Parasite-Derived Arginase Influences Secondary Anti-\textit{Leishmania} Immunity by Regulating Programmed Cell Death-1–Mediated CD4+ T Cell Exhaustion

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The breakdown of L-arginine to ornithine and urea by host arginase supports \textit{Leishmania} proliferation in macrophages. Studies using arginase-null mutants show that \textit{Leishmania}-derived arginase plays an important role in disease pathogenesis. We investigated the role of parasite-derived arginase in secondary (memory) anti-\textit{Leishmania} immunity in the resistant C57BL/6 mice. We found that C57BL/6 mice infected with arginase-deficient (arg^−) \textit{L. major} failed to completely resolve their lesion and maintained chronic pathology after 16 wk, a time when the lesion induced by wild-type \textit{L. major} is completely resolved. This chronic disease was associated with impaired Ag-specific proliferation and IFN-γ production, a concomitant increase in programmed cell death-1 (PD-1) expression on CD4+ T cells, and failure to induce protection against secondary \textit{L. major} challenge. Treatment with anti-PD-1 mAb restored T cell proliferation and IFN-γ production in vitro and led to complete resolution of chronic lesion in arg^− \textit{L. major}–infected mice. These results show that infection with arg^− \textit{L. major} results in chronic disease due in part to PD-1–mediated clonal exhaustion of T cells, suggesting that parasite-derived arginase contributes to the overall quality of the host immune response and subsequent disease outcome in \textit{L. major}–infected mice. They also indicate that persistent parasites alone do not regulate the quality of secondary anti-\textit{Leishmania} immunity in mice and that the quality of the primary immune response may be playing a hitherto unrecognized dominant role in this process.

C utaneous leishmaniasis causes disfiguring lesions that often develop as a result of secondary bacterial infections. In majority of cases, primary infections are generally limited to localized cutaneous lesions that heal spontaneously after several months, resulting in life-long immunity (1–3). This infection-induced immunity is dependent on a small number of persistent parasites that act as a source of continuous Ag stimulation to the host immune cells, leading to maintenance of effector-like memory cells (1, 2, 4). Infection-induced immunity is lost if infected mice are manipulated to completely clear their parasites, such as seen during an exclusive Th1 response to low-dose infection or following anti–IL-10R Ab treatment (5, 6). In addition, recrudescence (relapse) of disease can occur when the host’s immune system becomes compromised (4, 7, 8). Thus, whereas the contribution of the host immune response to parasite persistence and maintenance of secondary immunity is known, little is known about the role played by parasite-derived factors in this process.

The outcome of infection with \textit{Leishmania major} depends in part on the activation status of infected host macrophages (9–12). In infected animals, IFN-γ produced by CD4+ Th1 cells classically activates macrophages, leading to increased expression of inducible NO synthase (iNOS) (13–15). This enzyme acts on its substrate L-arginine to produce NO that is essential for parasite clearance (2, 14). In contrast, alternative activation of macrophages that favors parasite proliferation in infected cells. Alternative macrophage activation is accompanied by increased expression of arginase, which catalyzes the formation of ornithine from arginine, leading to polyamine synthesis (10, 11, 14, 16, 17). iNOS and arginase are reciprocally regulated (13–15, 18), and the two enzymes compete directly for their common substrate, L-arginine, and indirectly because some of their intermediate products inhibit each other at several metabolic points (14, 16, 19, 20). Additionally, arginine catabolism may lead to metabolic stresses also leading to shifts in the immune response (21).

\textit{Leishmania} also express an arginase enzyme (22, 23) related to the mammalian arginases 1 and 2 (23). Importantly, parasite-derived arginase is not stage specific, as expression has been detected in both amastigotes and promastigotes at similar levels (23). It has been proposed that parasite-derived arginase is a virulence factor, which may act to deprive iNOS of L-arginine availability, thereby limiting host NO production (24). Indeed, the proliferation and survival of \textit{Leishmania} arginase-deficient mutants is completely dependent on exogenous polyamine supplementation in vitro (22). In the susceptible BALB/c mice infected with arginase gene–deficient (arg^−) \textit{L. major}, lesion pathology and parasite burden were significantly reduced compared with those infected with wild-type (WT) parasites. Interestingly,

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Abbreviations used in this article: arg^−, arginase gene–deficient; BMDC, bone marrow–derived dendritic cell; DC, dendritic cell; dLN, draining lymph node; DTH, delayed-type hypersensitivity; iNOS, inducible NO synthase; PD-1, programmed cell death-1; SLA, soluble leishmanial Ag; Tcm, central memory–like T cell; Tem, effecter memory–like T cell; WT, wild-type.

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there was no significant difference in the production of NO by macrophages infected with arg and WT L. major (25), suggesting that parasite-derived arginase does not limit host NO production but enhances the establishment of a favorable environment for parasite survival and proliferation through increased polyamine synthesis. Previously, the contribution of arginase in the pathogenesis of cutaneous leishmaniasis has been investigated by using pharmacologic inhibitors (9–11). Because mammalian and Leishmania arginase show considerable homology (23), such approach does not permit the understanding of the specific role of parasite-derived arginase in disease pathogenesis. The availability of arginase mutant parasites provides an excellent resource for evaluating the relative contributions of parasite-derived arginase in a way not feasible by pharmacological means.

