Understanding IL-12 responsiveness during Leishmania amazonensis infection

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Understanding IL-12 responsiveness during

*Leishmania amazonensis* infection

by

Amanda Ellen Ramer

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Abstract

Cutaneous leishmaniasis is a vector-borne disease caused by intracellular protozoan parasites of the genus *Leishmania*. C3H mice challenged with *L. major* develop a polarized Th1 response and subsequently heal, whereas *L. amazonensis* challenge leads to chronic lesions with high parasite loads. This work demonstrates that infection with *L. amazonensis* creates a population of antigen-responsive, CD44\(^{hi}\) CD4\(^+\) T cells that proliferate and produce IL-2 but do not polarize to an effector phenotype and exhibit limited IFN-\(\gamma\) production in response to IL-12. CD44\(^{hi}\) CD4\(^+\) T cells from *L. amazonensis*-infected mice fail to accumulate in culture as compared to cells from *L. major*-infected mice. Neutralization of IL-2 promotes IL-12-mediated enhanced proliferation of CD44\(^{hi}\) CD4\(^+\) T cells from *L. amazonensis*-infected mice, indicating that IL-2 limits cell accumulation in response to IL-12 in vitro. Following multiple antigen stimulations, CD44\(^{hi}\) CD4\(^+\) T cells from *L. amazonensis*-infected mice sustain higher CD25 expression than cells from *L. major*-infected mice, a phenotype regulated by IL-2 and similar to regulatory T cells. To recapitulate continual in vitro culture of CD4\(^+\) T cells with IL-12 in vivo, multiple doses of IL-12 were administered to mice during the first two weeks of *L. amazonensis* infection. Although mice administered IL-12 developed a Th1 response early during infection, that response was not maintained and the mice failed to heal their infections. IL-12 treatment also failed to promote a sustained source of antigen-specific IL-12 from B cells in these mice, indicating that the failure of IL-12-treated *L. amazonensis*-infected mice to heal their infection is not due to an inability to mount a Th1 response, but rather from a lack of sustained, antigen-specific IL-12 production from B cells. Together, these results indicate that although CD4\(^+\) T
cells from *L. amazonensis*-infected mice respond to IL-12 by enhancing IFN-γ production, IL-2 suppresses their ability to enhance proliferation in response to IL-12. We believe this incomplete IL-12 responsiveness contributes to the chronicity of *L. amazonensis* infection.
Leishmaniasis

Epidemiology and ecology

Leishmaniasis is a vector-borne disease caused by intracellular protozoan parasites of the genus *Leishmania*. Leishmaniasis is a burden to both people and mammalian animals; it is endemic in 88 countries around the world, including countries in South America, southern Europe, Asia and the Middle East (39). *Leishmania* infection can result in a spectrum of diseases from self-limiting to disseminating cutaneous lesions, mucocutaneous lesions or fatal visceral disease. The ultimate course of disease is determined by both the species of the infecting *Leishmania* organism and the host's susceptibility (70). The various clinical presentations of leishmaniasis can be caused by nearly two dozen distinct *Leishmania* species (35). The yearly incidence of cutaneous leishmaniasis is estimated to be between 1 and 1.5 million cases and 500,000 cases for visceral leishmaniasis (also known as *kala azar*, which is Hindi for black sickness or fever). An estimated 350 million people around the globe are at risk for leishmaniasis with 12 million people currently infected. Dogs and rodents serve as the primary reservoirs for maintaining and disseminating *Leishmania* parasites in endemic areas (39).

While leishmaniasis does not often affect citizens of the United States, it is worth noting that cases of leishmaniasis have been reported in military personnel who have served in Iraq and other countries in the Middle East where leishmaniasis is endemic (44, 93, 94).
Human cutaneous leishmaniasis caused by *L. mexicana* has been reported in Texas (115), and cases of canine visceral leishmaniasis have been reported across the country (43, 52, 160, 169).

Leishmaniasis is primarily transmitted via the bite of a female sandfly of the *Lutzomyia* spp. in the New World and the *Phlebotomus* spp. in the Old World. Approximately 30 species of sandflies serve as *Leishmania* vectors (90). Less conventional methods of transmission include syringes, blood transfusion, transplacental spread and organ transplantation (33, 95, 121, 141). *Leishmania* parasites are dimorphic parasites that present two principle morphological forms. Found within the intestinal tract of the sandfly vector, the promastigote parasite is characterized by a long, slender body (15-30 μm by 2-3 μm) and a long, free anterior flagellum. Once the parasite is within parasitophorous vacuoles of mammalian macrophages or dendritic cells it transforms into the amastigote stage, which is characterized by a round or oval body about 2-6 μm in diameter with no exterior flagellum (35). Upon taking a blood meal from an infected mammal, the parasitized macrophages containing the amastigote form of the parasite are ingested. The parasites migrate to the midgut of the sandfly where they transform into promastigotes within 12 to 18 hours, attach themselves to the gut epithelia via lipophosphoglycan (LPG, a promastigote surface molecule) and multiply. Over the course of six to seven days, the promastigotes undergo metacyclogenesis, a process by which they become infective to a mammalian host. This transition is characterized by different morphological stages as well as a biochemical change in LPG structure that allows the promastigotes to detach from the gut and migrate forward towards the proboscis of the sandfly (162). When the infected sandfly takes another blood meal, the metacyclic promastigotes are inoculated into the skin and infection is initiated in
the mammalian host. Once inside either a macrophage or dendritic cell, the parasite enters a parasitophorous vacuole, transforms into an amastigote and replicates via binary fission. Infection is presumably spread when infected macrophages lyse due to their heavy parasitic burden, releasing amastigotes that are taken up by additional macrophages or dendritic cells (Figure 1).

**Human leishmaniasis**

Humans infected with *Leishmania* typically present with disease that is classified as cutaneous, mucocutaneous or visceral leishmaniasis. Localized cutaneous leishmaniasis (LCL) caused by *L. tropica* and *L. major* occurs primarily in the Mediterranean basin of Europe and the Middle East. New World *Leishmania* parasites belonging to either the *L. braziliensis* or *L. mexicana* complexes are responsible for LCL endemic in Central and South America. Patients afflicted with New World LCL usually present with a single primary lesion on unclothed parts of the body that are easily bitten by sandflies; patients with Old World LCL instead can develop multiple primary lesions in these areas (28). Most Old World LCL lesions caused by *L. major* self-cure within two to four months, while those caused by *L. tropica* take slightly longer. Healing for New World patients is variable, as healing after infection with *L. mexicana* is rapid (three months) but slow after infection with *L. braziliensis* and *L. panamensis* (125). Diffuse cutaneous leishmaniasis (DCL) is a variant of LCL in which lesions are disseminated and resemble lepromatous leprosy. Often caused by *L. aethiopica* in the Old World and *L. amazonensis* in the New World, DCL typically begins as a single primary lesion that metastasizes to other areas of the skin including the
Figure 1. Life cycle of *Leishmania major* infection. *Leishmania* parasites are transmitted by the bites of infected female sandflies, which inject a small number of infectious-stage, metacyclic promastigotes into the skin. These forms are opsonized efficiently by serum components and taken up by macrophages, where they reside in phagolysosomes and transform into replicating amastigotes. Infected macrophages are taken up by sandflies during blood feeding; they are lysed in the fly midgut, releasing parasites that transform into rapidly dividing, non-infectious-stage promastigotes. These forms undergo a process of attachment to the midgut wall, release and anterior migration that is accomplished by their differentiation to non-dividing, metacyclic promastigotes that can be transmitted when the sandfly takes another blood meal. Figure and legend reprinted by permission from Macmillian Publishers Ltd: *Nature Reviews Immunology*, Sacks, D. and N. Noben-Trauth. 2(11): 845-58, copyright 2002.
limbs and face. Post-kala-azar dermal leishmaniasis (PKDL) primarily occurs after infection with \textit{L. donovani}, which is endemic in East Africa and India. Post-kala-azar dermal leishmaniasis manifests itself in 20 to 50\% of patients recovering from visceral leishmaniasis. Most of these lesions heal spontaneously over a few months (28).

Mucocutaneous leishmaniasis (MCL), most commonly reported in Central and South America, results from \textit{L. braziliensis} or \textit{L. panamensis} infection. Mucosal dissemination occurs in a small percentage of these infections, developing one to five years after cutaneous leishmaniasis has healed. The mucosal disease often results in a perforated nasal septum and destructive inflammatory lesions that can obstruct the pharynx or larynx and produce considerable disfigurement (125). These patients rarely self-cure and treatment is necessary (28).

Kala-azar or visceral leishmaniasis (VL) is a systemic disease caused by dissemination of members of the \textit{L. donovani} complex, including \textit{L. donovani} and \textit{L. infantum} in the Old World and \textit{L. chagasi} in the New World. However, \textit{L. infantum} and \textit{L. chagasi} have been shown to be genetically identical (113). Patients typically present with fever, weakness, wasting, anemia, hepatomegaly and splenomegaly, and is fatal if left untreated (28, 125). An emerging concern is \textit{Leishmania}/HIV co-infection, as dually infected patients have been reported in 28 countries, including Spain, Italy, France and Portugal. In these countries, 25 to 70\% of adult cases of VL are associated with HIV infection and 1.5 to 9\% of patients with AIDS suffer from newly acquired or reactivated VL. Co-infection cases are being reported more frequently and it is thought that the number of cases will continue to rise due to the urbanization of VL (38). Viscerotropic leishmaniasis is a variant of VL caused by \textit{L. tropica}, a species of Leishmania once thought to only cause
cutaneous leishmaniasis. Several cases of viscerotropic leishmaniasis were diagnosed in soldiers who served in Saudi Arabia during Operation Desert Storm. These individuals presented with symptoms that were distinct from typical VL, such as malaise, intermittent diarrhea and abdominal pain (109).

Treatment of the various forms of cutaneous leishmaniasis is intended to accelerate parasitological cure, reduce scarring and prevent dissemination or relapse. In contrast, the intent of VL treatment is to decrease mortality. Despite the intent, the most efficacious chemotherapeutic agent for all forms of leishmaniasis is the pentavalent antimonial compound, sodium stibogluconate (trade name Pentostam). In use for over 50 years, this compound is thought to inhibit amastigote glycolytic activity and fatty acid oxidation. Another pentavalent antimonial compound, meglumine antimoniate (trade name Glucantime) is often the drug of choice to treat patients in South America (28). Both Pentostam and Glucantime are derivatives of stibonic acid; more specifically, the heavy metal antimony (Sb(V)) has been joined via oxygen atoms to the carbon chain of glucose. Although both drugs are highly effective, there are multiple disadvantages to treatment, including a parenteral mode of administration, long duration of therapy and frequent toxic side-effects (70). Recent reports exist of antimonial resistance, particularly in the Bihar State of India (182). Patients infected with resistant strains are treated with amphotericin B, a microlide antibiotic that is very toxic unless administered in its expensive liposomal form (32). Miltefosine may be another viable alternative to antimonial therapy. Originally developed as an anti-cancer drug, this alkylphospholipid can be administered orally and has been recently approved for use in India, Germany and Columbia. As with amphotericin B, however, cost remains a barrier to treatment (125). Undoubtedly, the most desirable method of treating
leishmaniasis would be prevention via vaccination. To date, no effective vaccine for leishmaniasis exists (181).

**Murine cutaneous leishmaniasis**

*L. major and the Th1/Th2 paradigm*

Infection of various inbred strains of mice with *L. major* is an extensively studied and well-characterized animal model of cutaneous leishmaniasis (155, 161). The study of these mouse models has generated much knowledge regarding host-pathogen relationships and the factors required for a positive disease outcome. Early studies demonstrated that host genetic background was critical in determining resistance or susceptibility to infection. Susceptible strains of mice (BALB/b, BALB/c, BALB/k and SWR/J) develop large ulcerated lesions that do not heal. In contrast, infection of resistant strains of mice (129, A/J, AKR, B10.D2, C3H, C57BL/6, C57BL/10, CBA, DBA and NZB) with *L. major* produces small lesions that resolve over time (40, 64, 73, 128). Pioneering studies in this field demonstrated that CD4^+^ T cells were necessary for resistance and that the production of IFN-γ in vivo correlated with resistance (74, 103, 163). These data correlated with earlier in vitro work indicating that interferon gamma (IFN-γ) is the critical effector molecule in murine leishmaniasis that activates macrophages to kill intracellular parasites (126, 127).

A more precise role for CD4^+^ T cells during experimental *Leishmania* infection was discovered in 1986 when Mosmann and Coffman published their groundbreaking work describing two distinct CD4^+^ T cell clones, T helper 1 (Th1) and T helper 2 (Th2) cells. Th1 cells were found to produce interleukin 2 (IL-2) and IFN-γ in response to antigen plus antigen-presenting cells (APCs) or stimulation with concanavalin A while Th2 cells
produced IL-4 and IL-5 (122). The dual role of these cells in vivo has been best illustrated in the mouse model of *L. major* infection. Using adoptive cell transfer, protection against *L. major* infection was obtained with Th1 cells whereas Th2 cells promoted disease (167). Additional work demonstrated that resistant mice (C57BL/6) possess high levels of IFN-γ mRNA in their draining lymph nodes and spleens while susceptible mice (BALB/c) express high levels of IL-4 mRNA (68). In vivo depletion of IFN-γ caused resistant mice to become susceptible to *L. major* infection while neutralization of IL-4 made susceptible mice resistant (18, 68). These initial studies of the Th1/Th2 paradigm during *L. major* infection have provided an important background for many additional studies that have facilitated the understanding of T helper cell development.

As of 2002, more than 6,000 papers had been published on Th1 and Th2 cells (102). The preceding twenty years of immunologic research have delineated the development of these CD4$^+$ T cells, including roles for cell surface receptors, signaling molecules and transcription factors. The commitment of a naïve T cell to a Th1 phenotype begins when IFN-γ binds the IFN-γ receptor and activates STAT1, a transcription factor that in turn induces the expression of the master regulator of Th1 differentiation, T-bet (185, 186). T-bet, a member of the T-box family of transcription factors, can be detected in naïve T cells, but its expression is dramatically increased in developing and committed Th1 cells (58). Szabo et al demonstrated that T-bet expression is necessary for resistance to *L. major* infection, as T-bet knock-out mice fail to heal their infections (186). T-bet is thought to further perpetuate Th1 development by repressing Th2 cytokine expression (185). Because binding of IFN-γ to its receptor down-regulates expression of the IFN-γ receptor β chain, IL-12 becomes important for perpetuating the Th1 phenotype (9). IL-12 is a heterodimer
composed of two disulfide-linked subunits designated p35 and p40 (178). The IL-12 receptor is composed of two chains, IL-12 receptor β1 (IL-12Rβ1) and IL-12Rβ2. Each subunit alone binds IL-12 with low affinity; co-expression of both subunits is necessary for high-affinity IL-12 binding and signaling (29, 30, 150). T-bet can induce expression of the IL-12 receptor β2 (IL-12Rβ2) subunit and facilitate IL-12 responsiveness in CD4⁺ T cells (1, 123, 184). Production of IL-12 by APCs including dendritic cells promotes development of a Th1 response by binding its receptor and activating the STAT4 signaling pathway to perpetuate IFN-γ production to influence the differentiation of naïve T cells, thereby closing the positive feedback loop associated with Th1 development (11, 88, 192).

Other transcription factors and cytokines are also involved in the establishment and maintenance of a Th1 response, albeit in varying degrees and stages of the response. Expression of the transcription factor Hlx is induced by T-bet and functions to promote heritable Th1 gene induction and IFN-γ production (123, 213). The transcription factor ERM is also induced during Th1 cell differentiation, specifically by IL-12 (136). The cytokines IL-18, IL-23 and IL-27 also have been reported to be involved in promoting a Th1 response (135, 147, 188).

The commitment of a CD4⁺ T cell to the Th2 lineage results from IL-4 binding to its receptor and subsequent signaling via the STAT6 pathway (170, 187). Th2 cells preferentially express certain transcription factors, including c-MAF, which regulates the expression of IL-4 (71). Upstream of c-MAF is GATA3, a transcription factor required for the induction and maintenance of the Th2 phenotype (211, 212). Like T-bet, GATA-3 can be detected in naïve CD4⁺ T cells; however, its expression increases significantly only in Th2 cells (58). GATA-3 induces its own expression via an auto-regulatory loop and promotes
transcription of genes encoding Th2 cytokines such as IL-4 and IL-5 via chromatin remodeling (98, 137, 211), while decreasing IFN-γ production and IL-12 responsiveness (46, 138, 195). Other molecules implicated in Th2 development include the transcription factor MEL18 (91), the post-transcriptional regulators FOG1 (friend of GATA1) (214) and ROG (repressor of GATA) (117) and possibly the Tec family tyrosine kinase Itk (119).

The cell-mediated immune response to L. amazonensis

Infection of L. major-resistant mice with certain New World species of Leishmania, particularly L. amazonensis and L. mexicana, creates a very different disease outcome than does infection with L. major. As discussed previously, C57BL/6 and C3H mice heal a L. major infection; however, these mice develop chronic disease when infected with either L. amazonensis or L. mexicana. These vast differences in disease outcome can be partially explained by evolution. Based on comparisons of DNA sequences, a divergence between the Leishmania complexes L. major, L. mexicana (of which L. amazonensis is a member) and L. donovani is estimated to have occurred between 40 to 80 million years ago (116). This is similar to the divergence observed between mice and humans. Old World and New World species which both cause cutaneous disease are as different from one another as they are from species causing visceral disease (116). In addition to evolutionary differences, considerable differences in virulence factors also exist between L. major and New World species. Lipophosphoglycan (LPG), a complex glycophasphatidylinositol-anchored phosphoglycan molecule found on the surface of all Leishmania promastigotes, is known virulence factor for L. major but not for L. mexicana (76, 175). In contrast, the cathepsin L-like cysteine protease B enzymes are considered virulence factors for the L. mexicana
complex but not for *L. major* parasites (4, 34, 36). The metalloprotease gp63 has been identified to be important for both *L. major* and *L. amazonensis* virulence (85, 168).

Just as BALB/c mice are susceptible to *L. major* infection, they, too, are susceptible to *L. amazonensis* infection, developing progressive, ulcerative lesions and systemic disease. Initially, infection of BALB/c mice with *L. major* results in production of both IFN-γ and IL-4. A polarized Th2 response eventually prevails, mediated by a predominance of Vβ4+Vα8+ CD4+ T cells which recognize LACK (*Leishmania* homolog of receptors for activated C kinase) antigen and produce IL-4 (86, 156, 173). In contrast, *L. amazonensis*-infected BALB/c mice maintain similar percentages of IFN-γ- and IL-4-producing CD4+ T cells throughout infection rather than developing a polarized Th2 response and fail to develop skewed T cell receptor usage among CD4+ T cells (80). The predominant cytokine produced by these mice is IL-2 rather than IFN-γ or IL-4. Adoptive transfer of a Th2 cell line to BALB/c mice subsequently infected with *L. amazonensis* exacerbated disease based on lesion size and parasite burden, indicating that a polarized Th2 response negatively influences disease outcome (80). Additional studies aimed at determining the mechanism by which BALB/c mice are susceptible to *L. amazonensis* infection included administering IFN-γ and genetic deletion of IL-10—neither of which changed disease outcome (16, 140). Administration of either anti-IL-4 or anti-TGF-β has been reported to heal *L. amazonensis*-infected BALB/c mice, but these mice still exhibited lesions and detectable parasites (2, 15), suggesting that the susceptibility of BALB/c mice to *L. amazonensis* is mediated via mechanisms other than the development of a polarized Th2 response.

