluoride membranes (BioRad Laboratories, Hercules, CA). Membranes were incubated with primary Abs (diluted 1:2,000 in PBS containing 5% nonfat milk) at 4°C overnight, washed, and incubated with HRP-conjugated rat anti-mouse IgG (1:3,000) for 1 h. Blots were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ).

2.10. Immunofluorescence

Cells were fixed in 4% formaldehyde-phosphate-buffered saline for 10 min at room temperature, permeabilized with ice-cold acetone-methanol (1:1) for 30 min at -20°C, and stained sequentially with rat anti-mouse CD11c, rabbit anti-mouse p-STAT1 or p-STAT2 mAbs. Alexa 488-conjugated goat anti-rabbit IgGs and Alexa 594-conjugated goat anti-rat IgGs were used as secondary Abs. Nuclei were visualized by using DAPI as a counter-stain. The fluorescence-positive cells were counted at 5 random fields (about 200 cells in total) under a Zeiss LSM 510 laser-scanning confocal microscope (Zeiss, Thornwood, NY) in the UTMB Optical Imaging Core Facility.

2.11. Gene array and quantitative real-time PCR

Total RNA was extracted from $1\sim 2 \times 10^6$ DCs using the RNeasy system (Qiagen, Valencia, CA). Over 10 µg of total RNA was subjected to JAK/STAT Signaling Pathway Oligo GEArray analyses (SuperArray Bioscience Corporation, Frederick, MD). Real-time RT-PCR was performed at the UTMB Sealy Center for Cancer Cell Biology Core Facility, using 20 × assay mixture of primers, TaqMan MGB probes (FAM dye-labeled) for mouse IL-12p35 and IL-12p40 (P/N 4331182), pre-developed 18S rRNA (VIC dye-labeled, as an endogenous control), and TaqMan assay reagent (P/N 4319413E), all of which were purchased from Applied Biosystems (Foster City, CA). Separate-tube (single plex), real-time PCR was performed with 40 ng cDNA for both target genes and the endogenous control, using Taqman Gene expression master mix (P/N 4370074). The PCR cycling parameters were: UNG activation 2 min 50°C, AmpliTaq activation 95°C for 10 min, denaturation 95°C for 15 sec, and annealing/extension 60°C for 1 min (repeat 40 cycles) on ABI7000 (Applied Biosystems). Duplicate C_T values were analyzed in Microsoft Excel using the comparative $C_T(\Delta\Delta C_T)$ method, as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta C_T}$) was obtained by normalization to endogenous reference (18s) and relative to control sample.

2.12. Statistical analysis

The difference between the two groups was determined by Student's t-test. A one-way ANOVA test was used for multiple group comparisons. The tests were performed using GraphPad Prism, version 4.00, for Windows (GraphPad Software, San Diego, CA, USA). Statistically significant values are as follows: *, $p < 0.05$ and **, $p < 0.01$, respectively.

3. Results

3.1. *L. amazonensis* infection modulates DC function and cytokine production

We have previously shown that profound impairment in cytokine/chemokine expression during *La* infection (Ji et al., 2003) was mostly due to intrinsic defects in DC activation (Xin et al., 2007), and, thus, we sought to explore the underlying mechanisms in the present study. First, we confirmed that DCs infected with *La* promastigotes (Pm) produced significantly lower levels of IL-12p40 and IL-10 than did control cells infected with *L. major* (Spl. 1A). Since the

JAK/STAT signaling pathway is important for DC differentiation/maturation and innate immunity (Shuai and Liu, 2003), we speculated that weak activation and dysregulation of this pathway may account for insufficient DC activation and T cell priming during *La* infection (Ji et al., 2003; Xin et al., 2007). Analyses of the expression of genes in the JAK/STAT signaling pathway using Oligo GEArray revealed that nearly 55 out of 120 tested genes were induced in both groups of infected DCs in comparison to control (data not shown). However, while more than 2.5-fold of induction was observed for Jak2, Stat1, NOS2 (nitric oxide synthase 2), IL-2 receptor γ chain, ISG15 (interferon-stimulated gene-15), and Oas1a (2'-5' oligoadenylate synthetase 1A) genes in *L. major*-infected DCs, none of these genes reached 2.5-fold induction following infection with *La* promastigotes (Spl. 1B). These results confirm and extend our recent report, suggesting that *La* promastigotes are intrinsically less competent in activating DCs than are *L. major* promastigotes.

To better understand how DCs respond to *La* parasites, we focused on the disease-forming amastigotes and first examined the DC activation and maturation status. After 24 h of infection with promastigotes, there was up-regulation of CD40, CD83, CD86 and MHC-II molecules on the cell surface of CD11c⁺ DCs; however, compared with control DCs, amastigote infection significantly down-regulated the expression of these surface markers (Fig. 1A). The impairment in DC activation during amastigote infection was confirmed in DC-T cell co-culture studies, where *in vitro* priming of naïve CD4⁺ T cells and restimulation of primed CD4⁺ T cells were tested (Fig. 1B). Together, these studies suggest functional defects in amastigote-infected DCs.

To further validate this observation, we examined the cytokine profiles in two groups of infected DCs in the absence and presence of secondary stimuli that are known to strongly induce IL-12 production and JAK/STAT signaling. While infection with *La* promastigotes (at 5:1 and 10:1 doses) triggered IFN- γ -primed DCs to produce IL-12p40, similar infection with amastigotes failed to induce IL-12p40 production, even at a 5:1 infection ratio (Fig. 2A). In the absence of exogenous stimuli, DCs produced very low or marginal levels of IL-10 following infection with *La* promastigotes (Spl. 1A) and amastigotes (data not shown). In subsequent experiments, we have chosen to use a 10:1 infection ratio. When DCs were treated with potent DC stimuli (LPS, IFN- γ /LPS, or IFN- α /LPS), both forms of parasites consistently suppressed the production of IL-12p40 (Fig. 2B), IL-12p70 (Fig. 2C), and IL-6 (Fig. 2D), but increased the production of IL-10 (Fig. 2E). These results indicate that both developmental forms of *La* parasites can down-modulate DC function.