In addition to directly inhibiting NO production, thereby enhancing parasite proliferation, excessive host arginase activity contributes to nonhealing disease by causing suppression of T cell proliferation and effector cytokine response (26). This is consistent with the observation showing that host arginase 1 impairs T cell responses by depleting the bioavailability of L-arginine, a key amino acid critical for optimal cell division (21, 27). Indeed, deprivation of L-arginine has been associated with impaired T cell response observed in many pathological conditions, including asthma (28), psoriasis (29), and tuberculosis (30). However, a recent report found that in vivo inhibition of arginase activity has no effect on skin allograft rejection or systemic T cell proliferation (31).

Recent reports suggest that T cell exhaustion, which is characterized by the presence of Ag-specific T cells exhibiting poor effector functions including proliferation and cytokine responses (32), is a hallmark of many protozoan diseases including malaria (33), toxoplasmosis (34, 35), and leishmaniasis (36, 37). In a murine model of visceral leishmaniasis, CD8+ T cell exhaustion due to high programmed cell death-1 (PD-1) expression was shown to be responsible for severe disease outcome (37). Similarly, decreased CD8 T cell response and loss of effector cytokine production (including IFN-γ, TNF-α, and IL-2) was associated with the development of diffuse cutaneous leishmaniasis in patients infected with L. mexicana (38). Although T cell exhaustion has been mostly described for CD8+ T cells in leishmaniasis, no report has demonstrated CD4+ T cell exhaustion in this disease, although dysfunctional CD4+ T cell compartment has been also observed in other chronic infections (32).

In this report, we investigated the influence of parasite-derived arginase on host T cell responses in vivo. Surprisingly, we found that infection with L. major causes chronic infection in C57BL/6 mice and fails to protect infected mice against virulent challenge. This inability of L. major to induce protection was related to clonal exhaustion of CD4+ T cells resulting in impaired Ag-specific proliferation and IFN-γ production by T cells from arg−/−L. major−infected mice. Thus, contrary to the effects of host arginase, our findings show that parasite-derived arginase plays a critical role in enhancing the host immune response to the parasite, and its absence results in impaired CD4+ T cell response, leading to chronic disease.

Materials and Methods
Reagents

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and all Abs are from eBioscience (San Diego, CA). Media, penicillin/streptomycin, FBS, and glutamine were purchased from Invitrogen (Carlsbad, CA).

Mice

Six- to 8-wk-old female C57BL/6 mice were purchased from Charles River Laboratories (Montreal, Canada) or Central Animal Care Services, University of Manitoba. Female Thy1.1 C57BL/6 mice (6 to 8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mouse experiments were approved by the University of Manitoba Animal Care Committee in accordance with the regulation of the Canadian Council on Animal Care.

Parasites and infection

All parasites were derived from the WT Leishmania major line L3V3c15 (RHO/SS/U9/P). The generation and biochemical characteristics of homozygous null mutants lacking arginine, Δarg−/−HYG/Δarg−/PAC, referred to arg−/−HYG/Δarg−/PAC (referred to arg−/−ARG, are described elsewhere (39). Parasites were grown at 25 °C in M199 medium (HyClone, Logan, UT) supplemented with 10% heat-inactivated FBS, 2 μg/ml bopetin, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml hemin, 1 μg/ml biotin, 0.1 mM adenine, 40 mM HEPES (pH 7.4), 20 mM t-glutamine, and 50 mM putrescine (for arg−/−L. major). For infection, stationary-phase promastigotes were washed three times in PBS, and 2 million parasites (suspended in 50 μl PBS) were injected into the right hind footpad. Postinfection, the development and progression of footpad lesions was monitored weekly by measuring the diameter of infected footpad with calipers. Uninfected contralateral footpads served as controls. In some experiments, mice were challenged in the contralateral footpad with 2 million parasites 14 wk after the primary infection, and footpad thickness was measured in the challenged footpad 5 d later to quantify delayed-type hypersensitivity (DTH) response.

Generation and infection of bone marrow–derived dendritic cells

Bone marrow–derived dendritic cells (BMDCs) were differentiated from the femur and tibia of 6- to 8-wk-old C57BL/6 mice. Briefly, marrow cells were prepared, depleted of erythrocytes with ACK lysis buffer (150 mM NH4Cl, 10 mM KHCO3, and 0.1 mM Na2EDTA [pH 7.2–7.4]), seeded in 100 × 15-mm Petri dishes (catalog number 351029; BD Falcon) at 2 × 107/ml, and incubated at 37 °C in CO2 incubator. The cells were differentiated into immature dendritic cells (DCs) with recombinant murine GM-CSF (20 ng/ml; PeproTech, Indianapolis, IN). The culture media were changed twice on days 3 and 6, and on day 7, the nonadherent cells (DCs) were collected for in vitro experiments. Immature DCs expressed CD11c (>98%), and high levels of CD40, CD80, CD86, and MHC class II as assessed by flow cytometry. For infection, BMDCs were incubated with WT parasites for 5 h at a BMDC/parasite ratio of 1:10. The free parasites were washed away (three times washing with complete media), and infected cells were cultured at 37 °C for 24 h.