Rather than developing a progressive, ulcerative lesion as BALB/c mice infected with *L. amazonensis* do, *L. major*-resistant mouse strains (C57BL/6 and C3H) develop
comparatively smaller lesions that may or may not ulcerate. These lesions gradually increase in size during the first eight weeks post-infection and then plateau and maintain a relatively consistent size for several months. Ample evidence exists that the failure of these mice to heal their infections is not due to the presence of a Th2 response, but rather to the failure to mount an efficient Th1 response. Low to undetectable levels of both IFN-γ and IL-4 are found during in vitro recall responses of lymph node cells draining the site of *L. amazonensis* infection (2, 83). The poor Th1 response associated with *L. amazonensis* infection persists even in the absence of IL-4 or IL-10 (2, 82, 83). Mice that have been treated with anti-IL-4 antibody or are IL-4 deficient do not heal their infections or enhance IFN-γ production (2, 83). Administration of exogenous IL-12 to IL-4 knockout mice at the time of *L. amazonensis* infection also fails to promote healing (83). In the absence of IL-10, *L. amazonensis*-infected C57BL/6 mice do exhibit a slightly enhanced Th1 response, but it is not sufficient to resolve the infection, as there is only a modest reduction in the parasite burden of the IL-10 knockout mice and no significant difference in lesion size as compared to the wild-type mice (81, 82). IL-12 production has been shown to be limited during *L. amazonensis* infection (83, 152, 198). Attempts to promote healing by administration of exogenous IL-12 at the time of *L. amazonensis* infection have proven unsuccessful; a phenomenon initially attributed to the low level of IL-12Rβ2 expression on CD4+ T cells (83). More recent research has shown that mice chronically infected with *L. amazonensis* can upregulate both IL-12Rβ2 and T-bet mRNA expression after repeated administration of antigen-pulsed bone marrow-derived dendritic cells in the presence of IL-12 (198). These mice subsequently develop a population of Th1 CD4+ T cells but still exhibit impaired antigen-specific IFN-γ production and fail to reduce their parasite burden. Additional work has shown that the transfer of a Th1 cell line
prior to *L. amazonensis* infection offered some protection against a promastigote infection, but not an amastigote infection (151). Together, these results suggest that simply the development of a population of Th1 CD4\(^+\) T cells is not sufficient to heal a *L. amazonensis* infection; it is instead likely that appropriate dendritic cell functions including efficient IL-12 production must also be established in order to facilitate healing.

**The biology of IL-12**

IL-12 was first identified as a product of Epstein-Barr virus-transformed human B cell lines that could activate natural killer (NK) cells and induce IFN-\(\gamma\) production and T cell proliferation (92). IL-12 is a heterodimer composed of two disulfide-linked subunits designated p35 and p40 (178). The genes encoding these two subunits are not located on the same chromosome in either humans or mice, but when both genes are coordinately expressed within a cell, biologically active IL-12p70 is secreted (63, 206). In addition to dimerizing with the p35 subunit, p40 subunits can also form disulfide-linked homodimers (IL-12p40\(_2\)) (54, 104). While the exact biological function of IL-12p40\(_2\) is uncertain, it may function as an antagonist that binds to the IL-12 receptor, thereby preventing binding and signaling of IL-12p70 (53, 54, 104).

The major cellular sources of IL-12 are dendritic cells and macrophages (193). The initial production of IL-12 is a rapid process that occurs upon cellular recognition of pathogen associated molecular patterns (PAMPs) via Toll-like receptors (TLRs) (108). Products from microorganisms, including bacteria, intracellular parasites, fungi, double-stranded RNA, bacterial DNA and CpG-containing oligonucleotides are all considered PAMPs. Toll-like receptor ligands are not sufficient to induce efficient IL-12 production
alone; secondary signals including cytokines and direct cell-cell interactions are necessary as well (66, 108). IFN-γ enhances IL-12 production, creating a positive feedback loop during inflammatory Th1 responses. Additionally, continued production of IL-12 is necessary to prevent the loss of Th1 cells and maintaining a productive Th1 response (143, 144, 179). Priming of dendritic cells and macrophages with a bacterial stimulus also induces expression of CD40 and makes these cells responsive to interactions with the ligand for CD40 (CD40L) on activated T cells (164). Signals transduced via CD40 in turn enhance IL-12 production by inducing transcription of the genes encoding p35 and p40 (164). IL-12 is a powerful pro-inflammatory cytokine and mechanisms to regulate it are necessary to prevent immune-mediated pathological damage associated with an excessive inflammatory response. IL-10 potently inhibits IL-12 by blocking transcription of both p35 and p40 (7). Transforming growth factor-β (TGF-β) also negatively regulates IL-12 production by reducing IL-12p40 mRNA stability (41). IL-12 production is also inhibited by IFN-α, IFN-β and tumor necrosis factor alpha (TNF-α) and certain G protein-coupled receptors (22, 31, 108).

**IL-12 signaling**

The IL-12 receptor is composed of two chains, IL-12 receptor β1 (IL-12Rβ1) and IL-12Rβ2, and is organized as a dimer on the surface of the cell. Each subunit alone binds IL-12 with low affinity; co-expression of both subunits is necessary for high-affinity IL-12 binding (29, 30, 150). IL-12p40 interacts predominantly with the β1 subunit whereas IL-12p35 interacts primarily with the β2 subunit (150). Activated T and NK cells are the cell types that primarily express the IL-12 receptor. Expression of the IL-12 receptor is highly regulated on T cells; resting T cells do not express either subunit of the receptor, but
expression of both β1 and β2 chains are induced upon T cell activation (37). IL-12Rβ2 expression facilitates IL-12 responsiveness, which is central to establishing an optimal Th1 response. Th1 cells express both subunits of the IL-12 receptor; in contrast, Th2 cells fail to express IL-12Rβ2 (184).

The IL-12 receptor subunits lack intrinsic enzymatic activity; signal transduction is instead accomplished via two members of the Janus kinase family of tyrosine kinases, Jak2 and Tyk2 (133). Tyk2 associates with the β1 subunit and Jak2 binds the β2 subunit of the IL-12 receptor (10, 215). The β2 subunit appears to be the signal-transducing component of the high-affinity receptor complex, as it contains multiple tyrosine residues on its cytoplasmic domain (150). IL-12Rβ1 is thought to be more important for ligand binding rather than signaling because it possesses no intracellular tyrosine residues to initiate a signaling cascade (207). Upon ligand binding, the receptor-associated Jaks are activated via transphosphorylation (99). In turn, these activated Jaks phosphorylate tyrosines located on IL-12Rβ2; those phosphorylated tyrosines then form docking sites for STATs (signal transducers and activators of transcription). The STATs bind to the phosphorylated β2 subunit via SRC homology (SH2) domains and they themselves become phosphorylated by the Jaks. Phosphorylated STATs then homo or heterodimerize, translocate to the nucleus, bind specific DNA sequences and modulate gene expression (201). Reports indicate that STAT1, STAT3, STAT4 and STAT5 can all be activated by IL-12 (55, 78, 209). Most of IL-12’s effects, however, seem to be mediated via STAT4 homodimers, as STAT4 knock-out mice display impaired IFN-γ production and impaired Th1 polarization, the two hallmark activities mediated by IL-12 (88, 192) (Figure 2).
IL-12 receptor and signal transduction. The interleukin-12 (IL-12) receptor is composed of two chains, IL-12Rβ1 and IL-12Rβ2, both of which have extensive homology to gp130, the common receptor β-chain of the IL-6-like cytokine superfamily. They are type I transmembrane glycoproteins, with molecular weights of ~100 kDa (IL-12Rβ1) and ~130 kDa (IL-12Rβ2). Co-expression of IL-12Rβ1 and IL-12Rβ2 is required for the generation of high-affinity (50 pM) IL-12-binding sites, and the IL-12Rβ2 subunit functions as the signal-transducing component of the high-affinity receptor complex. An 85-kDa protein that is associated with IL-12Rβ1 is phosphorylated in response to IL-12 and might be another component of IL-12R. Signal transduction through IL-12R induces tyrosine phosphorylation, primarily of the Janus-family kinases JAK2 and TYK2, which, in turn, phosphorylate and activate signal transducer and activator of transcription 1 (STAT1), STAT3, STAT4 and STAT5. The specific cellular effects of IL-12 are due mainly to its ability to induce activation of STAT4. Figure and legend reprinted by permission from Macmillian Publishers Ltd: *Nature Reviews Immunology*, Trinchieri, G. 3(2):133-46, copyright 2003.
IL-12 signaling can also be negatively regulated by various mechanisms. The suppressor of cytokine signaling (SOCS) family of proteins hinders the actions of numerous cytokines; both SOCS1 and SOC3 have been shown to inhibit IL-12 signaling (45, 208). PIASx, a member of the PIAS (protein inhibitors of activated STATs) family also dampens IL-12 signaling by binding to STAT4 and preventing STAT4-dependent gene transcription (6).

**Functions of IL-12**

IL-12 promotes IFN-$\gamma$ production from T cells and NK cells via STAT4 signaling. Not only is IL-12 efficient at inducing IFN-$\gamma$ at low concentrations, but it also synergizes with stimulation of the TCR-CD3 complex, activation of the CD28 receptor, IL-2, IL-18 or IL-27 to elicit rapid and efficient IFN-$\gamma$ production (14, 25, 96, 124, 147). The synergistic effect of IL-12 and IL-2 is thought to involve both increased IFN-$\gamma$ mRNA stability and p38 MAP kinase activation (24).

IL-12 also elicits a direct proliferative response from activated T cell and NK cells, although an exact mechanism has not been described (146, 178). STAT4 deficient mice display defective IL-12-induced proliferation (88, 192). Reports indicate that IL-12 mediates proliferation and IFN-$\gamma$ production by utilizing different components of the Jak-STAT pathway. Using the IL-12-responsive T cell clone 2D6, Tyk2 activation was associated with STAT3 and STAT4 phosphorylation leading to IFN-$\gamma$ production, while Jak2 activation correlated with STAT5 phosphorylation and cellular proliferation (3, 180). This group observed somewhat similar results using activated lymph node T cells. IL-12 alone does not seem to induce proliferation of resting T cells, but it can enhance the proliferation of these
cells when stimulated with mitogens, alloantigens, anti-CD3 and phorbol esters (92, 146). As with IFN-γ production, IL-12 synergizes with CD28 costimulation to induce proliferation in resting T cells (96).

**IL-12 and Leishmania**

IL-12 is an important aspect of the protective immune response to *L. major*. After exogenous IL-12 was given to susceptible BALB/c mice at the time of infection, smaller lesion sizes and parasite burdens were observed, along with an enhanced Th1 response and a decreased Th2 response (69, 183). Additionally, neutralizing IL-12 in vivo during *L. major*-infection of resistant mice resulted in increased IL-4 production, decreased IFN-γ production and non-healing lesions (67, 72, 183). Development of this Th2 response in these anti-IL-12-treated mice was transitory, as discontinuing treatment facilitated a switch to a Th1 response and subsequent healing (72). Studies with mice deficient in either IL-12p35, IL-12p40, IL-12Rβ2 or STAT4 also failed to heal their *L. major* infections and developed Th2 responses despite having a resistant genetic background (23, 112, 176).

**Immunological tolerance**

Immunological unresponsiveness, or tolerance, may occur after *Leishmania* infection, thereby promoting chronic disease. Work by Vanloubeeck et al demonstrated a functional delayed-type hypersensitivity (DTH) response upon in vivo antigen challenge in mice chronically infected with *L. amazonensis* (198). However, less IFN-γ was produced by lymph node cells draining the site of antigen challenge upon in vitro antigen restimulation as compared to lymph node cells from *L. amazonensis*-infected mice that did not receive an
antigen challenge—an observation consistent with tolerance. Others have shown that regulatory T (T<sub>reg</sub>) cells have been shown to be necessary for the establishment of a chronic <i>L. major</i> infection with accompanying low parasite load (17). Infection of PD-L1 (programmed death 1 ligand 1) knock-out mice with <i>L. mexicana</i> resulted in decreased parasite burden and lesion development compared to wild-type control mice (101). PD-1 and PD-L1 are members of the B7-1/B7-2:CD28/CTLA-4 costimulatory family of molecules. Studies are just beginning to elucidate PD-L1 and PD-L2 functions. Some in vitro studies have demonstrated that PD-L1 and PD-L2 can inhibit T and B cell proliferation and cytokine production by promoting cell cycle arrest; others have shown that the PD-1 ligands enhance T cell activation (reviewed in (57)). Experiments using PD-1 knock-out mice have suggested a role for PD-1 in regulating tolerance because a proportion of PD-1 knock-out mice develop characteristics of autoimmune disorders (129, 130). Together, these studies indicate that immunological tolerance may contribute to the chronicity of <i>Leishmania</i> infection.

Tolerance may also contribute to the establishment of other chronic infections. Varying degrees of dysfunctional or unresponsive CD4<sup>+</sup> T cells are reported during persistent viral infections. The T cell phenotypes observed are consistent with immunological tolerance or nonresponsiveness. A report by Zajac et al demonstrated that chronic lymphocytic choriomeningitis virus (LCMV) infection of mice resulted in virus-specific CD8<sup>+</sup> T cells that expressed activation markers and proliferated <i>in vivo</i> but were unable to elicit any antiviral effector functions (210). Others have observed impaired proliferation and effector cytokine production of antigen-specific CD4<sup>+</sup> T cells present during chronic hepatitis C infection (194). Additional examples of dysfunctional T cell responses similar to these also exist during other chronic diseases, including host responses after infection with
Trypanosoma cruzi, Mycobacterium tuberculosis, human immunodeficiency virus, gamma herpes virus and influenza virus (5, 48, 79, 97, 205).

Immunology textbooks define tolerance as immunological nonresponsiveness of an activated lymphocyte. The development of tolerance, especially self-tolerance, is an important dynamic process that begins with thymic selection of T cells. Only thymocytes that have productively rearranged the β chain of the T cell receptor (TCR) and whose TCRs exhibit self-MHC restriction are permitted to mature via a process known as positive selection. Cells that do not bind self-MHC are eliminated by apoptosis. Cells that survive positive selection then undergo negative selection, a process by which T cells are deleted if they possess a high affinity for self-MHC plus self-antigen. Elimination of these self-reactive cells is important as they pose a threat of perpetuating an autoimmune response once mature. An estimated 99% of all thymocytes die within the thymus due to elimination during positive or negative selection, a phenomenon known as central tolerance (reviewed in (177)).

Central tolerance is an efficient process, but not necessarily a complete one, as T cells with a low affinity for self-antigen can survive negative selection, exit the thymus and potentially lead to autoimmune responses (105). A second line of defense, known as peripheral tolerance, is therefore necessary to supplement central tolerance (84, 159). Peripheral tolerance is also important for preventing detrimental immune responses to ubiquitous proteins such as inhaled or ingested environmental antigens and gut flora antigens. During immune responses to invading microorganisms, various mechanisms of peripheral tolerance also prevent excessive inflammatory responses and subsequent host tissue damage. The primary mechanisms for silencing these responses involve eliminating responding T cells via clonal deletion or rendering these T cells unresponsive (84, 159). For
many years, the term anergy has been loosely used to describe unresponsive cell populations both in vitro and in vivo. As reviewed by Schwartz (166), T cell anergy can be generally defined as a tolerance mechanism in which the T cell is intrinsically functionally inactivated following antigen encounter, but remains alive for an extended period of time in a hyporesponsive state. The survival time of the cell is less than that of a normal T cell life span. Anergic cells are often deficient in a variety of functions, including cell division and cytokine production. A key characteristic of anergic cells is that they are cell-autonomous and not maintained by another cell type. These characteristics of anergy are generalizations; to be any more specific about functional defects and mechanisms one must address the different known types of anergic T cells individually. To avoid overuse of the term “anergic,” unresponsive T cells observed in vitro will be described as anergic T cells whereas unresponsive T cells described in vivo will be referred to as adaptively tolerant throughout the remainder of this work.

Clonal anergy

Traditional clonal anergy primarily represents a category of cells experiencing growth arrest and usually arises when previously activated T cells are incompletely reactivated either by a weak stimulus or a lack of costimulation in vitro (166). Despite weak or incomplete activation of these T cells, the signal received is sufficient to induce new protein synthesis to initiate and maintain the anergic state (153). High levels of the cell-cycle inhibitor p27Kip1 are thought to be involved in maintaining anergy as cell cycle progression seems important for preventing anergy; however, conflicting reports do exist (21, 149). Upon restimulation with antigen in the presence of APCs, clonally anergic T cells exhibit severely restricted
proliferation (165). IL-2 production is also limited in these cells, while production of effector cytokines including IL-4 and IFN-γ is only slightly impaired (106, 111). Clonally anergic cells do not seem to require antigen to maintain their unresponsive state, as unresponsiveness in vitro can remain for weeks after the removal of antigen and APCs (166). Proliferation and IL-2 production by clonally anergic cells can typically be restored upon exposure to a phorbol ester such as phorbol 12-myristate-13-acetate (PMA) and ionomycin (47, 100). Addition of IL-2 to clonally anergic cells can also reverse anergy (19), as does stimulation with anti-OX40 antibody (12). Clonally anergic cells have only been described in vitro. A slightly different pattern of anergy was observed in vivo and subsequently described as adaptive tolerance.

Adaptive Tolerance

Adaptively tolerant T cells are mature cells that have adapted to persistent antigen stimulation by down-modulating (or tuning) their sensitivity to specific antigen restimulation (166). This behavior is thought to be important for maintaining tolerance to self-antigen in the periphery. Adaptive tolerance, also known as in vivo tolerance (145), was first observed in a population of T cells that remained following expansion and cell death induced by superantigen stimulation in vivo (89, 154, 157). Antigen-specific models were developed later in which T cells from a TCR transgenic mouse were adoptively transferred into either an antigen-bearing or an antigen-free recipient that received a subsequent antigen challenge (142, 158, 159). These and other studies have yielded much insight into the characteristics of adaptively tolerant T cells, which differ considerably from those of clonally anergic T cells. The presence of persistent antigen is necessary for maintenance of this tolerant phenotype.
Adaptive tolerance is initiated in naïve T cells, and these T cells proliferate and produce IL-2 in response to antigen stimulation. Adaptively tolerant T cells differentiate to various degrees and then down-regulate proliferation and production of effector cytokines, including IFN-γ and IL-4, in the presence of persistent antigen (171, 189). Once antigen is removed, these T cells regain the ability to proliferate and produce cytokines.