3.2. Down-regulation of IL-12 production by *La* parasites at gene transcription level

To investigate the source of IL-12 at the single cell level, we used CFSE-labeled parasites and examined IL-12p40 production among parasite-carrying (CFSE⁺) and by-stander (CFSE⁻) DCs. As shown in Fig. 3A, DC infection rates for pro- and amastigotes were 62% and 72%, respectively, regardless of the stimulation status of IFN- γ or LPS/IFN- γ . Although IL-12p40 was detectable in both pro- and amastigote-carrying CD11c⁺ DCs following treatment with IFN- γ or LPS/IFN- γ , the percentages of IL-12p40⁺ DCs were notably lower in CFSE⁺ DC subsets (5~6% and 15.6~21% in IFN- γ - and LPS/IFN- γ - stimulated cells, respectively) than in those of CFSE⁻ DCs (10.5~17.1% and 18~27.3% in IFN- γ - and LPS/IFN- γ -stimulated cells, respectively).

Given that the biologically functional IL-12 is composed of two subunits, p40 and p35, we conducted real-time RT-PCR analysis to determine the kinetics of IL-12 mRNA expression. DCs were pre-infected with *La* parasites for 6 h, and then stimulated with or without LPS/IFN- γ for an additional 2 h or 18 h. As shown in Fig. 3B, there was a 16-fold induction of IL-12p40 mRNA at 8 h of infection with promastigotes alone, but a less than 4-fold induction following infection with amastigotes. Notably, the expression levels in both groups dropped dramatically

at 24 h post-infection, confirming the transient induction of IL-12p40 gene during *Leishmania* infection (Gorak et al., 1998). The levels of IL-12p35 mRNA were below the detection limit in DCs infected with parasites alone. In the absence of parasite infection, the induction of IL-12p40 and IL-12p35 genes with a 2-h treatment of LPS/IFN- γ were more than 1300-, and 500-fold, respectively; however, induction of both genes was significantly downregulated by pre-infection with either promastigotes or amastigotes. Similar trends were observed at 18 h after LPS/IFN- γ stimulation (Fig. 3C). Together, these results indicate a transient induction of IL-12p40 gene transcription following infection with *La* promastigotes and a profound suppression of IL-12 gene and protein expression by both pro- and amastigotes in response to exogenous stimulation.

3.3. *L. amazonensis*, especially its amastigotes, suppress the activation but not nuclear translocation of phosphorylated STATs

To investigate the mechanisms by which *La* parasites interfere with DC activation, we examined STAT activation in DCs that were pre-infected with parasites for 6 h or 20 h and then briefly stimulated with IFN- γ , IFN- α , or LPS plus IFNs. As shown in Fig. 4A, parasite infection by itself did not trigger STAT phosphorylation. However, infection with *La* promastigotes or amastigotes for 6 h resulted in markedly reduced phosphorylation of STAT1 or STAT2 induced by IFN- γ , LPS/IFN- γ , or LPS/IFN- α , and the reduction of p-STAT1 or p-STAT2 was more pronounced in amastigote-infected DCs (80% ~ 90% lower than stimulated controls) than that in promastigote-infected DCs (40% ~ 50% lower than stimulated controls). Furthermore, the level of total STAT2 proteins in amastigote-infected DCs was dramatically reduced (to a level that was only 10% of that in control groups). Similar results were observed at 20 h post-infection (Fig. 4B). These results suggest infection of *La* parasites suppress STAT activation and induce degradation of STAT2 proteins. Immunofluorescence results, however, indicated that parasite infection did not block the nuclear translocation of IFN-induced p-STAT1 or p-STAT2 molecules (Fig. 4C), although the percentages of CD11c⁺ DCs with positive staining for nuclear p-STAT1 or p-STAT2 were significantly lower among infected DCs (Fig. 4D). Therefore, infection of *La* parasites, especially the amastigote form, markedly suppressed activation of STATs without affecting the nuclear translocation of p-STATs.

3.4. STAT2 degradation induced by live amastigotes is protease- and proteasome-dependent

To further examine whether the reduction of the STAT2 protein level was mediated by parasite- or host-dependent mechanisms, we heat-inactivated parasites (60 °C, 30 min) prior to infection of DCs. Heat-killed pro- or amastigotes no longer suppressed phosphorylation of STAT1 or STAT2, nor were they capable of reducing total STAT2 protein levels (Fig. 5A). We postulated that STAT2 protein was likely degraded via a mechanism(s) that involves parasite-derived protease(s) or proteasomes. To explore this possibility, we prepared fresh DC lysates (in lysis buffer without inhibitors) and incubated it with increasing doses of fresh pro- or amastigote lysates at 37 °C for 30 min. In some cases, parasite lysates were pre-incubated with a protease inhibitor (E64) or a proteasome inhibitor (MG132) on ice for 10 min. As shown in Fig. 5B, promastigote lysates did not affect the STAT1 or STAT2 protein levels in DC lysates. However, incubation with amastigote lysates decreased the STAT2, but not the STAT1 protein levels, in a dose-dependent manner, which was blocked by pretreating parasite lysates with E64 or MG132 (Fig. 5B and C). Furthermore, pretreatment of live amastigotes with MG132 could reverse the amastigote-induced suppression of STAT activation and degradation of STAT2 proteins (Fig. 5D). The lack of inhibitory effect for E64 on live amastigotes was probably due to its non-permeability to live cells (Cameron et al., 2004). Pretreatment of live amastigotes with a phosphatase inhibitor (bpV) had no demonstrable effects on parasite-induced reduction of p-STATs, suggesting a negligible role for parasite phosphatase(s) in de-phosphorylation of tested STATs (data not shown). Collectively, these results suggest that amastigote-derived

protease(s) or proteasome is responsible for degradation of STAT2 and suppression of STAT activation.

