Leishmania major Attenuates Host Immunity by Stimulating Local Indoleamine 2,3-Dioxygenase Expression

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Inflammation stimulates immunity but can create immune privilege in some settings. Here, we show that cutaneous Leishmania major infection stimulated expression of the immune regulatory enzyme indoleamine 2,3 dioxygenase (IDO) in local lymph nodes. Induced IDO attenuated the T cell stimulatory functions of dendritic cells and suppressed local T cell responses to exogenous and nominal parasite antigens. IDO ablation reduced local inflammation and parasite burdens, as did pharmacologic inhibition of IDO in mice with established infections. IDO ablation also enhanced local expression of proinflammatory cytokines and induced some CD4+ T cells to express interleukin (IL) 17. These findings showed that IDO induced by L. major infection attenuated innate and adaptive immune responses. Thus, IDO acts as a molecular switch regulating host responses, and IDO inhibitor drugs are a potential new approach to enhance host immunity to established leishmania infections.

Leishmania major is an obligate intracellular protozoan parasite that establishes itself within the phagolysosome of host phagocytic cells, primarily macrophages and dendritic cells (DCs). L. major infection in mice is a widely used model of human infection that has yielded critical insights into the immunobiology of leishmaniasis [1–3]. Indeed, the T111/T112 “helper” T cell paradigm emerged from studies on the murine L. major infection model [4]. Hence, L. major infection of B6 and BALB/c mouse strains drives predominantly T111 and T112 responses, respectively [5–7]. Although T111 responses are more effective than T112 in controlling L. major infections, parasites are not eliminated completely by B6 or BALB/c hosts [8–10]. Thus L. major parasites persist in immunocompetent hosts, and mechanisms that explain L. major persistence are poorly defined. Suffia and colleagues have reported that Foxp3+ regulatory T cells (Tregs) at L. major infection sites were parasite specific [11–13]. Tregs proliferated in response to L. major infected DCs and were restricted to sites of infection, and Treg survival depended on parasite persistence. These findings suggest that L. major parasites actively subvert host immune responses to suppress parasite-specific effector T cell responses.

DCs induced to express IDO—a potent T cell regulatory enzyme—are pivotal regulators of T cell responses and the functional status of Tregs in several physiologic settings of inflammation [14]. Thus, IDO suppresses T cell responses and activates Tregs in lymph nodes (LNs) draining sites of cutaneous melanoma growth and topical application of the tumor promoter phorbol myristate acetate [15–17]. IDO also induces and maintains potent suppressor activity of Tregs that attenuate natural and vaccine-induced tumor-specific immunity [18–20]. In these physiologic settings, rare but distinctive DC subsets expressing IDO suppressed effector T cell generation and activated Tregs to establish and...
promastigotes grew out after 7 days of incubation at 26°C. Mastigates, as described elsewhere [24–27]. Numbers of viable burdens were assessed by limiting dilution from footpad homogenates. Parasite experimental end points. Footpad lesions were measured with a direct-reading Vernier caliper (Thomas Scientific). Parasite burdens were assessed by limiting dilution from footpad homogenates, as described elsewhere [24–27]. Numbers of viable parasites were determined from the highest dilution at which promastigotes grew out after 7 days of incubation at 26°C.

**MATERIALS AND METHODS**

**Mice**

All mice were bred in a specific pathogen-free facility. IDO1-deficient (IDO1-KO) mice with BALB/c and C57BL/6 (B6) backgrounds [22] and Foxp3-GFP knock-in mice [23] were described previously. All procedures were reviewed and approved by the local Institutional Animal Care and Use Committee.

**Parasite Preparation, Footpad or Upper Thigh Inoculation, and Infection Protocol**

*L. major* clone V1 (MHOM/IL/80/Friedlin/MD) or transgenic (SP-OVA) promastigotes (from David Sacks at the National Institute of Allergy and Infectious Diseases) were grown as described elsewhere [24–27]. Infective-stage metacyclic promastigotes were isolated from stationary cultures by negative selection using peanut agglutinin (Vector Laboratories). Metacyclic promastigotes ($2 \times 10^5$) were inoculated (subcutaneously) in 1 hind footpad (or upper thigh), and mice were sacrificed and their spleens and popliteal (or inguinal) LNs were collected at experimental end points. Footpad lesions were measured with a direct-reading Vernier caliper (Thomas Scientific). Parasite burdens were assessed by limiting dilution from footpad homogenates, as described elsewhere [24–27]. Numbers of viable parasites were determined from the highest dilution at which promastigotes grew out after 7 days of incubation at 26°C.