**Regulation of T cell signaling in adaptive tolerance**

The hyporesponsiveness of adaptively tolerant T cells involves T cell receptor (TCR) desensitization rather than decreased expression of the TCR (189). Analyses of TCR signaling pathways in adaptively tolerant T cells have revealed a deficit in Zap70 kinase activity (27, 118). In turn, this defect results in decreased levels of phosphorylated LAT (linker of activated T cells) and phosphorylated phospholipase Cγ1 (PLCγ1). Together, these defects greatly impair the release of intracellular calcium, a characteristic shared by many examples of adaptively tolerant T cells (27, 190, 196). Adaptively tolerant T cells also fail to degrade IκB, which prevents signaling via the NF-κB pathway. In contrast, ERK1/2 phosphorylation is only slightly impaired in adaptively tolerant T cells, as is activation of the Ras/MAP kinase pathway (27).

Adaptively tolerant T cells also possess defects in IL-2 receptor signaling, as providing exogenous IL-2 does not reverse the tolerant phenotype of these cells as it does for clonally anergic T cells (166). Expression of the IL-2 receptor alpha chain (CD25) is highly variable among various models of adaptive tolerance. In some experiments, low doses of antigen are sufficient for CD25 expression, while high doses are necessary in others. Another study indicates that adaptively tolerant T cells do not re-express CD25 upon in vitro stimulation.
The exact mechanisms involved in aberrant IL-2 signaling are currently unknown for adaptively tolerant T cells.

CTLA-4 is a well-known regulator of T cells; however, the exact role of CTLA-4 in adaptive tolerance is controversial, as both affirmative (56, 145) and negative reports (50, 174) have been published. Work by Inobe and Schwartz indicates that involvement of CTLA-4 in adaptive tolerance is complex, as CTLA-4 slows CD4\(^+\) T cell responses such as proliferation and cytokine production but is not necessary for tuning mechanisms involved in adaptive tolerance (77). Additional work indicates that CTLA-4 could be mediating, in part, the defects in IL-2 signaling observed in adaptively tolerant T cells (202).

**Antigen load and the induction of adaptive tolerance**

Various models proposed by Grossman, Paul and colleagues predict how the strength and quality of an antigenic stimulation as well as the amount of antigen present during stimulation influence the behavior of a T cell. At any given time in a population of T cells, various states of maturation exist. These T cells possess different capacities for either self-renewal or differentiation; two competing cellular events that are regulated by recurrent interactions with antigen, APCs and other T cells (59, 60, 62). T cell differentiation is often considered in a linear fashion, beginning with the naïve state, ending with death and consisting of various stages of differentiation in between. Grossman and colleagues propose that T cells transition between a resting and activated state at each stage of differentiation based on interactions with antigen and APCs (Figure 3). If antigen and activated APCs are
Stage 1

Resting

Antigen and APCs regulate the balance between activation and self-renewal.

Cytokines and antigen regulate the balance between self-renewal and differentiation.

Activated

Differentiation and proliferation

Differentiation and proliferation

Differentiation and proliferation

Death

Naive

Resting

Self-renewal

Resting

Self-renewal

Resting

Self-renewal

Self-renewal

Activated

Activated

Activated

Activated

TCR

Activation

Differentiation, proliferation, and cytokine production

Peptide-MHC

Cytokines

Thymus

RTEs

Self-renewal

Activated

Activated

Activated

Activated

T cell

Activated T cell
present, T cells become activated and subsequently undergo either self-renewal or
differentiation. Activated T cells also secrete and respond to cytokines, which further
influence the balance between self-renewal and differentiation (60). In the absence of
polarizing cytokines, brief antigenic stimulation in vitro or in vivo results in proliferation of
non-polarized T cells. However, prolonged stimulation in the presence of IL-12 or IL-4
creates populations of polarized Th1 or Th2 cells, respectively (75). In vivo stimulation with
low doses of antigen induced undifferentiated, proliferating CD4⁺ T cells that produced IL-2
but not IFN-γ or IL-4. Upon culture in the presence of the appropriate polarizing cytokines,
these cells, known as T helper primed precursor cells, were able to differentiate into fully
committed Th1 or Th2 cells (200).

Under conditions of recurrent or continual antigen stimulation, effector cells may
limit their own numbers by changing the balance between self-renewal and differentiation.
The tunable activation threshold (TAT) model proposed by Grossman and Paul states that T
cell activation is a threshold phenomenon and that the threshold is tuned by the stimulatory
experience of the cell (61, 62). T cells are predicted to respond to perturbations in their
environment, such as to a change in the level of stimulation. Moreover, T cells are predicted
to adapt to continuous or recurrent stimulation by updating their threshold for activation,
thereby preventing resting T cells from becoming activated effector cells in response to
ambient interactions. Instead, resting lymphocytes only fully respond when a sufficiently
rapid increase occurs in their level of stimulation upon infection with a pathogen. For
example, autoreactive T cells in the periphery could become activated when presented with
self-antigen. However, because self-antigen is continually being processed and presented,
these cells have increased their threshold for activation to a level beyond what they normally
encounter, thus maintaining peripheral tolerance. In contrast, invading pathogens provide not only a source of antigen for presentation, but also provide a source of inflammatory mediators that produce a significant perturbation in a T cell's environment. This rapid increase in the amount of antigen presented promotes the development of a productive immune response.

When lymphocytes are subjected to recurrent stimulation, they either respond by differentiating or by adapting and becoming less responsive. As predicted by the TAT model, adaptively tolerant T cells have been shown to tune their activation threshold according to the amount of antigen present in their environment (171, 189). Work by Tanchot et al demonstrated that retransfer of adaptively tolerant T cells into a second antigen-bearing host results in a reduced in vivo proliferative response. Moreover, these cells slowly entered into what was described as a deeper state of unresponsiveness, evidenced not only by a decrease in cytokine production upon in vitro stimulation, but also by a weak in vivo proliferative response upon transfer into yet a third antigen-bearing host (189). Chronic infections such as tuberculosis or HIV or slow-progressing forms of cancer may provide yet another scenario for T cell activation under the TAT model. The gradual accumulation of antigen may elicit a weak response from T cells as they continually update their antigen threshold based on cues from their environment. Some characteristics of activation tuning by adaptively tolerant T cells is observed during persistent viral infection with LCMV, hepatitis C, human immunodeficiency virus, gamma herpes virus and influenza virus (5, 48, 79, 139, 194, 210) and other chronic diseases, including those caused by infection with Trypanosoma cruzi and Mycobacterium tuberculosis (97, 205). Interestingly, a recent report by Barber et al is the first of its kind to identify a specific mechanism of CD8+ T cell dysfunction during
chronic LCMV infection. PD-1 (programmed death-1) expression was found to be selectively enhanced on adaptively tolerant T cells; in vivo administration of antibodies that blocked the interaction of this inhibitory receptor with its ligand, PD-1L, enhanced T cells responses (13). Identification of this mechanism may represent a potential strategy for treating chronic viral infections.

In addition to interactions with antigen, APCs and other T cells, cytokines can also influence the T cell decision between self-renewal and differentiation. Just as the presence of polarizing cytokines including IL-12 and IL-4 at the time of antigenic stimulation can influence a cell’s decision to differentiate to a Th1 or Th2 phenotype, respectively, the presence of regulatory cytokines can influence differentiation towards a suppressive or regulatory phenotype (60) (Figure 3). These cytokines, including IL-10 and TGF-β, can either act upon APCs, which in turn direct the activities of T cells, or they can act directly on T cells themselves (26, 132). Should the former scenario be the case, an expansion of T<sub>reg</sub> cells may be observed. These T<sub>reg</sub> cells, in turn, suppress the proliferation and subsequent differentiation of other CD4<sup>+</sup> T cells (120, 148, 199). Alternatively, an increase in the concentration of regulatory cytokines may act directly on conventional antigen-specific T cells themselves, thereby conferring suppressive activity to those cells. This phenomenon could have a spreading (or “infectious”) character, as these cells may recruit other T cells stimulated at the same time to acquire a suppressor/regulatory function (60).

Regulatory T cells are an important mechanism of immune regulation. Unfortunately, the exact definition of what constitutes a T<sub>reg</sub> cell, where and how they develop and the antigenic specificity and mechanisms of actions are not readily defined. Bluestone and Abbas contend that T<sub>regs</sub> cells can be classified into two general categories,
natural and adaptive (or inducible) Tregs (20). Natural Tregs are CD25+ CD4+ T cells that develop in the thymus and function primarily to maintain self-tolerance. In contrast, the category of adaptive Tregs is a broad one and encompasses Treg cells that function to maintain homeostatic control over various adaptive immune responses that may or may not be involved in maintaining self-tolerance. Mechanisms of adaptive Treg suppression vary, and can include cell-cell contact, APC contact and cytokine-mediated suppression. Adaptive Treg cells may be derived from either natural Tregs or from classical T cell subsets. Although these cells are thought to arise after antigen stimulation, such as after organ transplantation or infection, whether or not these Tregs are antigen specific is unclear (20). Many have speculated that IL-2 promotes Treg development, as Tregs are absent in IL-2-deficient mice (51, 110). However, a recent report indicates that IL-2 is dispensable for Treg development but necessary for Treg maintenance (49).

A report by Duthoit et al demonstrates perhaps yet another type of adaptive regulatory cell—stimulation refractory CD4+ T cells (42). Wilde and Fitch first described that CD4+ T cells maintained in continuous culture with IL-2 became profoundly unresponsive to antigenic restimulation for a period after initial antigenic stimulation (203, 204). This refractory period was found to increase in length with increasing doses of IL-2 and was associated with enhanced activation-induced cell death (AICD) upon TCR re-engagement (191). Stimulation refractory CD4+ T cells are an activated T cell population that, like anergic cells and Tregs, can suppress the antigen-specific proliferative responses of naïve T cells (42). Naïve T cells that failed to proliferate in the presence of stimulation refractory CD4+ T cells did engage antigen and were partially stimulated as evidenced by enhanced CD69 expression; nevertheless, these naïve T cells failed to enter the cell cycle,
were blocked at the G0/G1 phase and failed to produce IL-2. Suppressed naïve T cell proliferation was observed even in the presence of high levels of IL-2, indicating that failure of naïve T cells to produce IL-2 in response to antigen was not the sole reason for the proliferation defect. Naïve T cells also appeared to be able to bind IL-2, as the high affinity IL-2 receptor was expressed on all T cells exhibiting suppressed proliferation (42). Norris et al observed a suppressive role for IL-2 dependent upon T cell activation status (131). In rested T cell cultures, IL-2 promoted antigen-specific proliferation and IL-2 production. For activated T cells, IL-2 inhibited antigenic reactivity by prolonging post-activational refractoriness and limiting subsequent IL-2 responsiveness and proliferation, although not via down-regulation of the high affinity IL-2 receptor (131). Stimulation refractoriness may function to limit not only the response of antigen specific T cells themselves, but also to limit bystander T cell activation. During an immune response, stimulation refractoriness may help prevent T cell adaptive tolerance and promote memory cell development as antigen load decreases (87). However, during chronic infections, it is possible that the continual antigenic stimulation of T cells may perpetuate a population of stimulation refractory T cells that suppress naïve T cell proliferation and differentiation, thereby failing to mount a productive CD4⁺ T cell response that decreases antigen load and resolves infection.

**Role of dendritic cells in promoting tolerance**

T cells can be rendered immunologically tolerant when primed by immature dendritic cells (65). During “steady-state” conditions (normal physiological conditions in which there is no infection or inflammation), dendritic cells reside in peripheral tissues as immature APCs; these DCs express low levels of MHC II, few costimulatory molecules and do not
secrete cytokines. However, these immature DCs are particularly adept at capturing antigens in their environment. When antigen is targeted to steady-state DCs, they upregulate MHC II and induce extensive T cell proliferation. However, those T cells do not polarize nor do they experience prolonged expansion or activation. These results indicate that in the absence of inflammation, steady-state DCs function primarily in maintaining peripheral tolerance (65).

Under certain conditions, steady-state DCs can become migratory, which is a characteristic of mature DCs. The details of this change are currently unknown but may involve tumor necrosis factor alpha (TNF-α) or IL-10. These DCs appear to enhance expression of MHC and costimulatory molecules and process and present antigen; however, no inflammatory signals are present during steady-state conditions to promote production of proinflammatory cytokines such as IL-12, thereby giving them a semi-mature phenotype. In turn, these semi-mature DCs become tolerogenic by inducing $T_{\text{reg}}$ cells (107). Similar to semi-mature DCs, pathogen-driven immature regulatory DCs do not support substantial effector T cell development, and instead induce IL-10$^+$ $T_{\text{reg}}$ cells. However, they are different from semi-mature DCs in that they are unresponsive to inducers of DC maturation such as lipopolysaccharide (LPS) or CD40 stimulation (172). These pathogen-induced immature DCs have been shown to develop in response to infection with *Plasmodium falciparum* and hepatitis C virus (134, 8). In contrast to pathogen-driven immature regulatory DCs, pathogen-mature regulatory DCs typically express high levels of MHC and costimulatory molecules, although CD40 expression is slightly reduced compared to mature immunogenic DCs. These DCs are potent at T cell stimulation, but they produce low levels of cytokines, including IL-12 family members, type I interferons, TNF-α, IL-6 and IL-10 (172). Pathogenic compounds that prime for pathogen-mature regulatory DCs include
lysophosphotidylserine from *Schistosoma mansoni* and filamentous haemagglutinin from *Bordetella pertussis* (114, 197). Together, these reports demonstrate a wide variety of DC phenotypes and functions in promoting various aspects of T cell tolerance.

**Overview of thesis and project objectives**

Infection of C3H mice provides an excellent experimental model for studying the host immune response associated with cutaneous leishmaniasis. C3H mice challenged with *L. major* develop a polarized Th1 response and subsequently heal. In contrast, infection of C3H mice with *L. amazonensis* results in chronic cutaneous lesions containing up to $10^8$ parasites and low to undetectable amounts of both IFN-γ and IL-4 after in vitro recall stimulation of draining lymph node cells. The objective of this dissertation was to characterize the dysfunctional adaptive T cell immune response associated with *L. amazonensis* infection. Specifically, the aims were to (1) determine if polarized, antigen-responsive or tolerogenic CD4$^+$ T cells develop during *L. amazonensis* infection, (2) assess both the in vitro and in vivo IL-12 responsiveness of CD4$^+$ T cells present during *L. amazonensis* infection and (3) understand the regulation of IL-12 responsiveness during *L. amazonensis* infection. Information generated from this dissertation has provided insight into why a productive adaptive immune response fails to develop during *L. amazonensis* infection. By understanding this dysfunctional immune response, the potential exists to develop new therapeutic strategies that will diminish parasite load and perpetuate a productive CD4$^+$ Th1 cell response. Perhaps, too, this information will not only aid in the fight against leishmaniasis but also against the many other chronic diseases caused by intracellular pathogens that burden our world.
References


86. **Julia, V., and N. Glaichenhaus.** 1999. CD4+ T cells which react to the *Leishmania major* LACK antigen rapidly secrete interleukin-4 and are detrimental to the host in resistant B10.D2 mice. Infect. Immun. 67:3641-4.


144. **Park, A. Y., B. D. Hondowicz, and P. Scott.** 2000. IL-12 is required to maintain a Th1 response during *Leishmania major* infection. J. Immunol. 165:896-902.


200. Wang, X., and T. Mosmann. 2001. In vivo priming of CD4 T cells that produce interleukin (IL)-2 but not IL-4 or interferon (IFN)-gamma, and can subsequently differentiate into IL-4- or IFN-gamma-secreting cells. J. Exp. Med. 194:1069-80.


Chapter 2

Antigen-responsive CD4⁺ T cells from C3H mice chronically infected with *Leishmania amazonensis* are impaired in their transition to an effector phenotype

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Abstract

C3HeB/FeJ mice challenged with *Leishmania major* develop a polarized Th1 response and subsequently heal, whereas *L. amazonensis* challenge leads to chronic lesions with high parasite loads at ten weeks post-infection. In this study, a comparison of draining lymph node cells from *L. amazonensis*- and *L. major*-infected mice at ten weeks post-infection showed an equivalent percentage of effector/memory phenotype CD4⁴⁺ CD4⁴⁺ T cells producing IL-2 and proliferating after antigen stimulation. However, these cells isolated from *L. amazonensis*-infected mice were not skewed towards either a Th1 or Th2 phenotype in vivo as evidenced by their unbiased Th1/Th2 transcription factor mRNA profile. In vivo antigen stimulation with added IL-12 failed to enhance IFN-γ production of CD4⁺ T cells from *L. amazonensis*-infected mice. Antigen stimulation of CD4⁺ T cells from *L. amazonensis*-infected mice in vitro in the presence of IL-12 resulted in production of only 10-15% of the IFN-γ produced by T cells from *L. major*-infected mice under identical

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conditions. These results suggest that the CD4+ T cell response during chronic *L. amazonensis* infection is limited during the transition from an early-activated CD4+ T cell population to an effector cell population, and demonstrate that these T cells have an intrinsic defect beyond the presence or absence of IL-12 during antigen stimulation.

**Introduction**

Experimental infection of C3H and C57BL/6 mice with the obligate intracellular protozoan parasite *Leishmania major* results in a CD4+ T cell population that produces IFN-γ, perpetuates a Th1 response and ultimately promotes resistance and a productive memory response (reviewed in (19-21); (28)). In contrast, infection of the C3HeB/FeJ, C57BL/6 or C57BL/10 strains of mice with *L. amazonensis* results in chronic cutaneous lesions containing up to 10^8 parasites and low to undetectable levels of both IFN-γ and IL-4 in the in vitro recall responses of draining lymph node (DLN) cells (1, 13, 15). CD4+ T cells from *L. amazonensis*-infected mice express low levels of IL-12Rβ2—a phenomenon that was found to be IL-4-independent (15). Additionally, the poor Th1 response associated with *L. amazonensis* infection has been shown to persist even in the absence of either IL-4 or IL-10 and also in the presence of exogenous IL-12 (14, 15, 26). The failure of *L. amazonensis*-infected mice to develop an effective Th1 response and heal their infections has prompted us to determine whether this CD4+ T cell defect is the result of an absence of antigen (Ag)-responsive effector/memory CD4+ T cells or an inability of Ag-responsive cells to progress to a productive a Th1 response.

We present evidence that the effector/memory phenotype (CD44^hi^) CD4+ T cells present during *L. amazonensis* infection exist in vivo as an unskewed T cell population as
evidenced by an unbiased T-bet and GATA-3 mRNA expression profile and are only partially responsive to IL-12 both in vitro and in vivo. However, these cells are not anergic as evidenced by their Ag-responsive and ability to proliferate and produce IL-2 to the same extent as CD44hi CD4+ T cells from L. major-infected mice. L. major-infected mice were included in our analyses as a control for a functional Th1 effector/memory response. Moreover, CD44hi CD4+ T cells exist in equivalent percentages in both L. amazonensis- and L. major-infected C3H mice. Our results indicate that the large parasite load and non-healing phenotype of L. amazonensis-infected mice occurs in the presence of an Ag-responsive CD4+ T cell population limited by an inability to progress from an early-activated phenotype to an efficient effector CD4+ T cell population. Furthermore, the data suggest that the failure of this cell population to become efficient Th1 effector cells is not due to the absence of IL-12 or the inability of the cells to respond to IL-12.