3.5. *L. amazonensis* amastigote infection suppresses IRF-1 and IRF-8 expression in DCs

IRFs are a family of transcription factors involved in the regulation of both innate and adaptive immunity (Taniguchi et al., 2001). IRF-1 and IRF-8/ICSBP can bind to the promoters of mouse IL-12p35 and p40 genes and activate IL-12 expression (Liu et al., 2003; Maruyama et al., 2003; Masumi et al., 2002). To determine whether *La* parasite-induced IL-12 suppression correlates with alterations in IRF-1 or IRF-8, we examined the expression of IRF-1 and IRF-8 following *La* infection. We found that infection with promastigotes did not suppress the expression of IRF-1 and IRF-8 after 4 h of stimulation with LPS/IFN- γ or LPS/IFN- α , whereas infection with amastigotes resulted in nearly 90% and 95% reductions in IRF-1 and IRF-8 expression, respectively, compared to positive controls (Fig. 6, lanes 4 and 7). Similar results were observed in DCs with 20 h of parasite infection. These results suggest that reduced expression of IRF-1 and IRF-8 may contribute to impaired IL-12 production in *La*-infected DCs.

3.6. Reduced activation of STAT3 and ERK1/2, and IL-10-independent IL-12 suppression in *L. amazonensis*-infected DCs

IL-10 is a type II cytokine that can downregulate IL-12 expression (Moore et al., 2001), although IL-10 induction in *La*-infected DCs is at the low level (Qi et al., 2001; Xin et al., 2007). To test whether IL-12 suppression in *La*-infected DCs was secondary to the induction of IL-10, we examined two signaling pathways, STAT3 and ERK1/2, which are known to be associated with IL-10 induction (Benkhart et al., 2000; Lucas et al., 2005). We found that both developmental forms, especially amastigotes, markedly suppressed the induction of p-STAT3 and p-ERK1/2 (resulting in respective reductions of nearly 70% and 40% of controls) following LPS/IFN stimulation, without affecting their total protein levels (Fig. 7). Actually, the use of an ERK1/2 inhibitor (U0126) did dramatically reduce IL-10 production, but still, the suppression of IL-12 in both parasite-infected DCs was not reversed, although a slight increase in IL-12 production was observed (data not shown). Therefore, STAT3 or ERK1/2 may be partially involved in the induction of IL-10. To further assess the direct role of IL-10 in infection-mediated IL-12 suppression, we used an IL-10-neutralizing mAb. While this mAb totally blocked IL-10 production and significantly enhanced IL-12 production in LPS- or LPS/IFN- γ -stimulated DCs, it had no effect on infection-induced suppression of IL-12 production (Fig. 8). Together, these results suggest that IL-12 suppression from *La*-infected DCs is not due to the induction of IL-10 but rather to live parasites themselves.

4. Discussion

The responses of DC to *Leishmania* parasites have been recently studied in both murine and human models. In contrast to induction of DC activation and upregulation of IL-12 production by *L. major*, non-healing *La* infection induced impaired DC maturation/activation and cytokine production, which correlated with profoundly impaired, multiple immune responses at an early stage of the infection (Favali et al., 2007; Ji et al., 2003; Xin et al., 2007). However, the precise molecular mechanism(s) by which *La* modulates DC function remains unclear. In the current study, we investigated DC responses to *La* pro- and amastigotes and found that the JAK/STAT signaling pathway was altered by both parasite forms. Consistent with our previous study (Xin et al., 2007), *La* promastigotes induced both a lower cytokine production and gene expression in the JAK/STAT signaling pathway when compared to those parameters in *L. major* promastigotes (Spl. 1). When the two developmental forms of *La* parasites were compared, we found that, while promastigote infection could stimulate the expression of DC surface markers and prime CD4⁺ T cells to some extent, infection with the disease-forming amastigotes failed

to activate DCs (Fig. 1). Similar results were previously reported in DCs infected with *L. mexicana* pro- and amastigotes (Bennett et al., 2001). A possible explanation for differential responses of DCs to these two stages of parasites forms is that lipophosphoglycan (LPG), the major surface molecules on promastigotes, can activate the TLR2-mediated signaling and, further, the near absence of LPG on amastigotes is responsible for the lack of DC activation (Beverley and Turco, 1998; de Veer et al., 2003). However, it is also possible that intracellular amastigotes have evolved additional strategies to down-regulate key molecules (e.g. MHC II) in DCs (De Souza Leao et al., 1995; Prina et al., 1993).

Infection with *L. major* or *L. donovani* pro- or amastigotes, DCs can induce IL-12p40 production *in vitro* and *in vivo* (Bennett et al., 2001; Gorak et al., 1998; Konecny et al., 1999). However, we found that *La* promastigotes only weakly induced IL-12p40 production, whereas infection of amastigotes was devoid of this response (Fig.3B). To determine whether the impairment/absence of IL-12 induction in *La*-infected DCs was due to the intrinsic defect of these parasites to trigger DC activation or as a result of the active suppression of DC responses, we used an established infection model in which DCs were pre-infected with either pro- or amastigotes for 6 h or 20 h, and then examined cytokine production, gene expression and signaling pathways in the presence of other stimuli (e.g. IFNs, LPS, and LPS plus IFNs). Our results suggest that *La* pro- and amastigotes differentially modulate IL-12 production at the transcriptional level, and that both forms of parasites are able of suppressing LPS/IFN- γ -induced IL-12 transcription.