In vitro coculture experiments with BMDCs and T cells from infected mice

T cells were purified from the spleens or draining lymph nodes (dLNs) of infected or naive mice by positive selection using CD90.2-coated microbeads and autoMACS separator system according to the manufacturer’s suggested protocols (Miltenyi Biotec, Auburn, CA). Purified (>98%) T cells were labeled with CFSE dye and cocultured with L. major−infected BMDCs for 5 d, and cell proliferation and intracellular IFN-γ production were assessed by flow cytometry.

Direct ex vivo and in vitro recall responses and intracellular cytokine staining

At various times postinfection, spleens and dLNs were harvested and made into single-cell suspensions. The cells were directly stimulated with PMA (50 ng/ml), ionomycin (500 ng/ml), and BFA (10 μg/ml) (all from Sigma-Aldrich) for 4–6 h and stained intracellularly for IFN-γ (as below). The remaining cells were resuspended at 4 × 106 cells/ml in complete medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin), plated at 1 ml/well in 24-well tissue-culture plates (Falcon), and stimulated with soluble leishmanial Ag (SLA; 50 μg/ml). After 72 h, the culture supernatants were collected and stored at −20°C until assayed for cytokines by ELISA. Some cells were used for intracellular cytokine staining as previously described (40). Briefly, cells were stimulated with PMA, ionomycin, and brefeldin A for 4–6 h, washed, fixed with 2% paraformaldehyde for 10 min, and then permeabilized with 0.1% saponin (Sigma-Aldrich) in staining buffer. The Journal of Immunology 3381 at Washington University School of Medicine Library on August 16, 2013 http://www.jimmunol.org/Downloaded from
**In vitro proliferation assays**

To measure proliferation, single-cell suspension of spleen or lymph node cells from WT or arg−/− L. major–infected mice were labeled with CFSE (Molecular Probes, Eugene, OR) as previously described (41). Labeled cells were resuspended at 10^6/ml, plated onto 96-well round-bottom plates (200 μl/well), and stimulated with or without SLA (50 μg/ml), anti-CD4 mAb (10 μg/ml), or anti-CD3 and anti-CD28 (1 μg/ml). In some experiments, CFSE-labeled cells were cocultured with infected BMDCs at a 100:1 (cells to infected BMDC) ratio. After 5 d, proliferation was analyzed by flow cytometry.

**Adaptive transfer studies**

Infected CD90.2+ mice (Thy1.2) were sacrificed at least 14 wk after primary infection, and single-cell suspensions were made of the spleens and dLNs. Forty million cells in 100 μl PBS were injected into the tail vein of naive CD90.1+ (Thy1.1) recipient mice. Cells from naive age-matched mice were used controls. In some experiments, CD90.2+ T cells were purified by positive selection using kits and reagents from Miltenyi Biotec according to the manufacturer’s suggested protocol. CD90.2-enriched cells were >98% CD3+ as assessed by flow cytometry (data not shown). To monitor proliferation in vivo, donor cells were sometimes labeled with CFSE dye prior to transfer. Mice were infected as described above after cell transfer.

**Treatment with anti–PD-1 mAb in vivo**

To investigate the role of PD1 in lesion chronicity, arg−/− L. major–infected mice were injected weekly (starting at week 14 postinfection) with rat anti-mouse PD-1 mAb (1 mg/mouse i.p.; BioXCell, West Lebanon, NH). Lesion size was monitored weekly for an additional 8 wk.

**Estimation of parasite burden**

At various times postinfection or challenge, mice were sacrificed, and parasite burden in the footpads of infected mice was quantified by limiting dilution analysis as previously described (42, 43). If the footpad was from mice infected with arg−/− L. major, the media was also supplemented with 50 mM putrescine.

**Cytokine ELISAs**

The levels of IL-4, IL-10, and IFN-γ in dLN or spleen cell-culture supernatant fluids were determined by sandwich ELISA using Ab pairs (BD Biosciences, San Jose, CA) according to the manufacturer’s suggested protocols. The sensitivities of the ELISA were 31.25 pg/ml (IL-4) and 15.65 pg/ml for both IL-10 and IFN-γ.

**Arginase activity assay**

Arginase activity in vitro was quantified as previously described by Corraliza et al. (44). Briefly, homogenate of infected feet was first centrifuged at low speed (500 rpm for 5 min) to remove large tissue debris, and the supernatant was then centrifuged again at high speed (3000 rpm for 15 min) to pellet the cells and amastigotes. Both pellets were incubated for 30 min in 2 ml lysis buffer (0.1 M Tris-HCl, 300 μM NaCl, 1 μM PMSF, and 1% Triton X), and the lysate was assayed for arginase as described by Miltenyi Biotec (44). Briefly, homogenate of infected feet was first centrifuged at low speed (500 rpm for 5 min) to remove large tissue debris, and the supernatant was then centrifuged again at high speed (3000 rpm for 15 min) to pellet the cells and amastigotes. Both pellets were incubated for 30 min in 2 ml lysis buffer (0.1 M Tris-HCl, 300 μM NaCl, 1 μM PMSF, and 1% Triton X), and the lysate was assayed for arginase as described previously (44).