Materials and Methods

Parasites and Antigens. Culture of L. amazonensis (MHOM/BR/00/LTB0016) and L. major (MHOM/IL/80/Friedlin) and preparation of parasite antigens were performed as previously described (14). In all experiments involving Ag stimulation, cells were stimulated with the matching freeze-thawed promastigote Ag. The parasite burden of infected footpads was determined using a limiting dilution assay as described (1) and expressed as the negative log of parasite titer.

Mice. Female C3HeB/FeJ mice (six to eight weeks of age) were either bred in-house or obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in a specific
pathogen-free facility. The Committee on Animal Care at Iowa State University approved all protocols involving animals. Mice were injected with $5 \times 10^6$ stationary phase promastigotes in 50 μl PBS in the left hind footpad. Lesion size was monitored with a dial micrometer and expressed as the difference in footpad thickness between the infected and uninfected feet. Between three and twelve mice were pooled per group for each experiment and were sacrificed at ten weeks post-infection. For the in vivo Ag challenge, *L. amazonensis*-infected mice at ten weeks post-infection were injected in the right hind footpad with 20 μg of *L. amazonensis* Ag ± 0.2 μg of IL-12 (Peprotech, Rocky Hill, NJ) in a total volume of 50 μl of PBS or with 50 μl of PBS alone. *L. major*-infected mice at ten weeks post-infection were injected in the right hind footpad with 20 μg of *L. major* Ag. Mice were sacrificed at 48 hours post-Ag challenge.

**Flow Cytometry and Proliferation Assay.** Memory phenotype of T cells in the draining lymph node (DLN) was assessed ex vivo using flow cytometry as described in (15). Cells were surface stained with FITC-labeled anti-CD4 (H129.19), PE-labeled anti-CD62L (MEL-14), Cychrome-labeled CD44 (IM7) or the appropriate isotype control. All antibodies were obtained from Pharmingen (San Diego, CA) unless stated otherwise. Cells were acquired on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and data were analyzed using CellQuest software (Becton Dickinson).

To evaluate intracellular cytokines, $1 \times 10^6$ DLN cells were plated per well in a 96-well U-bottom plate with 50 μg/ml of Ag in complete tissue culture medium (CTCM; DMEM containing 4.5 mg of glucose/ml, 2 mM L-glutamine, 100 U penicillin, 100 μg streptomycin/ml, 25 mM HEPES, 0.05 μM 2-mercaptoethanol and 10% fetal bovine serum).
After 18 h, cells were stimulated with PMA (50 ng/ml) and ionomycin (50 ng/ml) in the presence of brefeldin A (10 µg/ml) for 6 h. Cells were harvested, washed, stained with either FITC-or PE-labeled anti-CD4, Cyochrome-labeled anti-CD44 or the appropriate isotype controls and fixed. Intracellular cytokines were assayed as described in (15). Antibodies used included FITC-labeled IL-2 (JES6-5H4), PE-labeled IFN-γ (XMG1.2), PE-labeled IL-4 (11B11) or an appropriate isotype control. Cells were acquired as described above.

Cell division was assessed by flow cytometry using the dye carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) as previously described (15). One million cells were cultured per well of a 96-well U-bottom plate with or without 50 µg/ml Ag in CTCM. After 4 days, the cells were harvested, washed, stained with PE-labeled anti-CD4 and Cyochrome-labeled anti-CD44 or the appropriate isotype controls, fixed and acquired as described above.

**CD4⁺ T Cell Purification.** CD4⁺ T cells were purified from lymph nodes via either magnetic positive selection using anti-CD4 MicroBeads or magnetic depletion using a biotin-conjugated antibody cocktail and anti-biotin MicroBeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol. Cells were subjected to one to three passes through an AutoMACS cell sorter. The purity of the CD4⁺ T cells was routinely 90% or greater. CD44hi CD4⁺ T cells from the DLN were stained with FITC-labeled anti-CD4 and Cyochrome-labeled anti-CD44 and sorted with an Epics Altra Cell Sorter (Beckman Coulter, Fullerton, CA). The purity was of the CD44hi CD4⁺ T cells routinely 90% or greater.
Polarization Assay, Recall Responses and ELISAs. For polarization assays, spleen cells from naïve female C3HeB/FeJ mice were incubated with a lysing buffer (0.15 M ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM ethylenediaminetetra-acetic acid) to lyse red blood cells. Splenocytes were treated with mitomycin C (Sigma) at a final concentration of 50 μg/ml at 37°C for 20 min and washed five times with an excess of complete media. In a 96-well U-bottom plate, each well contained 1 x 10^5 purified CD4^+ T cells, 1 x 10^6 mitomycin C-treated splenocytes and 50 μg/ml of Ag in CTCM. All co-cultures were expanded for five days with either 2 ng/ml of IL-12 (Peprotech) and 10 μg/ml of anti-IL-4 (Pharmingen, Th1 conditions) or with no additional cytokines and antibodies (neutral conditions). Supernatants were harvested and assayed via ELISA for IFN-γ; sensitivity ranged between 39 and 156 pg/ml. All ELISA antibodies were purchased from Pharmingen and used according to manufacturer recommendations. Ag-pulsed mitomycin C-treated splenocytes alone were cultured under polarizing conditions to determine baseline amount of cytokine production.

For recall responses, 1 x 10^6 LN cells draining the site of Ag challenge of infected mice were cultured in each well of a 96-well plate with or without 50 μg/ml of Ag in CTCM. After 3-d, supernatants were assayed for IFN-γ via ELISA as described above.

Real-time RT-PCR. Real-time RT-PCR was performed on either CD44^hi CD4^+ or CD4^+ T cells as described in (26).

Statistical Procedure. Statistical analysis was performed using Statview (SAS, Cary, NC). When treatment groups were compared, the data were analyzed with the Fisher's Protected
Least Significant Difference (PLSD) post-hoc test. When two treatments within a group were compared, data were analyzed using a paired t-test. Differences were considered significant when p < 0.05.

Results

Both *L. amazonensis* - and *L. major*-infected mice have equivalent percentages of CD4$^+$ T cells with an effector/memory phenotype.

C3H mice infected with *L. major* develop transient lesions that subsequently heal with less than 100 parasites detectable by ten weeks post-infection. However, *L. amazonensis* infection of C3H mice results in chronic lesions with up to $10^8$ parasites detectable in the footpad at ten weeks post-infection (Figures 1A and B). Moreover, mice infected with *L. amazonensis* fail to mount an effective Th1 response and consistently demonstrate poor effector cytokine production in recall responses of the draining lymph node (DLN). Because of these findings, we hypothesized that these mice might have a reduced percentage of effector/memory phenotype CD4$^+$ T cells in the DLN when compared to *L. major*-infected mice. However, an ex vivo analysis of the DLN of mice at ten weeks post-infection revealed that both *L. amazonensis*- and *L. major*-infected mice had equivalent percentages of CD4$^+$ T cells that were CD44$^{hi}$ and CD62L$^lo$ (Figure 1C). Absolute numbers of cells per DLN averaged $59.6 \times 10^6$ for *L. amazonensis*-infected mice and $28.1 \times 10^6$ for *L. major*-infected mice, indicating that *L. amazonensis*-infected mice have, overall, more effector/memory phenotype CD4$^+$ T cells at ten weeks p.i. than do *L. major*-infected mice. Moreover, both groups of *Leishmania*-infected mice had significantly greater percentages of
Figure 1. Both *L. amazonensis*- and *L. major*-infected mice have equivalent percentages of CD44^hi^CD62L^lo^CD4^+^ T cells in the DLN. (A) Lesion development in C3H mice infected with either *L. amazonensis* or *L. major*. Data are represented as the mean ± the standard deviation of the mean of one representative experiment with eight mice per group. (B) Parasite burden in the feet of *L. amazonensis*- or *L. major*-infected mice at ten weeks post-infection. Data are represented as the mean ± the SEM of three separate experiments. * represents a statistically significant difference at p < 0.05 as determined by Fisher's PLSD test. (C) DLN cells were harvested from mice infected with either *L. amazonensis* or *L. major* for ten weeks or from age-matched uninfected controls and stained for flow cytometry as described in materials and methods. The dot plots are based on a live CD4^+^ gate and are representative of four separate experiments.
these T cells than uninfected mice, indicating that this increase was associated with infection. These data indicate that the susceptibility of C3HeB/FeJ mice to *L. amazonensis* is not due to an absence of effector/memory phenotype CD4$^{+}$ T cells in the DLN, and that the percentage of effector/memory phenotype CD4$^{+}$ T cells present in the DLN of *L. amazonensis*-infected mice is not limited in comparison to *L. major*-infected mice.

**CD4$^{+}$ T cells from *L. amazonensis*-infected mice respond to parasite Ag by producing IL-2 and proliferating but do not have a skewed Th1/Th2 response.**

The CD4$^{+}$ T cell response associated with *L. amazonensis*-infection has been previously characterized as defective in terms of proliferative responses, Ag-specific cytokine production and chemokine and IL-12Rβ2 mRNA expression (13, 15). Our observations of an effector/memory phenotype CD4$^{+}$ T cell population that increases in the DLN of mice chronically infected with *L. amazonensis* prompted us to specifically assess the functional capabilities of the CD4$^{+}$ T cell population. After a 24-hour Ag stimulation of DLN cells, no significant difference was seen between the cells isolated from either *L. amazonensis*- or *L. major*-infected mice in terms of the percentage of CD4$^{+}$ T cells that produced IL-2 as assessed by intracellular staining (Figure 2A). In addition, the ability of CD4$^{+}$ T cells to proliferate after four days of Ag stimulation was evaluated using CFSE labeling. We found that 73% of these cells from both *L. amazonensis*-infected mice and *L. major*-infected mice proliferated (Figure 2B). CD4$^{+}$ T cell proliferation in the presence of Ag was almost exclusively from CD4$^{+}$ T cells (Figure 2C). CD4$^{+}$ T cell IL-2 production in the presence of Ag was predominantly from this cell population as well.
Figure 2. The Ag-specific CD4^{hi} CD4^{+} T cell population from mice chronically infected with *L. amazonensis* is capable of producing IL-2 and proliferating. (A) DLN cells were harvested from infected mice at ten wks p.i., stimulated for 24 h with 50 \( \mu \)g/ml of Ag, stained with fluorescent antibodies against CD4, CD44 and IL-2 and then analyzed by flow cytometry (see materials and methods). Cells from *L. amazonensis*-infected mice were stimulated with *L. amazonensis* Ag and cells from *L. major*-infected mice were stimulated with *L. major* Ag. Data are represented as the mean ± the SEM of three separate experiments. (B) DLN cells were harvested at ten weeks p.i., labeled with CFSE, cultured with (Ag Stim) or without (No Stim) 50 \( \mu \)g/ml of their respective Ag for four days, stained with fluorescent antibodies against CD4 and CD44 and then analyzed by flow cytometry. Cells from uninfected mice were stimulated with *L. amazonensis* Ag; cells from infected mice were stimulated as described in A. Data are represented as the mean ± the SEM of five separate experiments and is expressed as the percentage of CD4^{hi} CD4^{+} T cells that are proliferating. * represents a statistically significant difference between No Stim and Ag Stim within a group at p < 0.05 as determined by a paired t test. (C) Cells were cultured and assayed as described in B. All dot plots are for cells simulated with 50 \( \mu \)g/ml Ag and are based on a live CD4^{+} gate and are representative of five separate experiments. Quadrant statistics are percentages and are calculated based on a live CD4^{+} gate.
Thus, CD44$^{\text{hi}}$ expression defines Ag-reactive cells with similar proliferative and IL-2-producing capabilities in both *L. amazonensis*- and *L. major*-infected mice. Although central memory and effector memory CD4$^+$ T cells have recently been defined by CD62L$^{\text{hi}}$ and CD62L$^{\text{lo}}$ expression, respectively, during *L. major* infection (28), these populations do not significantly differ between *L. amazonensis*- and *L. major*-infected mice. These results demonstrate that the CD4$^+$ T cell population responds to *L. amazonensis* infection in vivo by enhancing the percentage of cells that recognize parasite antigen and that these cells undergo at least the early events of T cell activation including upregulation of CD44 and proliferation. Based on these results, we were interested in determining whether there would be differences in T cell characteristics associated with a mature Ag-responsive cell population.

Numerous studies have characterized the transcription factors T-bet and GATA-3 as master regulators of the Th1 and Th2 lineage fates, respectively, and the commitment of a T cell population towards either phenotype is associated with an upregulation of one of these transcription factors during progression through the activation pathway (25, 29). To that end, we assayed CD44$^{\text{hi}}$CD4$^+$ T cells isolated ex vivo from the DLN of infected mice for T-bet and GATA-3 mRNA expression via real-time RT-PCR. Consistent with a Th1 effector phenotype, CD44$^{\text{hi}}$CD4$^+$ T cells from *L. major*-infected animals had a significant increase in T-bet expression compared to cells from uninfected animals. CD44$^{\text{hi}}$CD4$^+$ T cells from *L. amazonensis*-infected mice, however, expressed 51% less T-bet mRNA when compared to CD44$^{\text{hi}}$CD4$^+$ T cells from *L. major*-infected mice (Figure 3A) and there was no significant difference in T-bet mRNA expression between CD44$^{\text{hi}}$CD4$^+$ T cells from *L. amazonensis*-infected and uninfected mice. In contrast, GATA-3 mRNA expression was found to be
similar between the CD44hi CD4+ T cells from both *L. amazonensis*- and *L. major*-infected mice, and this expression level was significantly lower than that of CD44hi CD4+ T cells from uninfected mice (Figure 3B). Together, these results indicate an unbiased T helper phenotype of the CD44hi CD4+ T cells in vivo during *L. amazonensis* infection as indicated by the absence of enhanced T-bet or GATA-3 mRNA expression over uninfected levels.

Effector cytokine production by the Ag-responsive CD44hi CD4+ T cells was assessed after stimulating the DLN cells with Ag for 24 hours and determining IFN-γ- or IL-4-positive cells via intracellular staining. The percentage of CD44hi CD4+ T cells producing IFN-γ from *L. amazonensis* mice was found to be 53% less than cells from *L. major*-infected mice (Figure 3C). In addition, there was no significant difference in the percentage of IL-4-producing CD44hi CD4+ T cells from *L. amazonensis*-infected mice compared to cells from *L. major*-infected mice. As with IL-2 production and proliferation, effector cytokine production in the presence of Ag was almost exclusively from the CD44hi CD4+ T cell population, thus reinforcing that these cells constitute the Ag-specific CD4+ T cell population (data not shown). The ratio of IFN-γ-producing cells to IL-4-producing cells was greater than 10 to 1 for the CD44hi CD4+ population derived from *L. major*-infected mice. Although *L. amazonensis*-infected mice had more IFN-γ-producing than IL-4-producing CD44hi CD4+ T cells (3 to 1), the ratio was not as skewed towards a Th1 response as that of cells from *L. major*-infected mice. This 3 to 1 ratio reflects the absence of a productive Th1 immune response in *L. amazonensis*-infected mice rather than an enhanced Th2 phenotype. Collectively, these intracellular staining results closely reflect the Th1/Th2 transcription factor mRNA expression profile and indicate that the Ag-responsive CD4+ T cells associated with chronic *L. amazonensis* infection exist in vivo as an unskewed population.
Figure 3. CD44^{hi} CD4^{+} T cells from *L. amazonensis*-infected mice do not have a skewed Th1/Th2 response. At ten weeks p.i., CD44^{hi} CD4^{+} T cells from the DLN of *Leishmania*-infected or control mice were sorted and analyzed ex vivo for (A) T-bet and (B) GATA-3 mRNA expression via real-time RT-PCR. Data are represented as the mean ± the SEM of four separate experiments. (C) DLN cells were harvested at ten weeks p.i., stimulated for 24 h with 50 μg/ml of their respective Ag as described in Figure 2A, stained with fluorescent antibodies against CD4, CD44 and either IFN-γ or IL-4 and then analyzed by flow cytometry. Data are represented as the mean ± the SEM of six separate experiments. * represents a statistically significant difference between indicated groups at p < 0.05 as determined by Fisher’s PLSD test.
Despite in vivo Ag-responsiveness, CD4\(^+\) T cells present in *L. amazonensis*-infected mice exhibit limited IL-12 responsiveness.

Considering that IL-12 expression is necessary for the development and maintenance of a CD4\(^+\) Th1 phenotype in vivo during *L. major*-infection (reviewed in (20)), and that IL-12 production has been shown to be limited during *L. amazonensis* infection (13, 15, 26), we determined if the CD4\(^+\) T cell population of mice chronically infected with *L. amazonensis* could respond to IL-12 in vivo in the presence of Ag. Since a delayed-type hypersensitivity reaction has long been utilized as a technique to evaluate memory CD4\(^+\) Th1 cell responses in vivo (reviewed in (18)), we infected mice in the left hind footpad with *L. amazonensis* for ten weeks and then injected the right hind footpad with either *L. amazonensis* Ag, Ag plus IL-12 or PBS. At 48 hours post-Ag challenge, there was a significant increase in the percent of CD44\(^{hi}\) CD4\(^+\) T cells present in the lymph node (LN) draining the site of Ag challenge over the PBS-injected controls in *L. amazonensis*-infected mice (Figure 4A), again demonstrating that CD44\(^{hi}\) CD4\(^+\) T cells do respond to Ag in vivo. However, the recall responses of the LN cells draining the site of Ag challenge showed no significant enhancement in IFN-\(\gamma\) production regardless of the presence or absence of IL-12 at the time of Ag challenge (Figure 4B). In contrast, high levels of IFN-\(\gamma\) were obtained from the recall responses of *L. major*-infected mice challenged with *L. major* Ag. To determine if the CD4\(^+\) T cells present in *L. amazonensis*-infected mice responded to the IL-12 treatment by altering Th1/Th2 transcription factor gene expression, real-time RT-PCR was used to analyze T-bet and GATA-3 mRNA expression in CD4\(^+\) T cells purified from the LN draining the site of Ag challenge. The presence of IL-12 at the time of in vivo restimulation did significantly enhance the T-bet to GATA-3 mRNA ratio over Ag-challenged *L. amazonensis*-infected
mice, but it was still significantly less than the ratio observed in L. major-infected mice challenged with Ag (Figure 4C). Data was expressed as the ratio of T-bet to GATA-3 mRNA as a previous study has shown that the relative expression of T-bet and GATA-3, rather than the expression of either transcription factor alone, was found to be more representative of the Th1/Th2 cytokine balance in a mixed population of cells (5). The more abundant GATA-3 mRNA expression than T-bet in the LN draining the site of Ag challenge results in a T-bet to GATA-3 mRNA ratio of less than one. However, this phenomenon is true for all samples, including the CD4⁺ T cells from L. major-infected mice that have a productive Th1 response. These results indicate that CD4⁺ T cells from L. amazonensis-infected mice can respond to Ag and IL-12 in vivo by enhancing accumulation. Additionally, an enhanced T-bet to GATA-3 mRNA ratio in CD4⁺ T cells draining the site of Ag challenge from L. amazonensis-infected mice is observed when IL-12 is present at the time of in vivo Ag stimulation, although it is unclear as to how IL-12 is influencing T-bet mRNA in these experiments. Taken together, our data indicate that the absence of IL-12 in vivo during Ag stimulation is not the sole reason for inefficient IFN-γ production from CD4⁺ T cells present during L. amazonensis infection.