IL-12 is a proinflammatory cytokine, and the expression of its two subunits, p40 and p35, are known to be transcriptionally regulated by NF- κ B and IRFs (Grumont et al., 2001; Liu et al., 2003; Maruyama et al., 2003; Masumi et al., 2002). Cameron *et al.* found that the inhibition of LPS-induced IL-12 production in *L. mexicana* amastigote-infected M Φ s was due to the degradation of I κ B and NF- κ B by amastigotes-derived cysteine peptidase B (Cameron et al., 2004). Another study showed that *L. major* amastigotes selectively inhibited p50/p65 translocation, but induced p50/c-Rel complex formation, which was correlated to the production of TNF- α and IL-10 from human monocytes (Guizani-Tabbane et al., 2004). Given that IRFs, including IRF-1 and IRF-8/ICSBP, are critical for the induction of IL-12, development of Th1 response, and control of *L. major* infection (Giese et al., 1997; Lohoff et al., 1997), we speculated that the modulation of DC JAK/STAT signaling by *Leishmania* parasites also contribute to the disease pathogenesis. In support of this view, it has been demonstrated that STAT1 in DCs, but not in T cells, is critically important for immunity to *L. major* (Johnson and Scott, 2007).

In this study, we have also provided evidence for the first time for the activation status of STATs and specific alterations of its related signaling pathways in murine DCs following infection with *La* pro- and amastigotes, respectively. It is evident that although infection with pro- or amastigotes alone could not induce the activation of STATs, infection of both parasite forms markedly suppressed the phosphorylation of STAT1 and STAT2 induced by IFNs or LPS plus IFNs. Notably, *La* amastigotes caused reductions of about 80%–90% in p-STAT1 and p-STAT2, respectively, and there was a nearly 90% reduction in the total STAT2 protein level (Fig. 4). However, infection with both developmental forms of parasites did not affect the nuclear translocation of phosphorylated-STAT1 or STAT2 molecules. Considering the important role of STAT2 in MHC II expression, this finding may potentially explain the impaired antigen presentation in *La* amastigote-infected DCs (Zhao et al., 2007). Although STAT1 α degradation was shown to be rescued by pretreatment of host cells with a proteasome inhibitor MG132 (Forget et al., 2005), we did not observe any rescue of amastigote-induced STAT2 degradation when DCs were pretreated with MG132 (data not shown). However, our studies confirmed that the parasite-derived factors were responsible for the suppression of STAT activation and total STAT2 degradation, because 1) only live, but not heat-killed

parasites, reduced p-STATs and total STAT2 levels, 2) the degradation of STAT2 protein was mediated by parasite-derived protease(s) and proteasome, which could be blocked by pretreatment of the parasite lysate with E64, a protease inhibitor, or MG132, a protease inhibitor. While the reduced p-STAT2 level in *La*-infected DCs could reflect the degradation of total STAT2 proteins by the parasites, the mechanisms by which *La* reduces phosphorylation of STAT1 are not known. Although various types of *Leishmania*-derived phosphatases, such as LPTP1, bisphosphatase and secreted acid phosphatase, have recently been identified (Naderer et al., 2006; Nascimento et al., 2006), our studies with phosphatase inhibitor (bpV)-pretreated parasites suggested a limited role for parasite-derived phosphatases in the dephosphorylation of STAT1 (Fig.5D). Whether *La* infection triggers the activation of a host cellular phosphatase(s) awaits further investigation. Together with previous findings of the cysteine peptidase-dependent degradation of MHC class II and I κ B molecules in *La*- or *L. mexicana*-infected M Φ s (Cameron et al., 2004; De Souza Leao et al., 1995), we conclude that parasite-derived protease/peptidase or proteasome may contribute to their virulence, as well as disease progression, by disrupting the early signaling events crucial for DC activation.

Of note, infection with *La* amastigotes, but not promastigotes, also ablated LPS/IFN-infected upregulation of IRF-1 and IRF-8 in DCs (Fig.6). At this stage, it is unclear whether these changes were due to the suppression of STAT activation or degradation. As IRF-1 and IRF-8 participate in TLR signaling via interaction with MyD88 (Negishi et al., 2006; Zhao et al., 2006), it will be interesting to determine in future studies whether regulation of IRF-1 and IRF-8 expression by *La* modulates TLR signaling and contributes to the pathogenesis of *La* infection and the different responses to the two parasite forms.

It has been reported that IL-10 can suppress DC activation and DC's APC function (Moore et al., 2001), and that the production of IL-10 from DCs and M Φ s is associated with activated STAT3 and ERK1/2 (Benkhart et al., 2000; Lucas et al., 2005). Of relevance to this study is the finding that lesion-derived *La* amastigotes can activate MAPK/ERK1/2 via the super-induction of IL-10 in M Φ s (Yang et al., 2007), apparently through the prominent effects of Fc γ R signaling because these amastigotes are known to be coated with anti-*Leishmania* IgG Abs (Wanasen et al., 2008). In this study, we have provided strong evidence that alteration in DC signaling pathways during infection with Ab-free amastigotes is not due to endogenous IL-10. First, infection with *La* pro- and amastigotes both reduced the phosphorylation of STAT3 and ERK1/2 without affecting the total levels of STAT3 and ERK1/2, suggesting a STAT3- and ERK1/2-independent mechanism in regulating IL-10 production in *La*-infected DCs. Second, the use of an ERK inhibitor and anti-IL-10 neutralizing Ab failed to reverse the suppression of IL-12 production in infected DCs, despite their effectiveness in blocking IL-10 production, which suggested that IL-12 suppression by *La* parasites was IL-10-independent. Although IL-10-deficient mice were more resistant to *L. major* (Brooks et al., 2006; Kane and Mosser, 2001), deletion of the IL-10 gene did not change the susceptibility of mice to *La* parasites (Jones et al., 2002), suggesting to us that an IL-10-independent mechanism in persistent infection is caused by *La* infection. Accordingly, this study supports the view that amastigotes in the *L. mexicana* complex (*La*, *L. mexicana*, and *L. pifanoi*) can directly suppress the activation and/or accelerate the degradation of signaling pathways via their peptidases/proteinases (Cameron et al., 2004), and that this delayed and suppressed DC activation is responsible for impaired innate and acquired immunity to parasite infection (Ji et al., 2003; Xin et al., 2007).