**Statistical analysis**

A two-tailed Student t test or ANOVA was used to compare means of lesion sizes, parasite burden, and cytokine production from different groups of mice. Significance was considered if p < 0.05.

**Results**

**Infection of C57BL/6 mice with arg−/− L. major results in chronic infection**

We previously showed that arg−/− L. major are impaired in their macrophage infectivity in vitro and their growth could be rescued by l-ornithine, agmatine, putrescine, or spermidine supplementation (39). In addition, their in vivo infectivity is also highly compromised because they fail to induce progressive disease in the susceptible BALB/c mice (25). In this study, we characterized the outcome of primary infection of C57BL/6 mice with arg−/− L. major. We used C57BL/6 mice because this strain heals WT L. major infection and generates life-long immunity, akin to most infected humans. C57BL/6 mice infected with WT L. major developed progressive lesions starting from 2 wk postinfection, which was associated with increased parasite burden (Fig. 1A, 1B). Lesions in arg−/− L. major–infected animals were slow to develop but surprisingly, although never reaching the severity seen in WT infection, remained stable until the termination of the experiment (>16 wk postinfection; Fig. 1A). Consistent with lesion sizes, parasite burden was significantly (p < 0.05) lower in arg−/− L. major–infected than WT-infected mice at 2, 4, and 8 wk postinfection (Fig. 1B). Interestingly, by 16 wk postinfection, both WT and arg−/− L. major–infected mice had comparable parasite burden despite the latter having slightly (but not significantly) bigger lesion size (Fig. 1A, 1B). The phenotypes seen were related strictly to loss of arg−/− alone rather than the process of genetic manipulation, because as previously shown in BALB/c mice (25), complementation of arg−/− L. major with ARG gene (arg+/+ARG; Add Back) fully restored the virulence of the parasites in C57BL/6 mice (Fig. 1A, 1B).

**Kinetics of immune response in C57BL/6 mice infected with arg−/− L. major**

We next compared the immune response in C57BL/6 mice following infection with WT and arg−/− L. major. At 1 and 2 wk postinfection, cells from dLNs were labeled with CFSE dye, stimulated with SLA for 5 d, and proliferation and intracellular cytokine (IFN-γ) production were assessed by flow cytometry. In some cases, the cell-culture supernatant fluids were also assayed for IL-4 and IFN-γ by ELISA. At 1 wk postinfection, there was no difference in proliferation and frequency of IFN-γ–producing CD4+ T cells (Fig. 2A, 2B, Supplemental Fig. 1A) in mice infected with WT and arg−/− L. major, and IL-4–producing cells were undetectable (data not shown). In contrast, Ag-specific proliferation and frequency of IFN-γ–producing CD4+ and CD8+ T cells were significantly (p < 0.05) lower in arg−/− L. major–infected mice at 2 wk postinfection (Fig. 2C, 2D, Supplemental Fig. 1B), which is consistent with the significantly lower parasite burden in these mice at this time point (Fig. 1B). The impaired proliferation and IFN-γ production by T cells from arg−/− L. major–infected mice at 2 wk was not related to reduction in the total numbers of CD4+ and CD8+ cells in their spleens or dLNs (data not shown).

At 4 and 8 wk postinfection, we found no significant difference in IL-4 production by ELISA (Fig. 3A), whereas the production of IFN-γ was still significantly (p < 0.05) lower in arg−/− L. major–infected than in WT-infected mice (Fig. 3B). Interestingly, despite having less parasite burden, the production of IL-10 was significantly (p < 0.05) higher in arg−/− L. major–infected than in WT-infected mice at 8 wk postinfection (Fig. 3C). In addition, despite the higher production of IFN-γ, the levels of arginase activity in the footpads (lesion) of mice infected with WT parasites was higher at 4 and 8 wk postinfection than in those mice infected with arg−/− L. major (Fig. 3D). Thus, unlike our findings in BALB/c mice in which there were no differences in cytokine responses (25), the production of IFN-γ by splenic and dLN cells from C57BL/6 mice infected with arg−/− L. major was significantly impaired later during infection despite the absence of progressive lesion development, parasite proliferation, and higher arginase production.