**Reagents and Antibodies**

Details of antibodies and protocols used to detect IDO and IL-6 by immunohistochemical staining were described previously [19, 21, 22, 28, 29].

1-Methyl-Tryptophan

For in vivo treatment, mice were provided with 1-methyl-[D]-tryptophan (D-1MT; NewLink Genetics) in drinking water at a final concentration of 100 μM (2 mg/mL) with sweetener (Nutrasweet) added to enhance palatability, as described elsewhere [18].

**Adoptive Transfer**

Mice were challenged in the upper thigh (subcutaneously) with $2 \times 10^5$ *L. major* (or SP-OVA) metacyclic promastigotes. Mice were vaccinated (subcutaneously) with 5 μg of CFA-free OVA$^{237–264}$ (OTI) or OVA$^{323–339}$ (DO11.10) in the same region. Mice were injected intravenously with $15 \times 10^6$ CFSE-labeled nylon-wool enriched splenocytes from OTI or DO11.10 mice. CFSE fluorescence was analyzed by flow cytometry (FACS) to measure T cell proliferation. Timelines for infection, peptide vaccination, T cell transfers, D-1MT treatments, and experimental end points are provided in figures.

**Flow Cytometry**

Cells were stained with the following antibodies: CD4-PerCP, CD25-PE, CD8-PE (BD Biosciences-Pharmingen) and analyzed using a FACSCalibur or FACS-Canto flow cytometer (BD Biosciences). Fc binding was blocked using an anti–CD16/CD32 cocktail (BD Biosciences). Intracellular cytokines were detected by staining live draining LN (dLN) cells with antibodies (1/200–1/400) against cell surface markers, then cells were fixed and permeabilized (20 min) using Cytofix-Cytoperm (BD Biosciences) and incubated with (1/50; 4°C; 6–16 h) anti-cytokine monoclonal antibodies (mAbs) (IL-10, interferon IFN γ [BD Biosciences]; IL-17 [eBioscience]). Between 20 and $400 \times 10^3$ cells/sample were analyzed using Cell-Quest software, and experiments were replicated at least 3 times. FACS data shown are representative of individual experiments in which replicated experiments yielded comparable results.

**MLRs**

CD11c$^+$ cells enriched by magnetic activated cell separation (MACS) or single-cell suspensions were co-cultured with nylon wool–enriched BM3, OT-1, DO11.10 responder T cells, and proliferation was assessed after 72 h in a thymidine incorporation assay [28]. Wells were pulsed with 0.5 μCi tritiated thymidine ($[^3]H$)-TdR for the last 6 h. TdR incorporation was measured using the BetaPlate system (Wallac).

**Cytokine Production**

Single-cell suspensions of pooled dLNs (popliteal) were adjusted to $10^6$ cells/mL in medium (Roswell Park Memorial Institute [RPMI], 5% FCS, 2 mM L-glutamine, and antibiotics) and were incubated (in triplicate) with lysates of *L. major* promastigotes (subjected to 3 freeze/thaw cycles) or RPMI only at 37°C for 48 h, and cytokines in culture media were detected using a multiplex bead system (Luminex) according to the manufacturer’s instructions.

**Statistical Analysis**

Statistical analyses of dual comparisons were made using the unpaired Student’s *t* test. Groups within a single experiment were compared by analysis of variance. Experiments were repeated at least 3 times unless otherwise stated.