In summary, our results showed that while *La* promastigotes are capable of inducing weak and transient DC activation, infection with its amastigotes failed to induce DC activation and such infected DCs are incompetent in priming naïve and stimulating primed CD4⁺ T cells. The suppression of IL-12 production in these infected DCs is correlated with the suppressed activation/expression of STAT and of the IRF molecule. At least some of these alterations are

due to direct effects from live amastigotes and are mediated by protease- and proteosome-dependent mechanisms. Although infection with *La* parasites can induce IL-10 production via STAT3- and ERK1/2-independent mechanisms, endogenous IL-10 is not a prerequisite for the suppression of IL-12. Although the detailed mechanisms remain poorly defined as to how parasite components travel through the PV membrane to interact with host components, it is conceivable that minimizing DC activation and down-modulating the responsiveness of infected DCs to other inflammatory stimuli at the infection site can alter DC migration, T cell priming, and Th1 cell development, which ultimately affects the outcome of the infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by a NIH grant AI043003 to LS. The authors wish to thank Dr. Janice Endsley for helpful discussion and critiques and Mardelle Susman for manuscript preparation.

Reference

- Baldwin T, Henri S, Curtis J, O'Keeffe M, Vremec D, Shortman K, Handman E. Dendritic cell populations in *Leishmania* major-infected skin and draining lymph nodes. *Infect Immun* 2004;72:1991–2001. [PubMed: 15039319]
- Belkaid Y, Von Stebut E, Mendez S, Lira R, Caler E, Bertholet S, Udey MC, Sacks D. CD8+ T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with *Leishmania* major. *J Immunol* 2002;168:3992–4000. [PubMed: 11937556]
- Benkhart EM, Siedlar M, Wedel A, Werner T, Ziegler-Heitbrock HW. Role of Stat3 in lipopolysaccharide-induced IL-10 gene expression. *J Immunol* 2000;165:1612–1617. [PubMed: 10903771]
- Bennett CL, Misslitz A, Colledge L, Aebischer T, Blackburn CC. Silent infection of bone marrow-derived dendritic cells by *Leishmania mexicana* amastigotes. *Eur J Immunol* 2001;31:876–883. [PubMed: 11241293]
- Bertholet S, Debrabant A, Afrin F, Caler E, Mendez S, Tabbara KS, Belkaid Y, Sacks DL. Antigen requirements for efficient priming of CD8+ T cells by *Leishmania* major-infected dendritic cells. *Infect Immun* 2005;73:6620–6628. [PubMed: 16177338]
- Beverley SM, Turco SJ. Lipophosphoglycan (LPG) and the identification of virulence genes in the protozoan parasite *Leishmania*. *Trends Microbiol* 1998;6:35–40. [PubMed: 9481823]
- Brooks DG, Trifilo MJ, Edelmann KH, Teyton L, McGavern DB, Oldstone MB. Interleukin-10 determines viral clearance or persistence in vivo. *Nat Med* 2006;12:1301–1309. [PubMed: 17041596]
- Cameron P, McGachy A, Anderson M, Paul A, Coombs GH, Mottram JC, Alexander J, Plevin R. Inhibition of lipopolysaccharide-induced macrophage IL-12 production by *Leishmania mexicana* amastigotes: the role of cysteine peptidases and the NF-kappaB signaling pathway. *J Immunol* 2004;173:3297–3304. [PubMed: 15322192]
- De Souza Leao S, Lang T, Prina E, Hellio R, Antoine JC. Intracellular *Leishmania amazonensis* amastigotes internalize and degrade MHC class II molecules of their host cells. *J Cell Sci* 1995;108 (Pt 10):3219–3231. [PubMed: 7593283]
- de Veer MJ, Curtis JM, Baldwin TM, DiDonato JA, Sexton A, McConville MJ, Handman E, Schofield L. MyD88 is essential for clearance of *Leishmania* major: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. *Eur J Immunol* 2003;33:2822–2831. [PubMed: 14515266]
- Dutta S, Ray D, Kolli BK, Chang KP. Photodynamic sensitization of *Leishmania amazonensis* in both extracellular and intracellular stages with aluminum phthalocyanine chloride for photolysis in vitro. *Antimicrob Agents Chemother* 2005;49:4474–4484. [PubMed: 16251285]