**In vitro recall immune response in mice infected with arg−/− L. major**

The preceding results show that the primary immune response to arg−/− L. major is significantly altered in comparison with that of WT L. major. Not only did T cells from arg−/− L. major–infected mice show marked reduction in Ag-specific proliferation, but also their ability to produce IFN-γ production was reduced.
Therefore, we examined the quality of recall response in mice infected with \( \text{arg}^2 \) \( \text{L. major} \) to assess whether their ability to develop infection-induced immunity may also be altered. At 16 wk postinfection, Ag-specific proliferation of total CD3+ (data not shown) and CD4+ (Fig. 4A) T cells from the dLNs and spleens of \( \text{arg}^2 \) \( \text{L. major} \)-infected mice were significantly \((p < 0.05)\) lower than those from WT-infected mice. In addition, the percentage of proliferating CD4+ T cells that express IFN-\( \gamma \) was also significantly \((p < 0.05)\) lower in dLNs and spleens from \( \text{arg}^2 \) \( \text{L. major} \)-infected mice than those from WT \( \text{L. major} \)-infected mice (Fig. 4B). Consistent with the flow data, cells from \( \text{arg}^2 \) \( \text{L. major} \)-infected mice produced significantly \((p < 0.01)\) less IFN-\( \gamma \) in culture supernatant fluids than cells from WT \( \text{L. major} \)-infected mice (Fig. 4C). In contrast, cells from \( \text{arg}^2 \) \( \text{L. major} \)-infected mice produced significantly \((p < 0.05)\) more IL-10 than those from WT \( \text{L. major} \)-infected mice (Fig. 4D). Interestingly, coculture of WT \( \text{L. major} \)-infected BMDCs with purified T cells from \( \text{arg}^2 \) \( \text{L. major} \)-infected mice did not restore their proliferation and IFN-\( \gamma \) production (Fig. 4E, 4F), suggesting that the impaired in vitro recall responses of cells from \( \text{arg}^2 \) \( \text{L. major} \)-infected mice is T cell intrinsic and not due to defects in APCs.

**Impaired in vivo recall immune response in mice infected with \( \text{arg}^2 \) \( \text{L. major} \)**

To further rule out the effect of differences in APCs in the impaired recall response in mice infected with \( \text{arg}^2 \) \( \text{L. major} \), we...
Adoptively transferred highly purified T cells from Thy1.2 (CD90.2) mice infected with WT or arg L. major into naive congenic (Thy1.1, CD90.1) recipients and monitored the proliferative behavior of the donor (Thy1.2+) T cells following L. major challenge.

Five days after challenge, the proliferation of donor CD3+ (Fig. 5A) or CD4+ (Fig. 5C) T cells from arg L. major-infected mice was significantly ($p < 0.05$) lower than those from WT-infected mice. This was true whether donor cells were recovered from dLNs or spleens (data not shown) of recipient mice. Furthermore, the frequency of IFN-$\gamma$-producing cells within the proliferating donor CD3+ or CD4+ T cells in mice that received cells from WT L. major--infected mice was significantly greater than those from arg L. major--infected mice (Fig. 5B, 5D, left panels). In contrast, the frequency of IFN-$\gamma$-negative cells within the proliferating donor cells (i.e., CFSElowIFN-$\gamma$+) in recipient mice was comparable, suggesting that only the IFN-$\gamma$--producing effector cells are affected (Fig. 5B, 5D, right panels). Thus, similar to in vitro recall response, proliferation and IFN-$\gamma$ production by cells from arg L. major--infected mice are impaired in vivo following secondary encounter with Leishmania Ag.

Quantitative differences in memory T cells in mice infected with WT and arg L. major

To determine whether there are quantitative differences in numbers of memory T cells generated in mice infected with WT and arg parasites, we collected spleens and dLNs at 16 wk postinfection and assessed the expression of CD62L and CD44 on CD3+ T cells directly ex vivo by flow cytometry. CD44 is a surface protein that is important for lymphocyte extravasation into inflammatory sites, and its upregulation is a marker of previous T cell activation and hence is expressed by all memory T cells (45, 46). CD62L is a lymph node–homing receptor for lymphocytes, which allows them to enter the high endothelial venules, and its downregulation is a prerequisite for effector T cells to exit the lymph nodes into tissues to mediate effector functions. By using these markers, we could discriminate between central memory–like T cells (Tcm; CD44hiCD62Lhi) and effectors and/or effector memory–like T cells (Tem; CD44hiCD62Llo). Both WT and arg L. major--

**FIGURE 3.** Cytokine and arginase responses in mice infected with arg and WT L. major. At various times postinfection, infected mice were sacrificed, and single-cell suspensions of dLNs were cultured with SLA (50 µg/ml), and, after 72 h, the culture supernatant fluids were collected and assayed for IL-4 (A), IFN-$\gamma$ (B), and IL-10 (C) by ELISA. Also, the infected footpads were homogenized in Triton X, and the total arginase activity was determined as described in the Materials and Methods (D). Data are presented as means ± SE and representative of three independent experiments (n = 3 to 4 mice) with similar results. *$p < 0.05$, **$p < 0.01$. KO, Knockout.

**FIGURE 4.** Impaired in vitro recall response in arg L. major--infected mice. Sixteen weeks postinfection, dLN cells from WT and arg L. major--infected mice were labeled with CFSE dye and restimulated in vitro with SLA (A–D), infected or uninfected BMDCs (E, F), or plate-bound anti-CD3/anti-CD28 mAb (as positive controls) for 5 d as described in Materials and Methods. CD4+ T cell proliferation (A) and the frequency of proliferating and IFN-$\gamma$--producing CD4+ (CFSElowIFN-$\gamma$+; B, F) and CD3+ (E) were determined by flow cytometry. In addition, the production of IFN-$\gamma$ (C) and IL-10 (D) in culture supernatant fluids was determined by sandwich ELISA. Data presented are representative of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$. KO, Knockout.
infected mice had higher Tcm- and Tem-like populations than naive uninfected mice (data not shown). Interestingly, there was no significant difference in the percentage of Tcm-like population between WT and arg−infected mice. However, the percentage of Tem-like cells in arg−infected mice was significantly (p < 0.05) lower than in WT-infected mice (Supplemental Fig. 2A–C).