**RESULTS**

**Cutaneous L. Major Infection Induces IDO Expression in dLNs**

To test whether *L. major* infection induced IDO expression, we infected B6 mice with metacyclic promastigotes. After 2 weeks, inguinal LNs draining sites of cutaneous *L. major* infection contained large cohorts of infiltrating mononuclear cells...
expressing IDO (Figure 1A). Most FACS-sorted plasmacytoid DCs (pDCs, B220+CD11c+) from dLNs of L. major–infected mice expressed IDO, whereas very few myeloid DCs (mDCs, B220negCD11c+) expressed IDO (Figure 1A). Thus, cutaneous L. major infection induced dLN pDCs to express IDO. These findings are consistent with our previous studies on mice bearing cutaneous melanomas and mice treated with a topical tumor promoter (phorbol myristate acetate), in which skin dLN pDCs expressing IDO mediated potent T cell suppression [15–17].

L. Major Infection Inhibits T Cell Stimulatory Activity in Inflamed LNs

Because we did not have access to a flow cytometer approved for sorting live cells from L. major–infected mice, we prepared MACS-enriched (CD11c+) dLN DCs and evaluated their T cell stimulatory functions by culturing them with responder H-2Kb-specific CD8+ T cells from TCR-transgenic BM3 mice [28]. DCs from dLNs of infected B6 mice stimulated T cell proliferation, but responses were enhanced significantly (~50%) when the IDO-specific inhibitor D-1MT was added to cultures (Figure 1B). In contrast, D-1MT did not enhance robust T cell proliferation stimulated by DCs from dLNs of L. major–infected IDO1-KO mice (Figure 1B). Thus, induced IDO expression attenuated the T cell–stimulatory properties of DCs in dLNs of L. major–infected mice.

IDO Induced by L. Major Attenuates Local T Cell Responses to Exogenous Antigen

To further examine whether IDO induced by L. major infection attenuated local T cell responses, we evaluated the effects of L. major infection on in vivo T cell responses to ovalbumin peptide (SIINFEKL, OVA 257–264, 5 μg administered subcutaneously) vaccination. As depicted in Figure 2A, B6 mice were infected with metacyclic promastigotes (day 0), vaccinated with OVA-peptide to target inflamed inguinal LNs (day 10), and injected (intravenously) with MACS-enriched, CFSE-labeled, OVA-specific splenic CD8+ cells from OT-1 TCR transgenic mice (day 12). Two days later (day 14), dLN cells were stained with anti-CD8a mAbs and analyzed to detect donor OT-1 T cells (CD8a+CFSE+) and to evaluate their proliferation status by assessing CFSE staining profiles. As expected (Figure 2B), OT-1 T cells in inguinal LNs of nonvaccinated mice retained high levels of CFSE staining (96% CFSE high), and OT-1 T cells proliferated extensively in OVA-vaccinated mice leading to uniform dilution of CFSE staining (97% CFSE low). Absolute numbers of OT-1 cells in dLNs of OVA-vaccinated mice increased 4–5 fold relative to nonvaccinated mice (Figure 2C). No OT-1 proliferation was observed in contralateral inguinal LNs from OVA-vaccinated mice, confirming that OVA vaccination stimulated local T cell responses (data not shown).

In L. major–infected mice, OT-1 T cells in dLNs proliferated after OVA vaccination, but CFSE dilution was not as extensive as
in uninfected OVA-vaccinated mice (Figure 2B). Thus, 17% and 76% of OT-1 dLN T cells exhibited the lowest and intermediate CFSE dilutions, respectively, in dLNs of L. major–infected mice, whereas the corresponding proportions of dividing cells in dLNs of uninfected mice were 56% and 41%, respectively. Moreover, OVA-induced OT-1 clonal expansion in dLNs was significantly

Figure 2. Indoleamine 2,3 dioxygenase (IDO) induced by Leishmania major attenuates local T cell responses to exogenous OVA vaccination in B6 mice. A, Schematic of experimental design. B, Flow cytometric analyses of CFSE-labeled CD8+ OT-1 T cell responses to OVA peptide vaccination in inguinal LNs draining sites of L. major infection. Numbers are the percentages of divided (CFSElow) and undivided (CFSEhigh) OT-1 T cells; markers on dot plots include 96% of naive T cells in untreated mice (upper left panel). Markers on histograms subdivide divided OT-1 T cells as indicated. C, Numbers of divided and undivided OT-1 T cells in LNs of mice treated were calculated from FACS data shown in B. Data are representative of 4 experiments (see Supplemental Table 1). Ns, not significant.
IDO Induced by L. Major Attenuates Local T Cell Responses to Pathogen-Encoded Antigen

Next, we investigated whether IDO suppressed in vivo T cell responses elicited by a nominal parasite antigen encoded by an OVA-transgene incorporated into the L. major genome of the SP-OVA strain [30]. For this approach, we evaluated OVA-specific CD4\(^+\) T cell proliferation in dLNs of SP-OVA–infected BALB/c mice harboring cohorts of CFSE-labeled T cells from DO11.10 TCR transgenic mice (data not shown). Thus, L. major infection stimulated host inflammatory responses that attenuated T cell responses to exogenous antigen vaccination by inducing local IDO expression.