**CD4⁺ T cells from L. amazonensis-infected mice have limited responsiveness to IL-12 in vitro.**

Since other cells types including APCs may influence the function of CD4⁺ T cells, we wanted to determine if APCs from non-infected mice with or without IL-12 could promote a Th1 effector phenotype in T cells comparable to that of T cells derived from
Figure 4. CD4+ T cells present in *L. amazonensis*-infected mice respond to Ag but exhibit limited IL-12 responsiveness in vivo. Mice that had been infected in the left hind footpad with *L. amazonensis* for ten weeks were injected in the right (uninfected) hind footpad with either PBS, *L. amazonensis* Ag or Ag and IL-12; *L. major*-infected mice were challenged in their uninfected footpad with *L. major* Ag. After 48 h post-Ag challenge, LN cells draining the site of Ag challenge were harvested and (A) analyzed via flow cytometry for the percentage of CD44 hi CD4+ T cells present in the LN after Ag challenge. * represents a statistically significant difference between the indicated treatments at *p* < 0.05 as determined by a paired t test. (B) LN cells draining the site of Ag challenge were stimulated for three days in the presence (Ag Stim) or absence (No Stim) of 50 µg/ml of their respective Ag as described in Figure 2A; supernatants were assayed for IFN-γ via ELISA. * represents a statistically significant difference between No Stim and Ag Stim within a group at *p* < 0.05 as determined by a paired t test. ** represents a statistically significant difference from all other groups at *p* < 0.05 as determined by Fisher’s PLSD test. (C) CD4+ T cells were purified from the LN draining the site of Ag challenge and analyzed via real-time RT-PCR for T-bet and GATA-3 mRNA expression. Data are expressed as the ratio of T-bet to GATA-3 mRNA after each target had been normalized to GAPDH. * represents a statistically significant difference between Ag and Ag + IL-12 within a group at *p* < 0.05 as determined by a paired t test. ** represents a statistically significant difference from all other groups at *p* < 0.05 as determined by Fisher’s PLSD test. All data are represented as the mean ± the SEM of two (C) to three (A & B) separate experiments.
L. major-infected mice in vitro. We purified CD4+ T cells from the DLN of infected mice and co-cultured those cells with Ag-pulsed, mitomycin C-treated splenocytes from naïve mice for five days; the production of effector cytokines was then quantified via ELISA. Under neutral conditions (no polarizing cytokines or antibodies), CD4+ T cells from L. amazonensis-infected mice produced significantly less IFN-γ than those cells from L. major infected mice (Figure 5A). Under Th1 conditions (anti-IL-4 and rIL-12), CD4+ T cells from L. amazonensis-infected mice did respond to IL-12 by enhancing their production of IFN-γ in comparison to neutral condition values. Despite equivalent absolute numbers of CD44hi CD4+ T cells present in both L. amazonensis and L. major cultures, the CD4+ T cells from L. amazonensis-infected mice consistently produced only 10-15% of the IFN-γ produced by the CD4+ T cells purified from L. major-infected mice stimulated under identical conditions (Figure 5B). These data indicate that although CD4+ T cells from L. amazonensis-infected mice can respond to IL-12 in vitro, intrinsic defects prevent them from developing a Th1 phenotype equivalent to that of CD4+ T cells derived from L. major infected mice.

Discussion

In this study, we extend our understanding of the dysfunctional immune response associated with the high parasite load and persistent lesion that characterizes the chronic stage of L. amazonensis infection in immunocompetent mice. Our data indicate that the immune response consists of an Ag-responsive CD4+ T cell population that expresses CD44, proliferates and produces IL-2 as well as their counterparts from L. major-infected mice (Figures 1C and 2). However, the CD44hi CD4+ T cell population from L. amazonensis-
infected mice has an unskewed effector phenotype as reflected by low levels of both Th1/Th2 transcription factor mRNA expression, IFN-γ and IL-4 protein expression (Figure 3) and a limited responsiveness to IL-12 (Figures 4 and 5).

The ability of Ag-responsive CD44hi CD4+ T cells from *L. amazonensis*-infected mice to proliferate as efficiently as cells derived from *L. major* infected animals was surprising, as previous works have indicated a suppressed proliferative response in comparative studies (13, 15). These differences may be a result of assessing proliferation at different time points during infection, whereas the current studies were performed exclusively during the established chronic phase of disease. Other factors that may influence the proliferative
response of these cells could include Ag preparation and the amount of Ag used in re-
stimulation. The phenomenon of cell death could also account for these different
observations as a loss of live cells in culture would not influence the results of our analysis
using CFSE labeling but would lead to decreased tritiated thymidine incorporation.

This current report of an uncommitted CD4$^+$ T cell phenotype during \textit{L. amazonensis}
infection complements previous studies in which a mixed Th1/Th2 response was observed
throughout the course of \textit{L. amazonensis} infection (1, 12). Our studies specifically
characterize an Ag-responsive subpopulation of CD4$^+$ T cells from \textit{L. amazonensis}-infected
mice with low mRNA expression levels of the Th1 transcription factor, T-bet, in comparison
to similar cells from \textit{L. major}-infected mice. These findings describe a specific defect in the
T cell activation pathway that can account for the previously described inefficient IFN-$\gamma$
production and low levels of IL-12R$\beta$2 mRNA expression observed from CD4$^+$ T cells
during \textit{L. amazonensis} infection ((13, 15) and in results). Moreover, we show that CD44$^h_i$
CD4$^+$ T cells from \textit{L. amazonensis}- and \textit{L. major}-infected mice both express similar levels of
GATA-3 mRNA and IL-4 intracellular staining, indicating that this T cell population is also
not biased towards a Th2 response. Interestingly, CD44$^h_i$ CD4$^+$ T cells from uninfected mice
express more GATA-3 mRNA than those cells from \textit{L. amazonensis}-infected mice (Figure
3B). These observations are consistent with the phenomenon that GATA-3 mRNA
transcripts are high in naïve cells and then either decrease as cells polarize toward a Th1
phenotype or remain high if a Th2 phenotype is developed (8). Nonetheless, relatively low
levels of GATA-3 and T-bet mRNA expression indicate that CD44$^h_i$ CD4$^+$ T cells from \textit{L.
amazonensis}-infected mice are a population that has not committed to either a Th1 or Th2
phenotype in vivo.
Previous work has indicated that mice infected with *L. amazonensis* fail to increase the number of IL-12-producing cells as compared to uninfected controls (15). To compensate for this deficit, we restimulated CD4\(^+\) T cells in vivo by injecting both IL-12 and Ag into the contralateral (uninfected) footpad of chronically infected mice to test the ability of this uncommitted cell population to differentiate towards a Th1 population during antigen stimulation in the presence of IL-12. We observed a response to Ag by the CD4\(^+\) T cell population as indicated by an increased percentage of CD44\(^{hi}\) CD4\(^+\) T cells in the lymph node draining the site of Ag challenge when compared to PBS-injected controls (Figure 4A). Despite observing an enhanced T-bet to GATA-3 mRNA ratio in the CD4\(^+\) T cells of *L. amazonensis*-infected mice challenged with Ag in the presence of IL-12, the production of IFN-\(\gamma\) upon *in vitro* Ag-stimulation was unchanged (Figure 4B). One in vivo administration of IL-12 may not be sufficient to promote the development of a population of Th1 CD4\(^+\) T cells, and previous work has suggested that CD4\(^+\) T cells from C3H mice with an acute *L. amazonensis* infection may be unable to respond to IL-12 due to low levels of IL-12R\(\beta2\) mRNA expression (15). However, the limited in vitro IL-12 responsiveness observed in this study and the successful development of CD4\(^+\) T cells with a Th1 phenotype by repeated administration of IL-12 with Ag-pulsed bone marrow-derived dendritic cells to mice chronically infected with *L. amazonensis* indicate that the CD4\(^+\) T cell population present during chronic *L. amazonensis* infection is able respond to IL-12 to some extent (26). The relatively low levels of IFN-\(\gamma\) production both in vitro and in vivo in response to IL-12 suggests that transition to a Th1 phenotypic cell population is limited by intrinsic defects in the CD4\(^+\) T cells rather than simply the presence or absence of IL-12 during T cell activation.
Much attention has been given to the negative role of T regulatory (T<sub>reg</sub>) cells in infectious disease, where T<sub>reg</sub> cells limit productive immune responses and promote infection (reviewed in (4, 17)). However, recent work has shown that T<sub>reg</sub> cells are important in limiting the immunopathogenesis of <i>L. amazonensis</i> infection, although that beneficial effect is transitory (11). Previous work has shown that T<sub>reg</sub> cells are necessary for the establishment of a chronic <i>L. major</i> infection with accompanying low parasite load, and that T<sub>reg</sub> function was dependent on IL-10 (3). However, IL-10 knock-out mice infected with <i>L. amazonensis</i> still develop a chronic infection with a relatively high parasite load, suggesting that T cell-derived IL-10 is not entirely responsible for limiting an effective immune response (14).

Blocking TGF-β in BALB/c mice infected with <i>L. amazonensis</i> has been reported to facilitate healing (2). However, the effects of TGF-β are often conflicting, and may be due in part to its opposing effects on Th1 development in various mouse strains (9). Blocking TGF-β in vitro fails to enhance IFN-γ production in CD4<sup>+</sup> T cells isolated from <i>L. amazonensis</i>-infected C3H mice ((15), Ramer and Jones unpublished observations). Taken together, these observations indicate that it is unlikely for T<sub>reg</sub> cells, IL-10 or TGF-β to be responsible for the lack of a Th1 phenotype in CD4<sup>+</sup> T cells during <i>L. amazonensis</i> infection.

We believe that the limited effector functions of CD4<sup>+</sup> T cells present in <i>L. amazonensis</i>-infected mice may result from a combination of priming by immature or semi-mature dendritic cells and the presence of high antigen load. Chronic <i>L. amazonensis</i> infection is characterized by the absence of a robust inflammatory response, as evidenced by decreased IL-12 production and decreased mRNA expression of multiple inflammatory mediators and a high parasite load (13, 15, 26). Recent work has shown that inappropriately primed dendritic cells in vivo can support CD4<sup>+</sup> T cell clonal expansion but cannot prime an
effector response (23). Indeed, some persistent infections, including HIV, are thought to limit dendritic cell maturation and thus induce peripheral tolerance due to Ag capture and presentation by immature dendritic cells (24). In addition, activation of CD4\(^+\) T cells in the presence of high viral load or providing multiple Ag stimulations to CD4\(^+\) T cells results in diminished CD4\(^+\) effector responses (6, 10). Dysfunctional CD8\(^+\) T cell responses have also been described in chronic lymphocytic choriomeningitis virus and *Trypanosoma cruzi* infections of mice, as these cells are activated yet exhibit attenuated IFN-\(\gamma\) production and cytotoxic activity (16, 27). With these studies in mind, we suggest that chronic *L. amazonensis* infection could result from parasite resistance to macrophage killing which creates a persistent, high Ag load that impairs CD4\(^+\) T cell effector functions (7, 22). In turn, these dysfunctional T cells are incapable of promoting effective macrophage activation and subsequent parasite elimination.

Altogether, our data indicate that mice with chronic *L. amazonensis* infections do possess an Ag-responsive CD44\(^{hi}\) CD4\(^+\) T cell population that can proliferate and produce IL-2, but is impaired in the ability to efficiently produce IFN-\(\gamma\). These CD44\(^{hi}\) CD4\(^+\) T cells have an unbiased pattern of Th1/Th2 transcription factor mRNA expression in vivo and cannot be effectively polarized towards a Th1 phenotype either *in vitro* or *in vivo* in the presence of IL-12. Our observations implicate an impaired, unskewed CD44\(^{hi}\) CD4\(^+\) T cell population as a factor contributing to the chronicity of *L. amazonensis* infection.

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References


Chapter 3

IL-2 limits IL-12 responsiveness of CD4$^+$ T cells from *Leishmania amazonensis*-infected C3H mice in vitro

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Abstract

C3H mice infected with *Leishmania major* develop a polarized Th1 response whereas infection with *L. amazonensis* creates a population of antigen-responsive, CD44$^{hi}$ CD4$^+$ T cells that proliferate and produce IL-2 but do not polarize to an effector phenotype. Based on CD25 expression, CD44$^{hi}$ CD4$^+$ T cells from *L. amazonensis*-infected mice exhibit a resting (CD25 negative) then activated (CD25 positive) effector phenotype after primary and secondary antigen (Ag) stimulations in vitro, respectively. Although multiple Ag stimulations in the presence of IL-12 yield equivalent percentages of IFN-γ-producing CD44$^{hi}$ CD4$^+$ T cells in cultures of T cells derived from *L. amazonensis*- and *L. major*-infected mice, the total amount of IFN-γ produced by T cells from *L. amazonensis*-infected mice is 40% less than that produced by T cells from *L. major*-infected mice. This deficit in IFN-γ production results from a failure of CD44$^{hi}$ CD4$^+$ T cells from *L. amazonensis*-infected mice to accumulate in culture. Unlike CD44$^{hi}$ CD4$^+$ T cells from *L. major*-infected mice, CD44$^{hi}$ CD4$^+$ T cells from *L. amazonensis*-infected mice did not exhibit enhanced proliferation in response to IL-2 or IL-12. However, neutralization of IL-2 mediates
enhanced proliferation of CD44\textsuperscript{hi} CD4\textsuperscript{+} T cells from \textit{L. amazonensis}-infected mice in response to IL-12. These results indicate that IL-2 suppresses enhanced proliferation in response to IL-12 for activated effector CD4\textsuperscript{+} T cells.

Introduction

When C3HeB/FeJ mice are infected with \textit{Leishmania major}, they develop a classic Th1 response characterized by the production of IFN-\(\gamma\). These mice subsequently heal their infections and are considered resistant to \textit{L. major} infection. However, infection of these \textit{L. major}-resistant mice with \textit{L. amazonensis} results in chronic lesions that contain up to \(10^8\) parasites and low to undetectable levels of both IFN-\(\gamma\) and IL-4 in the recall responses of draining lymph node (DLN) cells. Susceptibility to \textit{L. amazonensis} infection is not due to the presence of a Th2 response but rather to the absence of an effective Th1 response. Previous work in our laboratory has shown that \textit{L. amazonensis}-infected mice possess equivalent percentages of CD44\textsuperscript{hi} CD4\textsuperscript{+} effector/memory T cells as do \textit{L. major}-infected mice (1). This T cell population is Ag responsive but remains unskewed towards either a Th1 or Th2 phenotype. While these T cells are able to enhance IFN-\(\gamma\) production in response to IL-12, the amount of IFN-\(\gamma\) produced is only 10-15\% of that produced in \textit{L. major} CD4\textsuperscript{+} T cell cultures. This work demonstrates that the failure of CD4\textsuperscript{+} T cells from \textit{L. amazonensis}-infected mice to become efficient Th1 effector cells is due to factors beyond the presence or absence of IL-12.

In this study, we continue to investigate the ability of the CD44\textsuperscript{hi} CD4\textsuperscript{+} T cell population from \textit{L. amazonensis}-infected mice to respond to IL-12. Previous work has demonstrated that CD44\textsuperscript{hi} CD4\textsuperscript{+} T cells from \textit{L. amazonensis}-infected mice produce IL-2 and
proliferate upon Ag stimulation (1); central memory CD4+ T cells exhibit a similar phenotype but are able to produce effector cytokines upon secondary Ag stimulation (2). Therefore, we also address the hypothesis that CD44hi CD4+ T cells from L. amazonensis-infected mice may be a type of central memory cell requiring multiple Ag stimulations in order to produce IFN-γ as efficiently as CD44hi CD4+ T cells from L. major-infected mice. We also report that CD44hi CD4+ T cells from L. amazonensis-infected mice exhibit a resting then activated phenotype after primary and secondary antigen stimulations, respectively, based on CD25 expression. While providing multiple antigen stimulations in the presence of IL-12 elicits equivalent percentages of IFN-γ-producing CD44hi CD4+ T cells in cultures derived from L. amazonensis- and L. major-infected mice, it does not result in equivalent total amounts of IFN-γ between the two cultures. Cell accumulation is limited in cultures of T cells derived from L. amazonensis-infected mice as compared to those of T cells derived from L. major-infected mice. CD44hi CD4+ T cells from L. major-infected mice exhibit enhanced proliferation in response to IL-12 whereas CD44hi CD4+ T cells from L. amazonensis-infected mice do not. However, IL-2 was found to suppress the IL-12-dependent enhanced proliferation of activated effector CD4+ T cells from L. amazonensis-infected mice.

**Materials and Methods**

*Parasites and antigens.* Culture of L. amazonensis (MHOM/BR/00/LTB0016) and L. major (MHOM/IL/80/Friedlin) and preparation of parasite antigens were performed as previously described (3).
**Mice.** Female C3HeB/FeJ mice (six to eight weeks of age) were either bred in-house or obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free facility. Mice were injected with $5 \times 10^6$ stationary phase promastigotes in 50 $\mu$l PBS in the left hind footpad. Between four and seven mice were used per group for each experiment and were sacrificed at four weeks post-infection. The IACUC at Iowa State University approved all protocols involving animals.

**CD4$^+$ T cell purification.** CD4$^+$ T cells were purified from lymph nodes via magnetic depletion (negative selection) using a biotin-conjugated antibody cocktail and anti-biotin MicroBeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol. Cells were subjected to two passes through an AutoMACS cell sorter. The purity of the CD4$^+$ T cells was routinely 90% or greater.

