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

Lijun Xin[a], Kui Li[a,b,c], Lynn Soong[a,c,*]

[a] *Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, USA*
[b] *Sealy Center for Cancer Cell Biology, University of Texas Medical Branch, Galveston, TX, USA*
[c] *Center for Biodefense and Emerging Infectious Diseases, Sealy Center for Vaccine Development, Institute for Human Infections and Immunity, University of Texas Medical Branch, Galveston, TX, USA*

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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. *La* amastigote-infected DCs were less mature and less potent antigen-presenting cells (APC) than their promastigote-infected counterparts, as judged 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 down-regulate early innate signaling events, resulting in impaired DC function and Th1 activation.

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; 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 *Leishmania 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 *Leishmania donovani* promastigotes (Gorak et al., 1998; von Stebut et al., 1998), C57BL/6 (B6) DCs infected with *Leishmania mexicana* amastigotes show no sign of activation and no detectable levels of IL-12 and other proinflammatory cytokines (Bennett et al., 2001). Likewise, *Leishnahia 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

* Corresponding author at: Department of Microbiology and Immunology, University of Texas Medical Branch, Medical Research Building 3.132, 301 University Blvd., Galveston, TX 77555-1070, USA. Tel.: +1 409 772 8149; fax: +1 409 747 6869.
E-mail address: lysoong@utmb.edu (L. Soong).

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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 a 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 secret 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 proteosome-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, USA).

2.2. Reagents and chemicals

Recombinant mouse IFN-γ was purchased from Leinco Technologies (St. Louis, MO, USA) and CHO-derived recombinant mouse IFN-α was purchased from Cell Sciences (Canton, MA, USA). LPS from *Salmonella enterica* serovar Typhimurium and CFSE (5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 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, USA).

2.3. Antibodies

The following fluorescence-conjugated, mouse-specific mAbs were purchased from eBioscience (San Diego, CA, USA): 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, USA). Rabbit anti-mouse ERK1/2 and p-ERK1/2 mAbs were purchased from Cell Signaling Technology (Danvers, MA, USA); rabbit anti-mouse p-STAT2 mAb was obtained from Upstate Biotechnology (Charlottesville, VA, USA); mouse anti-IRF-8/ICSBP mAb was obtained from Invitrogen Life Technologies (San Francisco, CA, USA) and mouse anti-actin mAb (Sigma) was obtained from Dr. Jiaren Sun (Department of Microbiology and Immunology, UTMB, TX, USA). Purified hamster anti-mouse CD11c (N418) was purchased from eBioscience. Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjuaged anti-hamster IgG were purchased from Molecular Probes (Eugene, OR, USA), and mounting medium with DAPI was obtained from Vector Laboratories (Burlingame, CA, USA).

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 μg/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, USA) at 33 °C (Dutta et al., 2005). To label parasites, promastigotes or amastigotes, were suspended at 5×10^7 ml^{-1} in PBS containing 5 μM 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 (1×10^8 ml^{-1} in PBS) were incubated in a 60 °C water bath for 30 min. To prepare parasite lysates, parasites (2×10^8 ml^{-1} 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 1×10^6 ml^{-1} for infection. Usu-