IDO Blocks Local Expression of Proinflammatory Cytokines during L. Major Infection

IDO blocked IL-6–dependent conversion of preformed, functionally inert Foxp3-lineage Tregs into T\(_{H17}\)–like effector T cells in physiologic settings of inflammation created by tumor growth and treatment with TLR9 ligands [16, 19]. We hypothesized that IDO induced by L. major also blocks conversion of Tregs into T\(_{H17}\) T cells in inflamed dLNs at sites of infection by inhibiting local IL-6 release. Consistent with this hypothesis, IL-6 was not detected in LNs draining sites of L. major infection in IDO-sufficient B6 mice, whereas IL-6 was detected in dLNs from L. major–infected IDO1-deficient (IDO1-KO) mice and B6 mice given D-1MT during infection (Figure 5A). To further evaluate the effects of IDO ablation on cytokine production, we prepared

IDO Promotes L. Major Infection and Associated Inflammatory Responses

To evaluate whether IDO ablation affected the course and extent of parasite infection and associated inflammatory responses to infection, we compared foot-pad swelling over time at the site of infection and parasite burdens at experimental end points in L. major–infected B6 mice, IDO1-KO mice, and B6 mice treated with IDO inhibitor (Figure 4). Infecting mice with L. major at the base of the hind leg induced footpad swelling (Figure 4A). In contrast, genetic ablation of IDO and continuous pharmacologic treatment with IDO inhibitor starting 2 days before infection (day -2) led to significant reductions in footpad swelling from day 5 after L. major infections were initiated until experimental end points at day 25 (Figure 4A). Thus, ablating IDO, which is an enzyme that mediates potent anti-inflammatory and T cell regulatory effects, paradoxically resulted in reduced local inflammatory responses to L. major infection. These outcomes implied that IDO promotes host responses to L. major infection that drive chronic local inflammation in IDO-sufficient hosts. Moreover, genetic and pharmacologic ablation of IDO during infection led to significant reductions (>60%) in parasite burdens in footpads at experimental end points (Figure 4B). Thus, IDO1-deficient hosts mounted more-effective immune responses to cutaneous L. major infection than did IDO-sufficient hosts, which led to improved control of L. major parasites and reduced local inflammation.

Next, we evaluated whether IDO inhibitor treatment affected the course of established L. major infections. For this approach, we administered oral D-1MT 14 days (day +14) after L. major infection. Foot-pad swelling (Figure 4A) and parasite burdens at experimental end points (Figure 4B) decreased significantly when mice with established L. major infections were exposed to oral D-1MT. Indeed, parasite burdens and associated local inflammatory responses were reduced to the same extents as in IDO1-deficient mice and in B6 mice treated continuously with D-1MT. Thus, D-1MT was effective in changing the course of established L. major infection.
cells from dLNs of B6 and IDO1-KO mice infected with *L. major* for 25 days, incubated them with lysates of *L. major* parasites for 48 h, and subjected culture supernatants to multiplex (Luminex) analysis to detect cytokines. IDO ablation led to significant increases in IL-17 and decreases in IL-10 production by dLN cells (Figure 5B). In contrast, production of IFN-γ, a classic T H1 cytokine, by dLN cells was not changed significantly when IDO was ablated. Consistent with these findings, D-1MT treatment led to significant reductions in the proportions of dLN CD4+ cells expressing IL-10 but had little effect on the proportions expressing IFN-γ (Figure 5C).