**Infection with arg− L. major does not protect against secondary virulent challenge**

The preceding results suggest that infection with arg− L. major may not induce the same level of protection as WT L. major infection. To test this, we challenged mice infected with WT and arg− L. major at 16 wk postinfection with virulent parasites in the contralateral footpad and, at 3 d after challenge, assessed footpad thickness for DTH response. As shown in Fig. 6A, mice that healed from WT infections mounted strong DTH response following challenge infection that was significantly (p < 0.01) higher than infected naive mice. In contrast, DTH response in mice infected with arg− L. major was significantly (p < 0.01) lower than that seen in mice that healed their WT L. major infection. Consistent with this low DTH response, arg− L. major–infected mice contain significantly (p < 0.01) higher parasite burden than their WT–L. major–infected counterpart mice at 3 wk postchallenge (Fig. 6B). Thus, the resulting chronic and persistent infection generated in C57BL/6 mice by arg− L. major is not sufficient to stimulate protective immunity upon secondary rechallenge infections.

To more carefully investigate the quality of secondary immunity in mice infected with arg− L. major and to exclude possible defects in non–T cell compartment, we adoptively transferred highly purified CD3+ T cells from spleens of naive or mice infected with WT and arg− L. major for 16 wk into naive C57BL/6 mice and challenged them with virulent L. major. The lesion size in mice that received cells from WT-infected mice was significantly (p < 0.05) lower than those that received cells from arg− L. major–infected or naive mice (Fig. 6C). Furthermore, parasite burden in mice that received cells from arg− L. major–infected or naive mice was >100-fold higher than those that received cells from WT L. major–infected mice (Fig. 6D). These results suggest that the inability of arg− L. major–infected mice to resist secondary virulent L. major challenge may be related to defects and/or impaired generation of cells that mediate infection-induced immunity (memory T cells) following primary infection.

**FIGURE 5.** In vivo recall response in arg− L. major–infected mice is impaired. Naïve Thy1.1 (CD90.1) mice received (by i.v. injection) CFSE-labeled, highly purified (>98% pure) splenic T cells (purified by autoMACS) from uninfected (naive) or WT and arg− L. major–infected (>16 wk) Thy1.2 (CD90.2) mice and challenged with 2 × 10^6 WT L. major the next day. At 7 d postchallenge, dLNs cells were stained for CD3 (A, B) and CD4 (C, D) and directly assessed for proliferation and IFN-γ production by flow cytometry after gating on CD90.2+ donor population. Bar charts (B, D) represent the percentages of proliferating cells that are also positive (CFSEloIFN-γ+, left panels) or negative (CFSEhiIFN-γ−, right panels) for IFN-γ. Data presented are representative of two independent experiments (n = 4 to 5 mice/group) with similar results. *p < 0.05. KO, Knockout.

**FIGURE 6.** Infection with arg− L. major does not protect against virulent L. major challenge. Uninfected (naive) and C57BL/6 mice infected with WT or arg− L. major (>16 wk) were challenge with 5 million WT parasites in their contralateral footpad, and DTH response was measured 72 h postchallenge (A). After 3 wk postchallenge, mice were sacrificed, and parasite burden was determined by limiting dilution (B). In some experiments, CD3+ T cells were purified from spleens of WT or arg− L. major–infected mice and adoptively transferred into naive mice that were then challenged with virulent L. major. Three weeks after challenge, lesion size was determined (C), and mice were sacrificed to determine parasite burden (D). Data presented are representative of four (A, B) and two (C, D) independent experiments (n = 3–5 mice/group) with similar results. *p < 0.05, **p < 0.01. KO, Knockout.
Clonal exhaustion contributes to impaired T cell response and chronicity of arg

Because arg

L. major infection causes nonresolving infection, we investigated whether the significantly lower proliferation and effector cytokine (IFN-γ) production by T cells from these mice was related to increased expression of PD-1, a molecule that has been associated with immune cell exhaustion during chronic infections including Leishmania (36, 37, 47–49). As shown in Fig. 7A, CD4+ T cells from spleens and dLNs of mice infected with arg

L. major express significantly higher levels of PD-1 than those from WT L. major–infected mice. Interestingly, there was no difference in the percentage of Annexin-V+ cells (Supplemental Fig. 3), suggesting that cells from arg

L. major–infected mice were not undergoing more apoptosis than those from WT L. major–infected mice as previously suggested in diffused cutaneous leishmaniasis (50).