**Mini-double cultures.** These minicultures were adapted from previously described protocols (4, 5) as a method to provide purified CD4$^+$ T cells with both a primary and secondary in vitro stimulation. All cultures were performed in duplicate or triplicate in 96-well U-bottom plates and in a total volume of 200 $\mu$l per well. $1 \times 10^5$ CD4$^+$ T cells purified from the draining lymph node of either *L. amazonensis*- or *L. major*-infected mice were cultured with $2 \times 10^5$ mitomycin C-treated, carboxyfluorescein diacetate succinimidyl ester- (CFSE) labeled naïve splenocytes and 50 $\mu$g/ml freeze-thawed *Leishmania* promastigote Ag (CD4$^+$ T cells isolated from *L. major*-infected mice were stimulated with *L. major* Ag and CD4$^+$ T cells isolated from *L. amazonensis*-infected mice were stimulated with *L. amazonensis* Ag) in complete tissue culture medium (CTCM; DMEM containing 4.5 mg of glucose/ml, 2 mM L-
glutamine, 100 U penicillin, 100 µg streptomycin/ml, 25 mM HEPES, 0.05 µM 2-mercaptoethanol and 10% fetal bovine serum). Feeder splenocytes were prepared by incubating spleen cells from naïve female C3HeB/FeJ mice with a lysing buffer (0.15 M ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM ethylenediaminetetra-acetic acid) to lyse red blood cells. After red blood cells lysis, splenocytes were labeled using CFSE (Molecular Probes, Eugene, OR) as previously described (6) and then treated with mitomycin C (Sigma, St. Louis, MO) at a final concentration of 50 µg/ml at 37°C for 20 min and washed five times with an excess of CTCM before co-culture with purified CD4^+ T cells. Cultures were maintained in the presence of no exogenous cytokine (neutral conditions), 2 ng/ml IL-12 (Peprotech, Rocky Hill, NJ), 10 ng/ml IL-2 (Peprotech), 10 µg/ml anti-IL-2 (S4B6, BD Pharmingen, San Diego, CA), 10 µg/ml control antibody (R35-95, Pharmingen) or various combinations thereof. CD4^+ T cells were rested for 48 hrs on day 3 by removing 100 µl of culture supernatant and replacing it with 100 µl of medium containing 2 x 10^5 fresh feeder splenocytes without Ag and, depending on the culture conditions, cytokine or antibody at the final concentrations described above. CD4^+ T cells were given a secondary restimulation on day 5 by removing 100 µl of culture supernatant and replacing it with 100 µl of medium containing 2 x 10^5 fresh feeder splenocytes with 20 µg/ml Ag and, depending on the culture conditions, cytokine or antibody at the final concentrations described above. Cultures were analyzed by flow cytometry or BrdU ELISA either 24 hrs or 3 days after the secondary stimulation. Culture supernatants were removed for IFN-γ ELISA on day 3 after the secondary stimulation. To determine the number of live CD44^hi CD4^+ T cells in culture as an assessment of cell accumulation, a known number of washed, surfactant-free white sulfate latex beads (Interfacial Dynamics Corporation, Portland, OR) were added to each well
prior to harvest. Cells were stained and analyzed via flow cytometry as described below. A bead ratio was generated by dividing the number of beads collected during FACS acquisition by the number of beads added to the well. The number of live CD44\textsuperscript{hi} CD4\textsuperscript{+} T cells collected was determined using a typical live lymphocyte gate and then divided by the bead ratio to calculate the number of live CD44\textsuperscript{hi} CD4\textsuperscript{+} T cells present in each culture well.

**Flow cytometry.** To evaluate intracellular IFN-\(\gamma\) 24-hr after secondary stimulation, T cells were stimulated with PMA (50 ng/ml) and ionomycin (50 ng/ml) in the presence of brefeldin A (10 \(\mu\)g/ml) for 6 hrs prior to harvest. Cells were harvested, washed, stained with Cychrome-labeled anti-CD44 (IM7, Pharmingen) or the appropriate isotype controls and fixed. Intracellular IFN-\(\gamma\) was assayed using PE-labeled anti-IFN-\(\gamma\) (XMG1.2, Pharmingen) as previously described (6). Cells were acquired on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and data were analyzed using Flowjo software (Tree Star, Ashland, OR). For all samples analyzed via flow cytometry, the CFSE-labeled feeder layer was excluded from the analysis of the purified CD4\textsuperscript{+} T cells from infected mice. Surface staining for CD25 (PC61, eBioscience, San Diego, CA) was performed as previously described (3). To evaluate BrdU incorporation 3 days after primary stimulation or 24-hr after secondary stimulation, T cells were pulsed with 50 mM BrdU (Sigma) during the last 12 hrs of culture. Cells were then harvested, surface stained and fixed as described in (3). Following washes in FACS buffer (0.1% BSA and 0.1% sodium azide in PBS) and FACS buffer with 0.1% saponin, cells were incubated with 10 U of DNase I (Pierce Biotechnology, Rockford, IL), 0.15 M NaCl and 2.4 mM MgCl\(_2\) in 100 \(\mu\)l volume at 37°C for 1 hr. Cells were washed again in FACS buffer with saponin and stained using the PE-labeled anti-BrdU antibody set
(Pharmingen) according to manufacturer’s protocol. Cells were washed again in FACS buffer with saponin and then FACS buffer before acquisition.

**ELISAs.** Supernatants were assayed via ELISA for IFN-γ; sensitivity ranged between 39 and 156 pg/ml. All IFN-γ ELISA antibodies were purchased from Pharmingen and used according to manufacturer recommendations. Ag-pulsed mitomycin C-treated splenocytes alone were cultured under similar conditions as CD4⁺ T cells to determine baseline amount of cytokine production. BrdU ELISAs were conducted using the Cell Proliferation BrdU Colorimetric ELISA Kit (Roche Applied Science, Indianapolis, IN). Cultures were pulsed with 10 mM BrdU for 12 hrs and then transferred to flat-bottom 96-well plates before continuing with the manufacturer’s protocol. Ag-pulsed mitomycin C-treated splenocytes alone were cultured under similar conditions as CD4⁺ T cells to determine baseline amount of BrdU incorporation. For some primary stimulation cultures, tyrphostin AG490 (LC Laboratories, Woburn, MA) was added 24 hrs prior to harvest in the amounts indicated in the figure legend to inhibit Jak2 and/or Jak3 signaling.

**Statistical Procedure.** Statistical analysis was performed using Statview (SAS, Cary, NC). When treatment groups were compared, the data were analyzed with the Scheffe’s post-hoc test. When two treatments within a group were compared, data were analyzed using a paired t-test. Differences were considered significant when p < 0.05.
Results

Providing multiple in vitro Ag stimulations to CD4$^+$ T cells from *L. amazonensis*-infected mice does not enhance IFN-γ production to levels equivalent to that of CD4$^+$ T cells from *L. major*-infected mice.

We have previously reported that *L. amazonensis*-infected mice possess a population of antigen-responsive CD44$^{hi}$ CD4$^+$ T cells that proliferate and produce IL-2 but is not skewed towards either a Th1 or Th2 phenotype. Moreover, this population of T cells does not produce IFN-γ as efficiently as T cells isolated from *L. major*-infected mice following in vitro Ag stimulation in the presence IL-12 (1). Over the past few years, several studies have characterized central memory CD4$^+$ T cells—cells that, upon Ag-restimulation, proliferate and produce IL-2, but do not produce effector cytokines until a second Ag stimulation is provided (reviewed in (2)). To determine if the CD44$^{hi}$ CD4$^+$ T cells present in *L. amazonensis*-infected mice are a type of central memory T cell population, multiple in vitro Ag stimulations were provided to purified CD4$^+$ T cells from *L. amazonensis*-infected mice using a previously described protocol with some modifications (5). Briefly, CD4$^+$ T cells were purified from the DLN of mice at 4 wks post-infection and co-cultured with mitomycin C-treated splenocytes from naïve mice (an APC feeder layer) and Ag for a primary Ag stimulation. CD4$^+$ T cells were rested on day 3 of culture by adding fresh APC feeder cells without Ag. A secondary stimulation was provided on day 5 of culture using fresh Ag-pulsed APC feeder cells. Because CD44$^{hi}$ CD4$^+$ T cells are present in equivalent percentages ex vivo in both *L. amazonensis* - and *L. major*-infected mice (1), each well began the culture period with the same number of CD44$^{hi}$ CD4$^+$ T cells. CD4$^+$ T cells from *L. major*-infected mice were used as a benchmark for a productive Th1 response. Following a second Ag
stimulation, the percentage of CD44^hi CD4^+ T cells producing IFN-γ in cultures of T cells isolated from *L. amazonensis*-infected mice was only 32% of the percentage of CD44^hi CD4^+ T cells producing IFN-γ in cultures of T cells isolated from *L. major*-infected mice (Figure 1A). Also, the amount of IFN-γ detected in the culture supernatants of T cells from *L. amazonensis*-infected mice was only 29% of that detected in the culture supernatants of T cells from *L. major*-infected mice (7.0 ± 2.1 ng/ml and 24.0 ± 6.4 ng/ml, respectively). These data indicate that a second Ag stimulation is not sufficient to enhance IFN-γ production from CD44^hi CD4^+ T cells isolated from *L. amazonensis*-infected mice to levels equivalent to CD44^hi CD4^+ T cells from *L. major*-infected mice.

A possible explanation for the enhanced IFN-γ production of CD44^hi CD4^+ T cells from *L. major*-infected mice could be that these cells have an enhanced activation status. To address this, we examined expression of the high-affinity IL-2 receptor (CD25) on CD44^hi CD4^+ T cells from *Leishmania*-infected mice. Ex vivo, 20% of the CD44^hi CD4^+ T cells from *L. amazonensis* and *L. major*-infected mice were CD25^+ (Figures 1B & C). That percentage did not increase significantly after a 3-day primary Ag-stimulation in vitro. After the 2-day rest, however, the percentage of CD25^+ CD44^hi CD4^+ T cells more than doubled for both cultures and continued to increase during the secondary stimulation. Three days after the secondary restimulation, over 80% of the CD44^hi CD4^+ T cells were CD25^+ in both cultures; however, the mean fluorescence intensity (MFI) of CD25 expression on CD44^hi CD4^+ T cells from *L. amazonensis* cultures was 237 ± 8.2 while those cells from *L. major* cultures had an MFI of 100 ± 5.7 (Figure 1D). These data indicate that, based on CD25 expression, CD44^hi CD4^+ T cells from *L. amazonensis*-infected mice exhibit a resting (CD25 negative) then activated (CD25 positive) effector phenotype after primary and
L. amazonensis

L. major

ex vivo primary stim rest 24h secondary stim 3d secondary stim

CD25

L. amazonensis

L. major

ex vivo primary stim rest 24h stim 3d stim

MFI

L. amazonensis

L. major

neutral

IL-2
secondary in vitro Ag stimulations, respectively. Neutralizing IL-2 in vitro significantly reduced CD25 expression for CD44hi CD4+ T cells from L. amazonensis-infected mice while the addition of exogenous IL-2 to the cultures significantly increased CD25 expression on CD44hi CD4+ T cells from L. major-infected mice (Figures 1E & F). Together, these data describe a population of CD4+ T cells from L. amazonensis-infected mice that sustain high, IL-2-dependent expression of CD25 following a secondary Ag stimulation.

CD44hi CD4+ T cells from L. amazonensis-infected mice enhance IFN-γ production in response to IL-12 but fail to accumulate in culture as well as those from L. major-infected mice.

Previous work from our lab evaluated IL-12 responsiveness of CD4+ T cells after a primary Ag stimulation, which would correspond to T cells with a resting effector phenotype, and showed that CD4+ T cells from L. amazonensis-infected mice cultured in vitro in the presence of IL-12 and Ag produced only 10-15% of the IFN-γ produced by L. major CD4+ T cells cultured under identical conditions (1). Given the different activation status of CD44hi CD4+ T cells during the primary and secondary stimulations, we wished to assess IL-12 responsiveness of activated effector cells during the secondary Ag stimulation. After maintaining CD4+ T cells in IL-12 throughout the entire culture period, the overall amount of IFN-γ produced by T cells isolated from L. amazonensis-infected mice was enhanced in response to IL-12, increasing from 7 ng/ml to 65 ng/ml, but that amount was only 55% of the IFN-γ produced by T cells isolated from L. major-infected mice (Figure 2A). The percentage of CD44hi CD4+ IFN-γ+ T cells, however, was equivalent in the presence of IL-12 (Figure 2B). Mean fluorescence intensities were also similar between both cultures, indicating
similar amounts of IFN-γ were being produced per CD44^{hi} CD4^{+} T cell in response to IL-12 in both *L. amazonensis* and *L. major* CD4^{+} T cell cultures (data not shown). These data indicate that although a second Ag stimulation in the presence of IL-12 is sufficient to elicit equivalent percentages of IFN-γ-producing CD44^{hi} CD4^{+} T cells in cultures of T cells derived from *L. amazonensis*- and *L. major*-infected mice, it does not result in equivalent absolute amounts of IFN-γ in both cultures.

One plausible explanation for this deficit in total IFN-γ produced by T cells from *L. amazonensis*-infected mice in response to IL-12 could be the failure of CD44^{hi} CD4^{+} T cells from *L. amazonensis*-infected mice to accumulate (including both to proliferate and survive) in culture. Cell accumulation was assessed using latex beads as described in materials and methods. The number of live CD44^{hi} CD4^{+} T cells in the cultures of T cells derived from *L. amazonensis*-infected mice was found to be only 59% of the number present in the cultures of T cells from *L. major*-infected mice in the presence of IL-12 (Figure 2C). A similar phenomenon was observed under neutral conditions as the number of live CD44^{hi} CD4^{+} T cells in the cultures of T cells derived from *L. amazonensis*-infected mice was found to be only 71% of the number present in the cultures of T cells from *L. major*-infected mice (data not shown). Considering that both cultures began with the same number of live CD44^{hi} CD4^{+} T cells, this data indicates that CD44^{hi} CD4^{+} T cells from *L. amazonensis*-infected mice do not accumulate in culture as well as those from *L. major*-infected mice. This phenomenon can account for the 60% deficit observed in the total amount of IFN-γ produced by T cells from *L. amazonensis*-infected mice in response to IL-12.
Figure 2. CD44^hi^CD4^+^ T cells from *L. amazonensis*-infected mice enhance IFN-γ production in response to IL-12 but fail to accumulate in culture as well as those from *L. major*-infected mice. CD4^+^ T cells were cultured in the presence of IL-12 as described in materials and methods. (A) Culture supernatants were harvested 3 days post-secondary Ag stimulation and assayed via ELISA. (B) T cells were harvested 24 hours post-secondary Ag stimulation and assayed via flow cytometry for CD44 and IFN-γ expression. (C) T cells were harvested as in B and assayed via flow cytometry for CD44 expression. The number of CD44^hi^CD4^+^ T cells was determined as in materials and methods; number of CD44^hi^CD4^+^ T cells present in cultures of T cells from *L. amazonensis*-infected mice is expressed as a percentage of the number of CD44^hi^CD4^+^ T cells present in cultures of T cells from *L. major*-infected mice. For all panels, data are represented as the mean ± the SEM of nine (A) or six (B & C) separate experiments. * represents a statistically significant difference at p < 0.05 as determined by Scheffe’s test.
Resting effector CD4^+ T cells from *L. major*-infected mice respond to IL-12 and IL-2 by enhancing proliferation whereas cells from *L. amazonensis*-infected mice do not during primary Ag stimulation.

CD44^hi^ CD4^+^ T cells from *L. amazonensis*-infected mice may fail to accumulate in vitro compared to T cells from *L. major*-infected mice either because of enhanced proliferation of *L. major* CD44^hi^ CD4^+^ T cells or because CD44^hi^ CD4^+^ T cells from *L. amazonensis*-infected mice experience increased cell death in culture. ELISA analysis of BrdU incorporation during the last twelve hours of the 3-day primary stimulation revealed that the presence of IL-12 significantly increased the BrdU incorporation (47% increase) of T cells isolated from *L. major*-infected mice (Figure 3A). While the presence of IL-12 also significantly increased BrdU incorporation in cultures of T cells from *L. amazonensis*-infected mice, the increase was only 18% greater than cultures with no IL-12. An analysis of BrdU incorporation via flow cytometry during the last twelve hours of the 3-day primary Ag stimulation revealed that the percentage of CD44^hi^ CD4^+^ T cells from *L. major*-infected mice incorporating BrdU increased almost two-fold in response to IL-12 while CD44^hi^ CD4^+^ T cells from *L. amazonensis*-infected mice failed to exhibit any enhanced proliferation in the presence of IL-12 (Figure 3B), indicating that the slight increase observed in BrdU incorporation as assessed via ELISA could likely be attributed to improved cell survival rather than proliferation. Proliferation was equivalent between T cells from *L. amazonensis*- and *L. major*-infected mice in neutral conditions (no addition of exogenous cytokines) when assessed by both ELISA and flow cytometry. Together, this data demonstrates that CD44^hi^ CD4^+^ T cells from *L. major*-infected mice respond to IL-12 in vitro by enhancing proliferation whereas those cells from *L. amazonensis*-infected mice do not.
Figure 3. CD44^hi^CD4^+^ T cells from *L. amazonensis*-infected mice are non-responsive to IL-2 and IL-12. (A) Cells were cultured in the presence or absence of IL-12 for 3 days post-primary Ag stimulation and pulsed with BrdU during the final 12 hours of culture. BrdU incorporation was assessed via ELISA. (B) Cells were cultured as in A. CD44 expression and BrdU incorporation were assessed via flow cytometry. (C) Cells were cultured in the presence or absence of IL-2 or (D) with either a neutralizing antibody against IL-2 or a control antibody for 3 days post-primary Ag stimulation and pulsed with BrdU during the final 12 hours of culture. BrdU incorporation was assessed via ELISA. For all panels, data are represented as the mean ± the SEM of four (A, C & D) or six (B) separate experiments. * represents a statistically significant difference between treatments within a group at p < 0.05 as determined by paired t test.
Considering IL-2 has been described as a T cell proliferative factor in vitro (reviewed in (7)), we assessed the effect of IL-2 on proliferation of T cells from *Leishmania*-infected mice during the primary Ag stimulation. T cells were maintained in the presence of IL-2 throughout the entire culture period, and BrdU incorporation during the last twelve hours of the 3-day primary Ag stimulation was assessed via ELISA. The addition of exogenous IL-2 to cultures of T cells derived from *L. major*-infected mice resulted in a significant increase in BrdU incorporation (49% increase) over neutral conditions (Figure 3C). While not significant (p = 0.062), a 25% increase in BrdU incorporation was observed for T cell cultures isolated from *L. amazonensis*-infected mice in response to IL-2. Neutralization of IL-2 in cultures of T cells isolated from *L. major*-infected mice T cells decreased BrdU incorporation by 32% while only decreasing BrdU incorporation by 17% in cultures of T cells from *L. amazonensis*-infected mice (Figure 3D). Altogether, these data indicate that resting effector T cells from *L. amazonensis*-infected mice are less responsive to both IL-2 and IL-12.

Cytokine-mediated proliferation primarily involves the Jak-STAT pathway. While it is well documented that Jak3 plays a critical role in activating Stat5 proteins via IL-2 (reviewed in (8)), the mechanism by which IL-12 promotes proliferation is less understood. Some reports indicate that IL-12 can signal through Jak2 and STAT5 to promote proliferation (9, 10). Despite the decreased responsiveness of *L. amazonensis* CD4+ T cells to IL-2 and IL-12, these cells still exhibit a basal level of proliferation in the presence of these cytokines. We wished to determine if CD4+ T cells from *L. amazonensis*-infected mice still require Jak2 and Jak3 for proliferation. AG490 is a member of the synthetically derived family of tyrosine kinase inhibitors (11-13). At low doses (1 to 25 μM), AG490 inhibits Jak2
signaling and at higher doses (> 25 μM), it inhibits both Jak2 and Jak3 signaling (13, 14). AG490 does not inhibit other known lymphocytic tyrosine kinases such as Lck, Lyn, Btk, Syk, Src, Jak1 or Tyk2 (13). AG490 was added to CD4⁺ T cells from *L. amazonensis*- and *L. major*-infected mice cultured in the presence of IL-12. Proliferation was assessed via ELISA for BrdU incorporation. Proliferation in response to IL-12 by CD4⁺ T cells from *L. major*-infected mice appeared to not be mediated by Jak2, as there was no significant decrease in BrdU incorporation compared at low doses of AG490 as compared to the DMSO control (Figure 4B). High doses of AG490 significantly decreased proliferation for CD4⁺ T cells derived from both *L. amazonensis*- and *L. major*-infected mice (Figures 4A & B). The similar response curves for cultures of T cells from both *L. amazonensis*- and *L. major*-infected mice suggest that Jak3 is an important mediator of proliferation for resting effector CD4⁺ T cells from both *L. amazonensis*- and *L. major*-infected mice. These data also indicate that even though CD4⁺ T cells from *L. amazonensis*-infected mice respond poorly to IL-2, they still utilize Jak3 to help mediate proliferation, perhaps in response to another member of the common gamma chain family of cytokines.