Additional evidence that IDO blocked local proinflammatory cytokine production emerged from flow cytometric analyses that were performed to detect cells expressing intracellular cytokines. No IL-17–expressing cells were detected in dLNs from B6 mice 14 days after *L. major* infection (Figure 6). In contrast, small cohorts of CD4+ T cells and CD4neg cells (3%–5%) expressed IL-17 in dLNs from *L. major*–infected B6 mice treated

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**Figure 3.** Indoleamine 2,3 dioxygenase (IDO) attenuates local T cell responses to nominal parasite antigens in BALB/c mice. A, Schematic of experimental design. B, Flow cytometric analyses of CFSE-labeled CD4+ DO11.10 T cell responses were performed as in Figure 2. Panels on the right show CD44 expression profiles on donor (CFSE+) DO11.10 T cells. Data are representative of 3 experiments (see Supplemental Table 1).
expression completely and prevented Tregs from converting into TH17-like T cells in dLNs following L. major infection.

**DISCUSSION**

In this study, we show that IDO induced locally during cutaneous L. major infection is a critical factor facilitating the standard course of infection by attenuating host proinflammatory and immune responses. Thus, IDO enhanced parasite burdens and promoted chronic inflammation, which is a hallmark of cutaneous L. major infection. Moreover, administering IDO inhibitor to mice with established infections reduced parasite burdens and associated inflammatory responses, suggesting that D-1MT treatment may offer a novel therapeutic approach for patients with chronic leishmaniasis.

The pattern and morphology of LN cells induced to express IDO following L. major infection is remarkably similar to the effects of local melanoma growth and topical application of the tumor promoter phorbol myristate acetate on IDO expression in LNs [15, 17]. In these settings, IDO-expressing B220+ DCs in dLNs mediated T cell suppression and activated LN Tregs. Recently, we reported that closely related “IDO-competent” CD19+ DCs in spleen were B-lymphoid cells that displayed phenotypic features of B cells and DCs [31]. L. major infection also induced B220+ dLN DCs to express IDO and to attenuate the T cell stimulatory properties of dLN DCs. IDO induced by L. major infection also attenuated local CD8+ and CD4+ T cell responses to exogenous (OVA) vaccination. Induced IDO activity also blocked CD8+ T cell responses to OVA expressed as a nominal (transgenic) parasite antigen, suggesting that IDO creates local immune privilege that prevents T cells from responding to parasite antigens.

IDO ablation led to increased IL-6 and IL-17 and decreased IL-10 production that correlated with reduced local inflammation and lower parasite burdens. In contrast, IDO ablation had little effect on IFN-γ production by CD4+ T cells, which is the hallmark of Th1 responses that predominate in L. major–infected B6 mice. IL-27 ablation also led to increased numbers of IL17+ CD4+ T cells, but L. major–infected IL-27–deficient mice developed more-severe lesions [32]. In L. major–susceptible BALB/c mice, in which Th1 responses predominate, IL-17 ablation resulted in smaller parasite lesions that failed to progress [33]. These contradictory indications for the role of IL-17 attest to the underlying complexity of host innate and adaptive immune responses to L. major infection. Conflicting roles for IDO in pathogenic disease progression and persistence have also been reported [34]. IDO may limit pathogen growth but fail to promote sterile clearance, and some pathogens may have evolved strategies to evade the antimicrobial effects of IDO, allowing them to benefit from the regulatory effects of IDO on host immunity. L. major infection promotes Treg differentiation and stabilizes Treg suppression with oral D-1MT and IDO1-KO mice. Most (>80%) of CD4+ cells expressing IL-17 in infected IDO-deficient mice did not co-express CD25 (Figure 6). However, most IL-17-expressing CD4+ dLN cells from D-1MT–treated, L. major–infected Foxp3-GFP knock-in mice were Foxp3-lineage (GFP+) cells (Figure 6). Thus, IDO blocked IL-17

![Figure 4. Indoleamine 2,3 dioxygenase (IDO) ablation reduces parasite burdens in Leishmania major infected B6 mice. A, Mean change in foot pad swelling due to local L. major infection over time (5 mice/group). Significant reductions (P<.001) in footpad swelling relative to control B6 mice are indicated by asterisks. B, Parasite burdens were assessed at experimental end points (day 25) by performing limiting dilution assays to estimate parasite numbers in footpads. Numbers of living parasites in the footpads of individual mice (5 mice/group) and the mean values for each group (horizontal bars) are shown. Asterisks denote significant reductions in parasite burdens due to IDO ablation relative to L. major–infected B6 control mice (P < .001). Data are representative of ≥3 experiments.](https://academic.oup.com/jid/article-abstract/203/5/715/894477)
and DCs expressing IDO activate Tregs in tumor-associated LN and in mice treated with TLR9 ligands to induce IDO [16, 19]. Thus, IDO may promote Treg differentiation and stabilize Treg suppression to attenuate host immune effector functions directed against L. major parasites to promote host resistance to L. major.