To determine whether the observed increased PD-1 expression contributed to impaired proliferative response and lesion chronicity of arg

L. major infection, we assessed proliferative responses of cells from arg

L. major–infected mice in the presence of anti–PD-1 mAb in vitro. Anti–PD-1 mAb significantly abolished the impaired CD4+ T cell proliferation and IFN-γ production by cells from arg

L. major–infected mice (Fig. 7B, 7C). Consistent with the flow data, the level of IFN-γ in cell-culture supernatant fluids of cells from arg

L. major–infected was similar to those from WT-infected mice (Supplemental Fig. 4A). In contrast, IL-10 production was significantly downregulated (Supplemental Fig. 4B). Strikingly, weekly treatment with anti–PD-1 mAb led to complete resolution of footpad lesions in arg

L. major–infected mice (Fig. 7D). Collectively, these results suggest that clonal exhaustion contributes to the poor proliferative and effector function of cells from arg

L. major–infected mice, and this may be responsible for their inability to protect against secondary L. major challenge.

Discussion

We recently reported that infection of highly susceptible BALB/c mice with arg

L. major results in attenuated pathology and decreased parasite burden while generating comparable immune response to that seen following WT L. major infection (25). Surprisingly, we found that infection of resistant C57BL/6 mice with arg

L. major resulted in a chronic disease in which lesions developed slowly and nonprogressively and were maintained for >16 wk, several weeks after WT-infected mice were completely healed (Fig. 1A). The failure to completely resolve cutaneous lesion in arg

L. major–infected mice was associated with impaired Ag-specific CD4+ T cell proliferation and IFN-γ production and failure to resist secondary challenge infection. We showed that clonal exhaustion of CD4+ T cells due to increased PD-1 expression was responsible for impaired T cell response and nonhealing disease observed in mice infected with arg

L. major. To our knowledge, this is the first demonstration of CD4+ T cell exhaustion in a parasitic infection.

Why does arg

L. major cause a chronic infection in B6 mice? In our previous study in the highly susceptible BALB/c mice, we proposed a “take rations for invasion” model whereby parasite-derived arginase plays a critical role during the early infection process (25). Thus, the de novo polyamine synthesis in Leishmania is of nutritional importance in the early colonization of the hostile macrophage environment. This model explains why the addition of exogenous polyamines to infected cells in vitro increases parasite proliferation but does not fully rescue the defect in arg

L. major (24). As parasites become established in the host cell, immune subversion mechanisms begin to direct the host response to allow a full-blown infection (51–53). In the susceptible BALB/c mice in which a detrimental Th2 response predominates, proliferation of arg

L. major may eventually overcome the early nutritional impairment (39). We propose that the significantly lower Th1 response within the first 2 wk post-infection in arg

L. major–infected C57BL/6 mice may only weaken parasite proliferation but insufficient to fully activate the immune system for effective parasite control.

Despite harboring comparable parasite burden at the site of infection after 16 wk, mice with arg

L. major were not protected against virulent challenge. Recovery from primary infection with L. major usually results in concomitant immunity (referred to as
Infection-induced immunity, which is dependent on persistent parasites (2, 6, 40, 54). Manipulations that result in complete parasite clearance result in loss of infection-induced immunity and susceptibility to secondary challenge (6, 55). We previously reported that L. major parasites lacking the phosphoglycan coats (termed lpg2L. major) are attenuated in vitro, persist indefinitely (56), and protect against virulent L. major challenge (57). The attenuation in virulence of phosphoglycan-deficient parasites is related in part to the induction of skewed early IFN-γ and a concomitant blunted IL-4 and IL-10 responses when compared with WT infection (57, 58). Although infection with argL. major results in attenuated pathology, the early immune response was significantly reduced compared with WT infection. Furthermore, Ag-specific proliferation and IFN-γ production were greater in WT-infected mice than in argL. major-infected mice, and this reduced immune response may be implicated in the deficient memory response and lack of protection against secondary challenge observed in argL. major-infected mice. These observations indicate that persistence of parasites by itself is simply not enough for maintenance of immunity against secondary challenge. Consistent with this proposal, a recent report shows that sphingolipid-deficient L. major parasites persist in infected mice without causing any overt pathology but fail to induce protection following virulent L. major challenge (59). Interestingly, deficiency of sphingolipids results in blunted T cell responses (59) akin to our observation in argL. major-infected mice. Collectively, these findings strongly suggest that the quality and/or magnitude of the early immune response may be critical in shaping the nature of the ensuing memory response.

To become memory T cells, naive T cells must first become activated, proliferate, and take on effector phenotype upon encountering their cognate Ags (60). Following Ag clearance and the subsequent immune contraction, a small number of these effector T cells acquire memory phenotype and remain in circulation as central and effector memory cells (60). Because the quality and magnitude of memory T cell response is directly proportional to the magnitude of primary response, the primary infection must generate sufficient clonal T cell expansion and contraction to ensure an effective memory T cell response (61). We found that the early immune response (proliferation and IFN-γ production) to argL. major was remarkably reduced compared with those induced by WT infection. This defect in early priming response following argL. major infection could contribute to impaired memory T cell generation, leading to impaired protection upon secondary WT challenge.