**IL-2 limits enhanced proliferation in response to IL-12 for activated effector CD4⁺ T cells present during secondary Ag stimulation.**

Synergy between IL-2 and IL-12 in terms of IFN-γ production and proliferation has been described (15, 16). During secondary stimulation, IL-2 and IL-12 act in concert to promote IFN-γ production from activated effector CD4⁺ T cells isolated from *L. amazonensis*-infected mice (Figure 5A). Despite this synergy, the IFN-γ produced by CD4⁺ T cells from *L. major*-
**Figure 4.** Jak3 is involved in proliferation of CD4\(^+\) T cells from *L. amazonensis*-infected mice. CD4\(^+\) T cells from *L. amazonensis*- (A) or *L. major*-infected mice (B) were cultured with IL-12 as described in materials and methods for 3 days post-primary Ag stimulation. AG490 or DMSO alone was added during the final 24 hours of culture; cells were pulsed with BrdU during the final 12 hours of culture. BrdU incorporation was assessed via ELISA. Data are represented as the mean ± the SEM of three separate experiments. * represents a statistically significant difference from the DMSO control treatment at p < 0.05 as determined by paired t test.

Infected mice is still significantly more than is produced by CD4\(^+\) T cells from *L. amazonensis*-infected mice (data not shown). As in the primary stimulation, CD44\(^{hi}\) CD4\(^+\) T cells from *L. amazonensis* assayed during the secondary stimulation for BrdU incorporation are still non-responsive to both IL-2 and IL-12 in terms of enhanced proliferation (Figure 5C). BrdU incorporation is significantly decreased in the presence of both IL-2 and IL-12 during the secondary stimulation. However, neutralization of IL-2 promotes enhanced proliferation of CD44\(^{hi}\) CD4\(^+\) T cells from *L. amazonensis*-infected mice in response to IL-12 (Figure 5D), but it does not limit IFN-γ production in response to IL-12 (Figure 5B). CD4\(^+\) T
cells from *L. amazonensis*-infected mice maintained in the presence of IL-2, IL-12 or IL-2 plus IL-12 throughout the entire culture period significantly increased cell accumulation while neutralization of IL-2 throughout the entire culture period significantly decreased cell accumulation (Figures 5C &D), indicating that these cytokines may promote cell survival since they do not enhance proliferation. Taken together, these results indicate that IL-2 suppresses enhanced proliferation but not enhanced IFN-γ production in response to IL-12 in activated effector CD4⁺ T cells.

**Discussion**

The results reported in this study extend our understanding of the CD44⁹⁹CD4⁺ T cell population present during *L. amazonensis* infection. While previous work has demonstrated that this cell population is capable of Ag responsiveness, proliferation and IL-2 production, it remains unskewed towards either a Th1 or Th2 phenotype. Additionally, IL-12 enhanced the IFN-γ production of these CD4⁺ T cells, albeit limited, demonstrating that the failure of CD4⁺ T cells from *L. amazonensis*-infected mice to become efficient Th1 effector cells is due to factors beyond the presence or absence of IL-12 (1). The data presented in this study indicate that IL-2 may be a factor involved in limiting the immune response during *L. amazonensis* infection as it limits enhanced proliferation of CD44⁹⁹CD4⁺ T cells in response to IL-12.

In this present study, we find that multiple in vitro Ag stimulations fail to promote equivalent IFN-γ production between CD4⁺ T cells from *L. amazonensis*- and *L. major*-infected mice, indicating that the CD44⁹⁹CD4⁺ T cell population present in *L. amazonensis*-infected mice does not exhibit the functional characteristics of central memory CD4⁺ T cells.
Figure 5. IL-2 suppresses enhanced proliferation in response to IL-12 for activated effector CD4\(^+\) T cells from *L. amazonensis*-infected mice. CD4\(^+\) T cells were cultured as described in materials and methods in (A) the presence or absence of IL-2, IL-12 or IL-2 and IL-12 or (B) with a neutralizing antibody against IL-2 or a control antibody in the presence or absence of IL-12. Culture supernatants were harvested 3 days post-secondary Ag stimulation and assayed for IFN-\(\gamma\) via ELISA. Data are represented as the mean ± the SEM of three separate experiments. Treatments with different letters are significantly different from one another at \(p < 0.05\) as determined by Scheffe’s test. (C & D) Cells were cultured as described in A & B. To assess proliferation, cells were pulsed with BrdU during the final 12 hours of culture; BrdU incorporation was assessed via ELISA 24 hours post-secondary Ag stimulation. To assess cell numbers, the number of live CD44\(^hi\) CD4\(^+\) T cells was determined as described in materials and methods; this procedure was performed to ensure that enhanced BrdU incorporation was indicative of enhanced proliferation and not a result of greater numbers of cells in culture. Data are represented as the mean ± the SEM of three separate experiments except for treatment with IL-2 plus IL-12, which is two experiments. * represents a statistically significant difference at \(p < 0.05\) as determined by Scheffe’s test.
Instead, multiple Ag stimulations in the presence of IL-12 did yield equivalent percentages of IFN-γ-producing CD44^hi CD4^+ T cells in both *L. amazonensis* and *L. major* T cell cultures. However, the total amount of IFN-γ produced by T cells isolated from *L. amazonensis*-infected mice was 40% less than that produced by CD4^+ T cells from *L. major*-infected mice; a deficit that correlated with a failure of CD44^hi CD4^+ T cells from *L. amazonensis*-infected mice to accumulate in culture (Figure 2). In contrast to CD44^hi CD4^+ T cells from *L. major*-infected mice, CD44^hi CD4^+ T cells from *L. amazonensis*-infected mice are less responsive to both IL-2 or IL-12 (Figure 3). However, neutralization of IL-2 allows enhanced proliferation of CD44^hi CD4^+ T cells from *L. amazonensis*-infected mice in response to IL-12 (Figure 5). Together, these results indicate that IL-2 suppresses enhanced proliferation in response to IL-12 for activated effector CD4^+ T cells from *L. amazonensis*-infected mice, thus limiting CD44^hi CD4^+ T cell accumulation in vitro.

Based on these data, the lack of equivalent amounts of IFN-γ between *L. amazonensis* and *L. major* CD4^+ T cell cultures can be explained by an IL-2-mediated reduction of in vitro accumulation of IFN-γ-producing CD44^hi CD4^+ T cells from *L. amazonensis*-infected mice. However, IL-2 neutralization does not enhance IFN-γ production in the presence of IL-12 relative to the control antibody-treated cultures (Figure 5B). We suggest that in an unskewed T cell population, such as that observed in *L. amazonensis*-infected mice, endogenously produced IL-2 functions to limit enhanced proliferation in response to IL-12, whereas in classical Th1 cells, including those from *L. major*-infected mice, endogenously produced IL-2 promotes IFN-γ production. In fact, neutralization of IL-2 in the presence of IL-12 resulted in similar amounts of IFN-γ produced by cultures of T cells from *L. amazonensis*- and *L. major*-infected mice (data not shown). This occurred not because of enhanced IFN-γ
production by T cells from *L. amazonensis*-infected mice, but because neutralizing IL-2 in the presence of IL-12 significantly decreased the amount of IFN-γ produced by CD4⁺ T cells from *L. major*-infected mice (data not shown).

During the primary in vitro stimulation, two methods of assessing BrdU incorporation were employed. Because previous work via FACS analysis demonstrated that greater than 90% of CD4⁺ T cells incorporating BrdU were CD44⁺, we chose to utilize the ELISA method for the majority of our BrdU incorporation experiments to increase the power of our analysis. Our BrdU ELISA results indicate that CD4⁺ T cells from *L. amazonensis*-infected mice are unresponsive to both IL-2 and IL-12. In contrast, a considerably greater increase in BrdU incorporation is observed for CD4⁺ T cells from *L. major*-infected mice in response to either IL-12 or IL-2 and more likely to represent both enhanced proliferation and survival.

In order for IL-12 to promote IFN-γ production, signal transduction through the IL-12 receptor must result in phosphorylation of the Janus kinase Tyk2, which subsequently phosphorylates and activates signal transducer and activator of transcription 4 (STAT4) (17, 18). In the current study, the ability of CD44⁺ CD4⁺ T cells from *L. amazonensis*-infected mice to enhance IFN-γ production but not proliferation in response to IL-12 indicates that these two activities may be mediated by separate signaling pathways in this CD4⁺ T cell population which diverge downstream of the IL-12 receptor. Although previous reports indicate that IL-12 mediates its proliferative effects via Jak2 signaling (9, 10), our results do not support that observation for CD4⁺ T cells derived from *L. major*-infected mice (Figure 4). Instead, our data indicate a role for Jak3 in mediating proliferation of resting effector CD4⁺ T cells from both *L. amazonensis*- and *L. major*-infected mice in both the presence and absence of IL-12 (Figure 4 and data not shown). Jak3 is predominantly involved in mediating
proliferative signals from cytokines acting through receptors containing the gamma common chain. Our results indicate that proliferation of CD4\(^+\) T cells from *L. amazonensis*-infected mice may be mediated by a member of the common gamma chain family of cytokines including IL-7, IL-15 or IL-21, and may be independent of IL-2 (Figure 3) and IL-4 (data not shown).

Our data demonstrates that CD4\(^{4hi}\) CD4\(^+\) T cells from both *L. amazonensis* and *L. major*-infected mice have low yet similar CD25 expression ex vivo and during the primary Ag stimulation and thus exist as “resting” effector cells during these periods. These CD4\(^{4hi}\) CD4\(^+\) T cells then transition to “activated” effector cells during the rest and secondary Ag restimulation as evidenced by their enhanced CD25 expression. Moreover, CD4\(^{4hi}\) CD4\(^+\) T cells from *L. amazonensis*-infected mice sustain a higher CD25 expression longer after secondary Ag restimulation than CD4\(^{4hi}\) CD4\(^+\) T cells from *L. major*-infected mice. Moreover, IL-2 regulates the expression of CD25 on CD4\(^{4hi}\) CD4\(^+\) T cells from *L. amazonensis*-infected mice, as neutralizing IL-2 decreases CD25 expression on these cells (Figure 1E). IL-2 seems to have two opposing functions in T cell biology—potentiating T cell proliferation and terminating T cell responses. IL-2 has been described as a potent stimulator of naïve T cell growth and proliferation in vitro (7). More recently, however, its role in vivo has been described as one for maintaining tolerance instead of amplifying T cell responses. The primary mechanism by which IL-2 elicits its tolerogenic influence in vivo is via regulatory T (T\(_{reg}\)) cells, most of which constitutively express the high affinity IL-2 receptor (19, 20). IL-2 has also been shown to promote a stimulation refractoriness phenotype in activated CD4\(^+\) T cells cultured in vitro in the presence of IL-2 (21-23). T cells cultured continuously in the presence of IL-2 were found to be unresponsive to antigenic
restimulation for a period after initial stimulation (21); furthermore, these stimulation refractory T cells suppressed the proliferative response of naïve T cells much like conventional T_reg cells (23). Our data suggest that CD44^{hi} CD4^+ T cells from *L. amazonensis*-infected mice may experience a similar IL-2-mediated refractoriness following in vitro restimulation, as neutralization of IL-2 promotes enhanced proliferation in response to IL-12. Work by Norris et al demonstrates that the inhibitory effect of IL-2 is dependent upon the activation status of the T cell, as IL-2 promotes antigen reactivity of resting but not activated T cells (22). Unlike the T cells described by Norris et al, CD44^{hi} CD4^+ T cells from *L. amazonensis*-infected mice are non-responsive to both IL-2 and IL-12 as resting effector cells. This difference may result because our study utilized CD4^+ T cells isolated from infected rather than naïve animals. Nevertheless, we also observed an inhibitory effect for IL-2 on activated T cells from *L. amazonensis*-infected animals.

Various models proposed by Grossman, Paul and colleagues predict that the strength and quality of an antigenic stimulation as well as the amount of antigen present during stimulation influence the behavior of a T cell. T cells possess different capacities for either self-renewal or differentiation—two competing cellular events that are regulated by recurrent interactions with antigen, APCs and other T cells (24-26). Under conditions of recurrent or continual antigen stimulation, effector cells may limit their own accumulation and differentiation by changing the balance between self-renewal and differentiation. The tunable activation threshold (TAT) model proposed by Grossman and Paul states that T cell activation is a threshold phenomenon and that the threshold is tuned by the stimulatory experience of the cell (25, 27). T cells are predicted to adapt to continuous or recurrent stimulation by updating their threshold for activation, thereby preventing resting T cells from
becoming activated effector cells in response to ambient interactions. Adaptively tolerant T cells have been shown to partially recapitulate this predicted behavior in vivo. Adaptively tolerant T cells are mature T cells that have adapted to persistent antigen stimulation by down-modulating (or tuning) their sensitivity to specific antigen restimulation (28). These T cells differentiate to various degrees and then down-regulate their production of effector cytokines, including IFN-γ and IL-4, in the presence of persistent antigen (29, 30). Some characteristics of activation tuning by adaptively tolerant T cells can also be observed following certain persistent viral infections. Chronic lymphocytic choriomeningitis virus (LCMV) infection of mice has been shown to result in virus-specific CD8$^+$ T cells that expressed activation markers and proliferated in vivo but were unable to elicit any antiviral effector functions (31, 32). We and others have observed impaired effector cytokine production of antigen-specific CD4$^+$ T cells present during chronic infections such as those caused by L. amazonensis, hepatitis C virus, Mycobacterium tuberculosis, human immunodeficiency virus and gamma herpes virus (1, 33-36).

In addition to Ag, cytokines can also influence the balance between self-renewal and differentiation (26). The presence of regulatory cytokines such as IL-10 or TGF-β can induce differentiation towards a suppressive or regulatory phenotype. These cytokines act upon and create tolerogenic antigen presenting cells, which then induce T$_{reg}$ cells. These T$_{reg}$ cells, in turn, suppress the proliferation and subsequent differentiation of other CD4$^+$ T cells (37-39). Alternatively, an increase in the concentration of regulatory cytokines may act directly on the conventional antigen-specific T cells themselves, thereby conferring suppressive activity to those cells. This phenomenon could have a spreading (or “infectious”) character, as these cells may recruit other T cells stimulated at the same time to
acquire a suppressor/regulatory function (26). We propose that high amounts of IL-2 exist during in vitro culture of CD44<sup>hi</sup> CD4<sup>+</sup> T cells from <i>L. amazonensis</i>-infected mice. In turn, IL-2 prevents enhanced proliferation and accumulation of IL-12 responsive CD4<sup>+</sup> T cells in the DLN. Due to the sustained, elevated expression of CD25 on CD44<sup>hi</sup> CD4<sup>+</sup> T cells from <i>L. amazonensis</i>-infected mice, it is tempting to speculate that these T cells are a type of regulatory T cell.

Chronic <i>L. amazonensis</i> infection is characterized by the absence of a robust inflammatory response, as evidenced by decreased IL-12 production and decreased mRNA expression of multiple inflammatory mediators (6, 40, 41), as well as by a high parasite load due to enhanced resistance of the parasite to macrophage-mediated killing (42, 43). We propose that the CD44<sup>hi</sup> CD4<sup>+</sup> T cells present in vivo during <i>L. amazonensis</i> infection have adapted to the continual Ag stimulation by becoming tolerant, and that continual antigen stimulation due to the high parasite load present during infection promotes CD4<sup>+</sup> T cell self-renewal. IL-2 from unpolarized CD4<sup>+</sup> T cells may in turn promote the development and survival of an antigen-specific regulatory T cell population that limits the differentiation and expansion of Th1 CD4<sup>+</sup> T cells even in the presence of IL-12.

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References


Chapter 4

*Leishmania amazonensis*-infected mice respond in vivo to exogenous IL-12 by developing a Th1 response but fail to create a population of antigen-responsive IL-12-producing B cells

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Abstract

Infection of C3H mice with *Leishmania major* results in a productive Th1 response and the mice subsequently heal their infections. In contrast, infection of C3H mice with *L. amazonensis* creates chronic cutaneous lesions; a susceptible phenotype thought to result from an inability of these mice to develop a Th1 response. We demonstrate that although administration of exogenous IL-12 to mice at the time of *L. amazonensis* infection promotes a Th1 response at two weeks post-infection, these mice do not maintain that Th1 response and fail to heal their infections. IL-12 is necessary for maintaining a Th1 response, we examined whether or not administration of exogenous IL-12 and the subsequent Th1 response that developed promoted endogenous IL-12 production. At two weeks post-infection, IL-12p40 production is enhanced in draining lymph node cell cultures from IL-12-treated *L. amazonensis*-infected mice compared to control mice, but by ten weeks post-infection there is no difference between the two groups. We found that CD19⁺ B cells are a source of antigen-specific IL-12 during late *L. major* infection in contrast to CD19⁺ B cells
present during chronic \textit{L. amazonensis} infection. IL-12 treatment does not promote a sustained source of antigen-specific IL-12 from B cells in these mice. Together, these results describe that the failure of IL-12-treated mice to heal their infections is not due to an inability to respond to IL-12 by developing a Th1 response. Instead, the chronicity of \textit{L. amazonensis} infection may result, in part, by a lack of sustained, antigen-specific IL-12 production from B cells.

**Introduction**

Experimental infection of inbred mice with various \textit{Leishmania} spp. that cause cutaneous leishmaniasis has facilitated an understanding of some of the immune mechanisms necessary for control of these intracellular protozoan parasites. C3HeB/FeJ and C57BL/6 mice are able to control an \textit{L. major} infection by developing a Th1 response, including both a sustained source of IL-12 and IL-12-responsive CD4$^+$ T cells that upregulate the IL-12 receptor $\beta 2$ (IL-12R$\beta 2$), express the Th1-associated transcription factor, T-bet, and produce IFN-$\gamma$ (13, 15, 24, 37). These mice ultimately heal their cutaneous lesions, which contain very few parasites by ten weeks post-infection (reviewed in (26, 28)). Infection of C3HeB/FeJ and C57BL/6 mice with \textit{L. amazonensis}, however, results in chronic cutaneous lesions that contain up to $10^8$ parasites by ten weeks post-infection and low to undetectable levels of both IFN-$\gamma$ and IL-4 in the \textit{in vitro} recall responses of draining lymph node (DLN) cells (1, 14, 17). The susceptible phenotype of these mice is described as a lack of a productive Th1 response rather than the presence of a Th2 response, as IL-4 and IL-10 knock-out mice fail to heal their infections (16, 17).
The development of a Th1 response is dependent upon the presence of IL-12 and the ability of CD4\(^+\) T cells to respond to IL-12 (10, 35, 36). Previous work has found that draining lymph node cells from \textit{L. amazonensis}-infected mice produced low amounts of IL-12 when compared with those from \textit{L. major}-infected mice and that they fail to express a functional IL-12R (17). That same study also showed that administration of IL-12 to \textit{L. amazonensis}-infected mice at the time of infection does not promote healing, presumably because of the failure to upregulate IL-12R\(\beta\)2 (17). A subsequent study has demonstrated that mice chronically infected with \textit{L. amazonensis} can respond to IL-12 when administered with antigen-pulsed bone marrow-derived dendritic cells as determined by enhanced T-bet and IL-12R\(\beta\)2 mRNA expression (38). This data suggests that the failure of IL-12-treated \textit{L. amazonensis}-infected mice to heal is not due solely to an inability to respond to IL-12. In this study, we have further investigated the IL-12 responsiveness of CD4\(^+\) T cells during in vivo administration of exogenous IL-12 and infection with \textit{L. amazonensis}.