Figure 5. Indoleamine 2,3 dioxygenase (IDO) regulates proinflammatory cytokine production at sites of Leishmania major infection. A, B6, IDO1-deficient (IDO1-KO) mice and B6 mice given oral 1-methyl-[D]-tryptophan (D-1MT) were infected with L. major. After 14 days, draining lymph nodes (dLNs) were sectioned and stained to detect interleukin (IL) 6 (original magnification, ×400). Data are representative of 3 experiments. B, Cells from dLNs of L. major infected B6 (closed bars) and IDO1-KO (open bars) mice (5 mice/group, day 25) were incubated for 48 h in the presence (−) or absence (+) of L. major antigens as indicated. Cytokines in cultures were detected using a Luminex multiplex analyzer. Asterisks indicate significant changes in cytokine production (P < 0.01, by Bonferroni test). B6 mice (+/- oral D-1MT) were infected with L. major, and after 14 days, dLN cells were stained with anti-CD4 mAb before fixation to detect intracellular IL-10 and interferon γ. Percentages are the proportion of CD4+ cells expressing cytokines. Data are representative of 2 experiments.
From a therapeutic perspective, a key finding was that IDO inhibitor treatment reduced parasite burdens when administered to mice with established L. major infections, revealing a constitutive requirement for IDO to maintain local conditions that favor parasite persistence and pathogenesis and suggesting that D-1MT treatment may help reduce parasite burdens in patients with leishmaniasis. Bourreau et al [36] reported that Foxp3 and IDO levels were correlated in infectious lesions from patients with acute and chronic cutaneous leishmaniasis, providing additional support for the notion that IDO promotes Treg-suppressive activity in patients.

L. major may also induce other cell types to express IDO. Macrophages, epithelial cells, and tumor cells express IDO at sites of infection and tumor growth [37–40]. Diametrically opposed roles have been proposed for IDO during infection. Early studies posited that IDO mediates host defense. In this model, IDO limits the spread of infectious pathogens by inhibiting proliferation of infected cells, such as macrophages [41]. However, the discovery that IDO suppressed T cell immunity raised the possibility of an alternative paradigm, that IDO attenuated host adaptive immunity by establishing immune privilege at sites of infection [14, 42]. Findings from the current study support a counter-regulatory role for IDO that benefits the pathogen, not the host. Although IDO may also contribute to host defense by constraining the initial infection, the finding that parasite burdens were significantly lower when IDO was ablated argues against a prominent role for IDO in host defense during cutaneous L. major infection.

Figure 6. Indoleamine 2,3 dioxygenase (IDO) ablation during Leishmania major infection leads to interleukin (IL) 17 expression in draining lymph nodes (dLNs). B6 and Foxp3-GFP mice (+/- oral 1-methyl-[D]-tryptophan [D-1MT] from day -2) and IDO1-deficient (IDO1-KO) mice were infected with L. major, and 14 days later, dLN cells were stained with anti CD4, anti-CD25 mAbs, and (after fixation) anti-IL-17 mAb. Dot plots show total dLN cells (upper panels) or gated CD4+ dLN cells (center and lower panels). Proportions of IL-17+ cells among total cells and gated CD4+ and GFP+ cells are indicated (upper, center, and lower panels respectively). Data are representative of 4 experiments (see Supplemental Table 1).
In summary, IDO plays a critical role in the host-pathogen interactions that drive chronic inflammation and persistent infection in L. major–infected mice, and IDO inhibitors currently undergoing phase I clinical trials in patients with cancer offer a potential new strategy to treat patients with leishmaniasis.

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**References**