- Favali C, Tavares N, Clarencio J, Barral A, Barral-Netto M, Brodskyn C. Leishmania amazonensis infection impairs differentiation and function of human dendritic cells. *J Leukoc Biol* 2007;82:1401–1406. [PubMed: 17890507]
- Forget G, Gregory DJ, Olivier M. Proteasome-mediated degradation of STAT1alpha following infection of macrophages with Leishmania donovani. *J Biol Chem* 2005;280:30542–30549. [PubMed: 15983048]
- Gabriele L, Fragale A, Borghi P, Sestili P, Stellacci E, Venditti M, Schiavoni G, Sanchez M, Belardelli F, Battistini A. IRF-1 deficiency skews the differentiation of dendritic cells toward plasmacytoid and tolerogenic features. *J Leukoc Biol* 2006;80:1500–1511. [PubMed: 16966383]
- Giese NA, Gabriele L, Doherty TM, Klinman DM, Tadesse-Heath L, Contursi C, Epstein SL, Morse HC 3rd. Interferon (IFN) consensus sequence-binding protein, a transcription factor of the IFN regulatory factor family, regulates immune responses in vivo through control of interleukin 12 expression. *J Exp Med* 1997;186:1535–1546. [PubMed: 9348311]
- Gorak PM, Engwerda CR, Kaye PM. Dendritic cells, but not macrophages, produce IL-12 immediately following Leishmania donovani infection. *Eur J Immunol* 1998;28:687–695. [PubMed: 9521079]
- Grumont R, Hochrein H, O'Keeffe M, Gugasyan R, White C, Caminschi I, Cook W, Gerondakis S. c-Rel regulates interleukin 12 p70 expression in CD8(+) dendritic cells by specifically inducing p35 gene transcription. *J Exp Med* 2001;194:1021–1032. [PubMed: 11602633]
- Guizani-Tabbane L, Ben-Aissa K, Belghith M, Sassi A, Dellagi K. Leishmania major amastigotes induce p50/c-Rel NF-kappa B transcription factor in human macrophages: involvement in cytokine synthesis. *Infect Immun* 2004;72:2582–2589. [PubMed: 15102766]
- Jackson SH, Yu CR, Mahdi RM, Ebong S, Egwuagu CE. Dendritic cell maturation requires STAT1 and is under feedback regulation by suppressors of cytokine signaling. *J Immunol* 2004;172:2307–2315. [PubMed: 14764699]
- Ji J, Sun J, Qi H, Soong L. Analysis of T helper cell responses during infection with Leishmania amazonensis. *Am J Trop Med Hyg* 2002;66:338–345. [PubMed: 12164286]
- Ji J, Sun J, Soong L. Impaired expression of inflammatory cytokines and chemokines at early stages of infection with Leishmania amazonensis. *Infect Immun* 2003;71:4278–4288. [PubMed: 12874303]
- Johnson LM, Scott P. STAT1 expression in dendritic cells, but not T cells, is required for immunity to Leishmania major. *J Immunol* 2007;178:7259–7266. [PubMed: 17513775]
- Jones DE, Ackermann MR, Wille U, Hunter CA, Scott P. Early enhanced Th1 response after Leishmania amazonensis infection of C57BL/6 interleukin-10-deficient mice does not lead to resolution of infection. *Infect Immun* 2002;70:2151–2158. [PubMed: 11895981]
- Jones DE, Buxbaum LU, Scott P. IL-4-independent inhibition of IL-12 responsiveness during Leishmania amazonensis infection. *J Immunol* 2000;165:364–372. [PubMed: 10861073]
- Kane MM, Mosser DM. The role of IL-10 in promoting disease progression in leishmaniasis. *J Immunol* 2001;166:1141–1147. [PubMed: 11145695]
- Kima PE. The amastigote forms of Leishmania are experts at exploiting host cell processes to establish infection and persist. *Int J Parasitol* 2007;37:1087–1096. [PubMed: 17543969]
- Konecny P, Stagg AJ, Jebbari H, English N, Davidson RN, Knight SC. Murine dendritic cells internalize Leishmania major promastigotes, produce IL-12 p40 and stimulate primary T cell proliferation in vitro. *Eur J Immunol* 1999;29:1803–1811. [PubMed: 10382742]
- Liu J, Cao S, Herman LM, Ma X. Differential regulation of interleukin (IL)-12 p35 and p40 gene expression and interferon (IFN)-gamma-primed IL-12 production by IFN regulatory factor 1. *J Exp Med* 2003;198:1265–1276. [PubMed: 14568984]
- Lohoff M, Ferrick D, Mittrucker HW, Duncan GS, Bischof S, Rollinghoff M, Mak TW. Interferon regulatory factor-1 is required for a T helper 1 immune response in vivo. *Immunity* 1997;6:681–689. [PubMed: 9208841]
- Lucas M, Zhang X, Prasanna V, Mosser DM. ERK activation following macrophage FcgammaR ligation leads to chromatin modifications at the IL-10 locus. *J Immunol* 2005;175:469–477. [PubMed: 15972681]
- Maruyama S, Sumita K, Shen H, Kanoh M, Xu X, Sato M, Matsumoto M, Shinomiya H, Asano Y. Identification of IFN regulatory factor-1 binding site in IL-12 p40 gene promoter. *J Immunol* 2003;170:997–1001. [PubMed: 12517966]