A third possibility that might contribute to failure of T cells from argL. major-infected mice to protect against secondary virulent challenge is T cell exhaustion, a dysfunction that is characterized by high expression of inhibitory receptors such as PD-1 and poor function at both effector and memory T cell levels (32). T cell exhaustion occurs during many chronic infections (47–49) and is a hallmark of many protozoan diseases including malaria (33), toxoplasmosis (34, 35), and leishmaniasis (36, 37). Exhaustion is characterized by increased expression of PD-1 molecule, a transmembrane receptor of the Ig superfamily that is expressed on thymocytes, mature T, and B cells following activation (62, 63). Interaction of PD-1 with its ligand PD-L1 or PD-L2 negatively regulates cytokine production and T cell proliferation. Recently, PD-1–mediated exhaustion of CD8+ T cells has been linked to increased susceptibility to L. donovani (37) and L. mexicana infections in humans (50) and mice (36). Although T cell exhaustion in leishmaniasis has been described for CD8+ T cells, CD4+ T cell exhaustion has not been demonstrated in this disease. We found that the expression of PD-1 on CD4+ T cells from mice infected with argL. major is significantly higher than those infected with WT parasites (Fig. 7A). We propose that the chronic nature of argL. major infection in C57BL/6 mice and the concomitant increase in PD-1 expression leads to T cell exhaustion (poor proliferation and IFN-γ production), resulting in impaired effector function and failure to protect against secondary L. major challenge. In line with this, we found that treatment of argL. major–infected mice with anti–PD-1 mAb leads complete resolution of chronic cutaneous lesion, increased recall response and resistance to secondary L. major challenge.

We were puzzled by the striking effects of in vivo anti–PD-1 mAb treatment given the relatively small (although significant) difference in PD-1 expression on CD4+ T cells from WT and argL. major–infected mice. In addition to blocking the interaction between CD4+PD-1+ cells with their ligands (PD-L1/PD-L2–expressing cells), thereby restoring CD4+ T cell proliferation and cytokine release (Fig. 7B, 7C), it is possible that this striking outcome (complete resolution of chronic lesion) may also be related to other indirect effects associated with PD-1 signaling. For example, a recent report showed that PD-1 triggering on monocytes enhances IL-10 production by these cells, which in turn inhibits CD4+ T cell response (64). Another report showed that CD4+PD-1+ cells produce large amounts of IL-10 and use this cytokine to mediate immunoregulatory properties (65). Because IL-10 is known to enhance susceptibility to cutaneous leishmaniasis (66–68), it is conceivable that anti–PD-1 mAb treatment may globally block IL-10 production by monocytes/macrophages and CD4+PD-1+ cells, resulting in enhanced resistance to L. major infection. In line with this, we found that anti–PD-1 mAb treatment significantly reduced IL-10 production by cells from both WT and argL. major–infected mice (Supplemental Fig. 4), leading to enhanced proliferation and IFN-γ production (Fig. 7B, 7C). In addition, in vivo anti–PD-1 mAb treatment was associated with transient increase in swelling of infected footpads in both WT and argL. major–infected mice (Fig. 7D), an effect that could be due to increased inflammation resulting from more effector T cell response, presumably related in part to decreased IL-10 regulatory activities.

In summary, we have shown that proliferation of argL. major is impaired in C57BL/6 mice. Although the early immune response is able to contain the infection, the persistent nature of the parasite results in a chronic, low-level infection resulting in nonresolving (chronic) lesion at the site of infection. We propose that the impaired early immune response to infection with argL. major (manifested as decreased proliferation and IFN-γ production) creates a poor priming environment for memory cell generation. This, combined with CD4+ T cell exhaustion due to chronic infection-induced expression of PD-1 molecules, results in failure of T cells from argL. major–infected mice to protect against secondary virulent L. major challenge. Intriguingly, these observations show that secondary immunity in L. major–infected mice does not only depend on the presence of persistent parasites. Our studies therefore present an interesting question as to what parasite factors determine a successful memory response capable of controlling L. major infection.

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Disclosures

The authors have no financial conflicts of interest.
References


Figure S1. Impaired antigen-specific proliferation in arg- L. major-infected mice. At 1 (A) and 2 (B) weeks after infection, WT and arg- L. major-infected mice were sacrificed and draining lymph node cells were labeled with CFSE dye. The cells were stimulated with SLA (50 μg/ml) for 72 hr, surface stained for CD4 (upper panels) and CD8 (bottom panels) and analyzed by flow cytometry. Histogram plot shows the percentage of proliferating CD4+ (upper panels) and CD8+ (lower panels) cells.

Figure S2. Quantitative differences in memory T cells in mice infected with WT and arg’ L. major. Draining lymph node cells from mice infected for 16 weeks with WT or arg’ L. major were stained with antibodies conjugated with different fluorochromes against CD3, CD4, CD62L and CD44 and analyzed by flow cytometry. Shown are shadow plots representing the percentages of CD62L and CD44 expressing cells after gating on CD3+CD4+ cells (A). Bar graphs show effector memory-like (CD44^highCD62L^lo, C) and central memory-like (CD44^highCD62L^lo,D), respectively.

Figure S3. Impaired immune response in arg’ L. major-infection is not due to increased apoptosis. Infected mice were sacrificed at 14 wk post-infection and the expression of Annexin-V on CD3+ splenic and dLN cells was assessed directly ex vivo by flow cytometry.
Figure S4. Blockade of PD-1 reverses impaired IFN-γ production and inhibits IL-10 production by cells in vitro. Splenocytes from mice infected with WT and arg⁻ L. major were restimulated in vitro for 5 days with SLA in the presence or absence of anti-PD-1 mAb (10 μg/ml) and the levels of IFN-γ (A) and IL-10 (B) in the culture supernatant fluids were determined by sandwich ELISA. *, p < 0.05; **, p < 0.01