- Masumi A, Tamaoki S, Wang IM, Ozato K, Komuro K. IRF-8/ICSBP and IRF-1 cooperatively stimulate mouse IL-12 promoter activity in macrophages. *FEBS Lett* 2002;531:348–353. [PubMed: 12417340]
- Moll H, Flohe S, Rollinghoff M. Dendritic cells in *Leishmania* major-immune mice harbor persistent parasites and mediate an antigen-specific T cell immune response. *Eur J Immunol* 1995;25:693–699. [PubMed: 7705398]
- Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001;19:683–765. [PubMed: 11244051]
- Naderer T, Ellis MA, Sernee MF, De Souza DP, Curtis J, Handman E, McConville MJ. Virulence of *Leishmania* major in macrophages and mice requires the gluconeogenic enzyme fructose-1,6-bisphosphatase. *Proc Natl Acad Sci U S A* 2006;103:5502–5507. [PubMed: 16569701]
- Nascimento M, Zhang WW, Ghosh A, Houston DR, Berghuis AM, Olivier M, Matlashewski G. Identification and characterization of a protein-tyrosine phosphatase in *Leishmania*: Involvement in virulence. *J Biol Chem* 2006;281:36257–36268. [PubMed: 17005559]
- Negishi H, Fujita Y, Yanai H, Sakaguchi S, Ouyang X, Shinohara M, Takayanagi H, Ohba Y, Taniguchi T, Honda K. Evidence for licensing of IFN- γ -induced IFN regulatory factor 1 transcription factor by MyD88 in Toll-like receptor-dependent gene induction program. *Proc Natl Acad Sci U S A* 2006;103:15136–15141. [PubMed: 17018642]
- Olivier M, Gregory DJ, Forget G. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. *Clin Microbiol Rev* 2005;18:293–305. [PubMed: 15831826]
- Prina E, Jouanne C, de Souza Lao S, Szabo A, Guillet JG, Antoine JC. Antigen presentation capacity of murine macrophages infected with *Leishmania amazonensis* amastigotes. *J Immunol* 1993;151:2050–2061. [PubMed: 8102156]
- Qi H, Popov V, Soong L. *Leishmania amazonensis*-dendritic cell interactions in vitro and the priming of parasite-specific CD4(+) T cells in vivo. *J Immunol* 2001;167:4534–4542. [PubMed: 11591781]
- Ramirez-Pineda JR, Fröhlich A, Berberich C, Moll H. Dendritic cells (DC) activated by CpG DNA ex vivo are potent inducers of host resistance to an intracellular pathogen that is independent of IL-12 derived from the immunizing DC. *J Immunol* 2004;172:6281–6289. [PubMed: 15128817]
- Remer KA, Apetrei C, Schwarz T, Linden C, Moll H. Vaccination with plasmacytoid dendritic cells induces protection against infection with *Leishmania* major in mice. *Eur J Immunol* 2007;37:2463–2473. [PubMed: 17705130]
- Sacks D, Noben-Trauth N. The immunology of susceptibility and resistance to *Leishmania* major in mice. *Nat Rev Immunol* 2002;2:845–858. [PubMed: 12415308]
- Schleicher U, Liese J, Knippertz I, Kurzmann C, Hesse A, Heit A, Fischer JA, Weiss S, Kalinke U, Kunz S, Bogdan C. NK cell activation in visceral leishmaniasis requires TLR9, myeloid DCs, and IL-12, but is independent of plasmacytoid DCs. *J Exp Med* 2007;204:893–906. [PubMed: 17389237]
- Shuai K, Liu B. Regulation of JAK-STAT signalling in the immune system. *Nat Rev Immunol* 2003;3:900–911. [PubMed: 14668806]
- Taniguchi T, Ogasawara K, Takaoka A, Tanaka N. IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol* 2001;19:623–655. [PubMed: 11244049]
- von Stebut E, Belkaid Y, Jakob T, Sacks DL, Udey MC. Uptake of *Leishmania* major amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-*Leishmania* immunity. *J Exp Med* 1998;188:1547–1552. [PubMed: 9782133]
- Wanasen N, Xin L, Soong L. Pathogenic role of B cells and antibodies in murine *Leishmania amazonensis* infection. *Int J Parasitol* 2008;38:417–429. [PubMed: 17959178]
- Xin L, Li Y, Soong L. 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* 2007;75:5018–5026. [PubMed: 17682041]
- Yang Z, Mosser DM, Zhang X. Activation of the MAPK, ERK, following *Leishmania amazonensis* infection of macrophages. *J Immunol* 2007;178:1077–1085. [PubMed: 17202371]
- Zaph C, Uzonna J, Beverley SM, Scott P. Central memory T cells mediate long-term immunity to *Leishmania* major in the absence of persistent parasites. *Nat Med* 2004;10:1104–1110. [PubMed: 15448686]

- Zhao J, Kong HJ, Li H, Huang B, Yang M, Zhu C, Bogunovic M, Zheng F, Mayer L, Ozato K, Unkeless J, Xiong H. IRF-8/interferon (IFN) consensus sequence-binding protein is involved in Toll-like receptor (TLR) signaling and contributes to the cross-talk between TLR and IFN-gamma signaling pathways. *J Biol Chem* 2006;281:10073–10080. [PubMed: 16484229]
- Zhao W, Cha EN, Lee C, Park CY, Schindler C. Stat2-dependent regulation of MHC class II expression. *J Immunol* 2007;179:463–471. [PubMed: 17579067]

Fig. 1.

Profound impairment in DC activation, maturation, and APC function following infection with *L. amazonensis* amastigotes. DCs were infected with *La* promastigotes (Pm, at a 10:1 ratio) or amastigotes (Am, at a 5:1 ratio) for 24 h. (A) Indicated cell surface markers were assayed by FACS. MHC II- and CD86-positive cells were gated on CD11c⁺ DCs. Parasite-infected DCs were cocultured with naïve (B) or *in vivo*-primed (C) CD4⁺ T cells for 3 days (see Materials and Methods). T cell proliferation was assayed through ³H-thymidine uptake assay. Shown are the representative results from three independent experiments. * ($P < 0.05$) and ** ($P < 0.01$) indicate statistically significant differences between control and infection groups or two infection groups.

Fig. 2.

Selective modulation of IL-12, IL-6 and IL-10 production following *L. amazonensis* infection in DCs. (A) BMDCs were infected with pro- or amastigotes (at indicated parasite-to-cell ratios) in the absence or presence of IFN- γ (100 ng/ml). IL-12p40 production at 24 h post-infection was measured by ELISA. Shown are the representative results from three independent experiments with similar trends. (B–E) DCs were infected with parasites (10:1) for 6 h and then stimulated with LPS (100 ng/ml), LPS plus IFN- γ (100 ng/ml) or LPS plus IFN- α (100 U/ml) for an additional 18 h. The levels of IL-12p40, IL-12p70, IL-6 and IL-10 in culture supernatants were assayed by ELISA. The representative data are shown as mean $\pm$ SD from one of at least three independent experiments with similar results. * ($P < 0.05$) and ** ($P < 0.01$) indicate statistically significant differences between control and infected groups.

Fig. 3. Intracellular IL-12p40 production and IL-12 gene expression from *L. amazonensis*-infected DCs. (A) BMDCs were either left untreated or infected with CFSE-labeled parasites (10:1) for 24 h in the absence or presence of IFN- γ or LPS/IFN- γ (see Materials and Methods). Intracellular IL-12p40 in gated CD11c⁺ DCs was measured by FACS. Shown are data from one of three independent experiments with similar results. The small-sized numbers represent the percentages of CFSE⁺ cells, and large-sized numbers represent the percentages of IL-12⁺ cells within non-infected or infected DCs in the corresponding boxes. DCs were infected for 6 h and then stimulated without (B) or with (C) LPS/IFN- γ for an additional 2 h or 18 h. The levels of IL-12p40 and IL-12p35 mRNA were determined by quantitative real-time RT-PCR. Results are shown from two independent repeats and presented as relative expression after being normalized with the 18S rRNA gene and the control group.

Fig. 4.

Suppressed STAT activation, but not p-STAT nuclear translocation, in *L. amazonensis*-infected DCs. BMDCs were infected with pro- or amastigotes for 6 h (A) or 20 h (B), and then treated with IFN- γ , LPS/IFN- γ or LPS/IFN- α for an additional 30 min. Protein levels for STAT1, STAT2 and p-STAT1, p-STAT2 were assayed. The densitometry analyses for p-STAT1, p-STAT2 and STAT2 in (A) were plotted as the percentage of suppression in infected groups relative to their corresponding controls following the same group orders with □ medium, ▨ IFN- γ , ■ LPS/IFN- γ , and ▩ LPS/IFN- α , respectively. Results are shown as mean $\pm$ SD from at least three independent repeats. (C) DCs were infected for 6 h, and then treated with IFN- γ (100 ng/ml) or IFN- α (100U/ml) for 30 min. After cell fixation, p-STAT1 and p-STAT2 (green)

were respectively stained together with CD11c marker (red) and DAPI (blue). The right lane showed nuclear translocation of p-STAT1 in Pm- or Am-infected DCs (red arrow). (D) The stained positive cells for p-STAT1 or p-STAT2 were counted in five random fields ($\times 200$). Shown are the representative results from three independent repeats. * ($P<0.05$), ** ($P<0.01$) indicate statistically significant differences between control and infection groups.

Fig. 5.

STAT2 degradation by live *L. amazonensis* amastigotes was protease- and proteasome-dependent. (A) BMDCs were infected with live or heat-killed (HK) pro- or amastigotes for 6 h, and then treated with IFN- γ for 30 min. The levels of STAT1, STAT2, p-STAT1, and p-STAT2 were assayed by immunoblot analysis. (B, C) Cell lysates were freshly prepared from DC cells or parasites without any inhibitors. DC lysates (100 μ g) were incubated with indicated amounts of parasites lysates at 37 °C for 30 min. Where indicated, pro- or amastigote lysates were pre-treated with E64 (10 μ M) or MG132 (10 μ M) on ice for 10 min. The levels of total STAT1 and STAT2 were assayed. (D) Amastigotes were pretreated with DMSO, MG132 (10 μ M), E64 (10 μ M) or bpV (10 μ M) for 1 h and then infected DCs for 6 h. After treated with LPS/IFN- γ for 30 min, DCs were used for preparing cell lysates and for detecting the levels of STAT1, STAT2, p-STAT1, and p-STAT2 by immunoblot analysis. Shown are the representative results from at least three independent repeats.

Fig. 6.

Inhibition of IRF-1 and IRF-8 expression in *L. amazonensis* amastigote-infected DCs. BMDCs were infected with pro- or amastigotes for 6 h or 20 h, and then treated with LPS/IFN- γ or LPS/IFN- α for an additional 4 h. The expression of IRF-1 and IRF-8 was determined by immunoblot analysis. The densitometry analyses were plotted as the percentage of suppression in infected group relative to their corresponding controls. Results are shown as mean $\pm$ SD from at least three independent repeats. * ($P < 0.05$) and ** ($P < 0.01$) indicate statistically significant differences between control and infection groups.

Fig. 7.

Inhibition of STAT3 and Erk1/2 phosphorylation in *L. amazonensis*-infected DCs. BMDCs were infected with pro- or amastigotes for 6 h, and then treated with IFN- γ or LPS/IFN- α for an additional 3 h (STAT3) or 30 min (ERK1/2). The levels of STAT3, ERK1/2 and phospho-proteins were assayed. Shown are the representative results from three independent repeats.

Fig. 8.

Addition of anti-IL-10 mAb did not reverse IL-12p70 suppression in *L. amazonensis*-infected DCs. BMDCs were infected with pro- or amastigotes for 6 h in the presence of neutralizing anti-IL-10 mAb or isotype control (5 μ g/ml), and then treated with LPS or LPS/IFN- γ for an additional 18 h. The levels of IL-12p70 and IL-10 in culture supernatants were measured by ELISA. The representative results are shown as mean $\pm$ SD from three independent repeats. N.D. indicated non-detectable. * ($P < 0.05$) indicate statistically significant differences between the control and IL-10 mAb-treated groups, or between the control and infected groups.