We provide evidence that \textit{L. amazonensis}-infected mice treated with IL-12 at the initiation of infection do develop a CD4\(^+\) Th1 response similar to that of \textit{L. major}-infected mice, yet this response is not maintained and the mice subsequently fail to heal their infection. Also in this study, we demonstrate that CD19\(^+\) B cells are a source of antigen-specific IL-12 during chronic \textit{L. major}-infection. Although IL-12 treatment of \textit{L. amazonensis}-infected mice promoted early endogenous IL-12 production by predominantly CD11c\(^+\) cells, these mice failed to develop a population of B cells that produced IL-12 in response to antigen (Ag). Our results indicate that the failure of \textit{L. amazonensis}-infected mice treated with exogenous IL-12 to heal is not due to an inability of the CD4\(^+\) T cells to respond to IL-12 or to an inability of CD11c\(^+\) cells to produce IL-12. Instead, chronic
infection may result, in part, because these mice fail develop a population of Ag-responsive, IL-12-producing B cells to help maintain the Th1 response.

Materials and Methods

Parasites and antigens. Culture of *L. amazonensis* (MHOM/BR/00/LTB0016) and *L. major* (MHOM/IL/80/Friedlin) and preparation of promastigote parasite antigens were performed as previously described (16). For amastigote parasite antigens, amastigotes were isolated from the footpads of infected C3H SCID mice, washed three times in PBS, resuspended in PBS, sonicated and subjected to three freeze-thaw cycles consisting of −80°C and 37°C. Protein concentration was determined using the bicinchoninic acid assay, final concentration was adjusted to 1 mg/ml and aliquots were stored at −80°C until use. In all experiments involving Ag stimulation, cells were stimulated with the matching freeze-thawed promastigote Ag. The parasite burden of infected footpads was determined using a limiting dilution assay as previously described (1) and expressed as the negative log of parasite titer.

Mice. Female C3HeB/FeJ mice (six to eight weeks of age) were either bred in-house or obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free facility. Mice were infected with 5 x 10⁶ stationary phase promastigotes in 50 μl PBS in the left hind footpad. At the time of infection, some *L. amazonensis*-infected mice were also injected with 0.2 μg of IL-12 (Peprotech, Rocky Hill, NJ). These mice received 0.2 μg of IL-12 in 25 μl of PBS intralesionally every other day for two weeks (a total of six injections) while control *L. amazonensis* - and *L. major*-infected mice received PBS injections of 25 μl. Lesion size was monitored with a dial micrometer and expressed as the difference
in footpad thickness between the infected and uninfected feet. Between three and five mice were pooled per group for each experiment and were sacrificed at either two or ten weeks post-infection as indicated in the figure legends. The IACUC at Iowa State University approved all protocols involving animals.

**Flow cytometry.** To evaluate intracellular IFN-γ, 1 x 10^6 draining lymph node (DLN) cells were plated per well in a 96-well U-bottom plate with 50 μg/ml of Ag in complete tissue culture medium (CTCM; DMEM containing 4.5 mg of glucose/ml, 2 mM L-glutamine, 100 U penicillin, 100 μg streptomycin/ml, 25 mM HEPES, 0.05 μM 2-mercaptoethanol and 10% fetal bovine serum). After 18 h, cells were stimulated with phorbol 12-myristate-13-acetate (PMA, 50 ng/ml) and ionomycin (50 ng/ml) in the presence of brefeldin A (10 μg/ml) for 6 h. Cells were harvested, washed, stained with FITC-labeled anti-CD4 (H129.19), Cyochrome-labeled anti-CD44 (IM7) or the appropriate isotype controls and fixed in paraformaldehyde. Intracellular cytokines were assayed as described in (17) using PE-labeled IFN-γ (XMG1.2) or an appropriate isotype control. All antibodies were obtained from Pharmingen (San Diego, CA) unless stated otherwise. Cells were acquired on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and data were analyzed using CellQuest software (Becton Dickinson).

**Measurement of cytokine responses.** For recall responses, 1 x 10^6 DLN cells were cultured in each well of a 96-well plate with or without 50 μg/ml of Ag in CTCM. After 3 d, supernatants were harvested and assayed via ELISA for IFN-γ; sensitivity ranged between 39 and 156 pg/ml. To assay IL-12p40 production, 1 x 10^6 DLN cells were cultured in each well
of a 96-well plate with or without 50 μg/ml of Ag in CTCM. After 24 hr, supernatants were harvested and assayed via ELISA for IL-12p40 with sensitivity to 39 pg/ml. All ELISA antibodies were purchased from Pharmingen and used according to manufacturer recommendations. The IL-12p40 ELISPOT protocol was adapted from Scharton-Kersten et al (30) and used the same pair of antibodies as used in the IL-12p40 ELISA. Briefly, Immulon II plates (Dynex Technologies, Chantilly, VA) were coated with antibody overnight at 4°C. The next day, plates were washed with sterile PBS and blocked with 5% fetal calf serum in PBS for 2 hours. Freshly isolated cells were added to the wells in CTCM and serially diluted. *Leishmania* Ag was added to some wells at a final concentration of 50 μg/ml. Plates were incubated overnight at 37°C and 5% CO₂ and then washed four times in PBS. The secondary antibody was added overnight at 4°C, followed by extensive washing with Tween/PBS and PBS the next day. Plates were developed by adding an alkaline phosphatase conjugate for 30 min at 37°C, washing and adding the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Fisher, Hanover Park, IL) diluted to 1 mg/ml in a solution of 0.1 M 2-amino-2-methyl-1-propanol (ICN Biomedicals, Aurora, OH) and 0.6% low-melt agarose (Fisher). Spots, which represent single IL-12p40-producing cells, were counted the next day using a platform microscope.

**CD11c depletion and B cell purification.** CD11c⁺ cells were depleted from lymph nodes via magnetic positive selection using anti-CD11c MicroBeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol. Cells were subjected to two passes through an AutoMACS cell sorter. B cells were purified from lymph nodes via magnetic depletion using the MagCellect B cell isolation kit (R & D Systems, Minneapolis, MN). Cells were
labeled with the biotin-conjugated antibody cocktail and streptavidin solution according to the manufacturer's recommendations, and then subjected to two passes through an AutoMACS cell sorter. The purity of the CD19\(^+\) B cells was routinely 95% or greater.

Statistical procedure. Statistical analysis was performed using Statview (SAS, Cary, NC). When the data were normally distributed, data were analyzed with either a Scheffe test when pair-wise comparisons were made between treatment groups or a paired t-test when two treatments within a group were compared. When data were not normally distributed, data were analyzed using a Mann-Whitney U test. Differences were considered significant when \( p < 0.05 \).

Results

Exogeneous administration of IL-12 promotes a Th1 response in \( L. \) *amazonensis*-infected mice.

A previous study has shown that the administration of exogenous IL-12 at the time of \( L. \) *amazonensis* infection does not promote healing (17). Additional work has demonstrated that mice chronically infected with \( L. \) *amazonensis* do possess a population of Ag-responsive CD44\(^{hi}\) CD4\(^+\) T cells that are unskewed towards either a Th1 or Th2 phenotype in vivo but can enhance IFN-\( \gamma \) production in response to IL-12 in vitro, although that enhancement is limited compared to CD4\(^+\) T cells from \( L. \) *major*-infected mice (25). To assess the extent of IL-12 responsiveness in vivo during \( L. \) *amazonensis* infection, mice were administered IL-12 at the time of infection and then every other day after infection for two weeks in the infected footpad. \( L. \) *major*-infected mice were used as a benchmark for a productive Th1 response.
At two weeks post-infection, the draining lymph node (DLN) was removed and cells were stimulated with Ag. After 24 hours, the percentage of CD44^{hi} CD4^{+} T cells producing IFN-\gamma from *L. amazonensis*-infected mice treated with IL-12 was similar to the percentage of CD44^{hi} CD4^{+} T cells producing IFN-\gamma from *L. major*-infected mice (Figure 1A). The percentage of CD44^{hi} CD4^{+} IFN-\gamma^{+} T cells was significantly greater in IL-12-treated *L. amazonensis*-infected mice as compared to *L. amazonensis*-infected control mice. Similarly, recall responses of DLN cells showed a significant enhancement in IFN-\gamma production from the *L. amazonensis*-infected mice treated with IL-12 compared to the controls (Figure 1B). This increased amount of IFN-\gamma production was similar to amounts produced by DLN cells from *L. major*-infected mice. This data indicates that exogenous IL-12 treatment at the time of *L. amazonensis*-infection is sufficient to drive a Th1 response.

**L. amazonensis*-infected mice treated with IL-12 do not maintain a Th1 response.**

Since treatment with IL-12 has been shown not to mediate healing of *L. amazonensis*-infected mice (17), we examined whether or not IL-12-treated *L. amazonensis*-infected mice sustain the Th1 response that is observed at two weeks post-infection. In contrast to the two weeks post-infection (Figure 1A), the percent of CD44^{hi} CD4^{+} T cells producing IFN-\gamma after a 24-hr Ag stimulation was not significantly different between IL-12-treated and control *L. amazonensis*-infected mice at ten weeks post-infection (Figure 2A). Both of these groups had significantly less CD44^{hi} CD4^{+} IFN-\gamma^{+} T cells than the *L. major*-infected mice (data not shown and as reported in (25)). Recall responses of DLN cells at ten weeks post-infection showed no difference in IFN-\gamma production between IL-12-treated and control
Figure 1. *L. amazonensis*-infected mice respond to exogenous IL-12 treatment by developing a Th1 response. (A) DLN cells were harvested at two weeks p.i., stimulated for 24 h with 50 μg/ml of Ag, stained with fluorescent antibodies against CD4, CD44 and IFN-γ and then analyzed by flow cytometry as described in materials and methods. Cells from *L. amazonensis*-infected mice were stimulated with *L. amazonensis* Ag and cells from *L. major*-infected mice were stimulated with *L. major* Ag. Data are represented as the mean ± the SEM of three separate experiments. * represents a statistically significant difference from all other groups at p < 0.05 as determined by a Scheffe test. (B) DLN cells harvested at two weeks p.i. were stimulated for three days in the presence (Ag Stim) or absence (No Stim) of 50 μg/ml of their respective Ag as described in (A); supernatants were assayed for IFN-γ via ELISA. Data are represented as the mean ± the SEM of four to five separate experiments for *L. major* - and *L. amazonensis* -infected groups, respectively. * represents a statistically significant difference between No Stim and Ag Stim within a group at p < 0.05 as determined by a Mann-Whitney U test. Ag stim groups with different superscripts are significantly different from one another at p < 0.05 as determined by a Mann-Whitney U test.

*L. amazonensis*-infected mice (Figure 2B). DLN cells from *L. major*-infected mice produced significantly more IFN-γ than either group of *L. amazonensis*-infected mice. Although treatment of *L. amazonensis*-infected mice with IL-12 promoted a significant reduction in lesion size as compared to *L. amazonensis* control mice in these experiments, the lesions were still significantly larger than those of *L. major*-infected mice at ten week post-infection (Figure 2C). The parasite burden in the infected footpad of IL-12-treated *L. amazonensis*
infected mice was not significantly different from either the *L. amazonensis*- or *L. major*-infected mice (Figure 2D). Together, this data indicates that while *L. amazonensis*-infected mice do respond to exogenous IL-12 treatment by developing a Th1 response early during infection, that response is not maintained over time and the mice fail to heal their infection to the same extent as *L. major*-infected mice.

**B cells are a source of IL-12 during chronic *L. major* infection but not during chronic *L. amazonensis* infection.**

IL-12 expression is necessary for the development and maintenance of a CD4\(^+\) Th1 phenotype *in vivo* during *L. major* infection (reviewed in (28)). Although previous work has demonstrated that the number of IL-12p40-producing cells present in the DLN of *L. major*-infected mice is significantly greater than in *L. amazonensis*-infected mice early during infection (17), we found similar numbers of DLN IL-12-p40-producing cells in both *L. amazonensis*- and *L. major*-infected mice ex vivo at ten weeks post-infection (Figure 3A). Recent work has shown that Ag-specific IL-12p40 can be detected in vitro following Ag stimulation of DLN cells isolated from *Toxoplasma gondii*-infected mice (11). Upon in vitro Ag stimulation of DLN cells from *L. major*-infected mice, a significant increase in the number of IL-12p40-producing cells was observed compared to the unstimulated controls. In contrast, DLN cells from *L. amazonensis*-infected mice fail to increase IL-12p40 production in response to parasite stage-specific Ag stimulation. Similar results were obtained when DLN cell culture supernatants were assayed via ELISA for IL-12p40 (Table 1). Upon observing an Ag-specific increase in the number of IL-12p40-producing cells in the *L. major*
Figure 2. IL-12 treated *L. amazonensis*-infected mice do not maintain their Th1 response and subsequently fail to heal their infection. (A) DLN cells harvested at ten weeks p.i. were cultured and analyzed as described in Figure 1A. Data are represented as the mean ± the SEM of two separate experiments. (B) DLN cells harvested at ten weeks p.i. were cultured as described in Figure 1B and supernatants were assayed for IFN-γ via ELISA. Data are represented as the mean ± the SEM of two to three separate experiments for *L. major*- and *L. amazonensis*-infected groups, respectively. * represents a statistically significant difference between No Stim and Ag Stim within a group at p < 0.05 as determined by a paired t test. Ag stim groups with different superscripts are significantly different from one another at p < 0.05 as determined by a Scheffe test. (C) Lesion size in C3H mice infected with either *L. amazonensis* ± IL-12 or *L. major* at ten weeks p.i. Individual mouse measurements are graphed from four experiments for a total of 13 mice per group. Long line represents the group mean and short lines represent the standard deviations of the mean. Groups with different superscripts are significantly different from one another at p < 0.05 as determined by a Mann-Whitney U test. (D) Parasite burden in the feet mice infected with *L. amazonensis* ± IL-12 or *L. major* at ten weeks post-infection. Data are represented as the mean ± the SEM of three to four separate experiments for *L. major*- and *L. amazonensis*-infected groups, respectively. Groups with different superscripts are significantly different from one another at p < 0.05 as determined by Scheffe’s post-hoc test.
DLN cell cultures, we wished to determine the cell population responsible for this increase. Ag stimulation of dendritic cell-depleted DLN cell cultures from *L. major*-infected mice still resulted in a significant increase in the number of IL-12p40-producing cells (Figure 3B). Harris et al has demonstrated that B cells from mice infected with *T. gondii* produce IL-12 in response to in vitro Ag stimulation (11). Cultures of purified B cells from *L. major*-infected mice significantly increased the number of IL-12p40-producing cells in response to Ag stimulation whereas cultures of B cells from *L. amazonensis*-infected mice did not (Figure 3C). Similar results were obtained when B cell culture supernatants were assayed via ELISA for IL-12p40 (Table 2). These data demonstrate that B cells are a source of Ag-specific IL-12 production during chronic *L. major* infection and not during chronic *L. amazonensis* infection.

**Administration of exogenous IL-12 does not promote a population of Ag-specific, IL-12p40-producing B cells during *L. amazonensis* infection.**

Considering that IL-12-treated *L. amazonensis*-infected mice fail to maintain a Th1 response (Figure 2), and that B cells are a source of Ag-specific IL-12 during chronic *L. major*-infection (Figure 3C), we determined if administering exogenous IL-12 to *L. amazonensis*-infected mice promoted endogenous IL-12p40 production. At two weeks post-infection, the number of IL-12p40 producing total DLN cells was similar between IL-12-treated *L. amazonensis*-infected mice and *L. major*-infected mice, and both groups had more IL-12p40-producing total DLN cells than the *L. amazonensis*-infected control mice (Figure 4A). Similar results were observed when supernatants from DLN cells stimulated for 24
Figure 3. CD19\(^+\) B cells are a source of IL-12 during chronic *L. major* infection but not during chronic *L. amazonensis* infection. (A) DLN cells were harvested at ten to sixteen weeks post-infection and either left unstimulated or stimulated with either promastigote or amastigote antigen overnight; the number of IL-12p40-producing cells was determined via ELISPOT. Data are represented as the mean ± the SEM of two separate experiments for uninfected mice, four experiments for *L. amazonensis*-infected mice and six experiments for *L. major*-infected mice. * represents a statistically significant difference between No Stim and either Promastigote or Amastigote Ag Stim within a group at p < 0.05 as determined by a paired \(t\) test. (B) CD11c-depleted DLN cells were harvested and assayed as in A. Data are represented as the mean ± the SEM of two separate experiments for uninfected mice, four experiments for *L. amazonensis*-infected mice and five experiments for *L. major*-infected mice. * represents a statistically significant difference between No Stim and Amastigote Ag Stim within a group at p < 0.05 as determined by a paired \(t\) test. (C) Purified B cells were harvested and assayed as in A; number of experiments and statistical analysis are same as in B.
Table 1. IL-12p40 (in pg/ml) detected in total lymph node cell cultures 24 hours post-Ag stimulation

<table>
<thead>
<tr>
<th>Experiment</th>
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<th>Promastigote Ag</th>
<th>Amastigote Ag</th>
</tr>
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<td>106</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>L. major</td>
<td>159</td>
<td>180</td>
<td>292</td>
</tr>
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<td>103</td>
<td>102</td>
<td>135</td>
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<td>254</td>
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<td></td>
<td>L. major</td>
<td>308</td>
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</tr>
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<td>L. amazonensis</td>
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<td></td>
<td>L. major</td>
<td>82</td>
<td>122</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Uninfected</td>
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<td>ND</td>
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Mean difference from No Stim (p values in parentheses)

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<tr>
<th>Infection</th>
<th>Promastigote Ag</th>
<th>Amastigote Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. amazonensis</td>
<td>-26.33 (0.346)</td>
<td>5.00 (0.788)</td>
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<tr>
<td>L. major</td>
<td>132.50 (0.112)</td>
<td>123.75 (0.008)</td>
</tr>
<tr>
<td>Uninfected</td>
<td>7.33 (0.448)</td>
<td>18.67 (0.192)</td>
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</tbody>
</table>

* indicates no sample
ND indicates non-detectable; for statistical analysis, ND samples were given a value of 38 because the detection limit of the ELISA was 39 pg/ml
Table 2. IL-12p40 (in pg/ml) detected in CD19<sup>+</sup> B cell cultures 24 hours post-Ag stimulation

<table>
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<tr>
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<th>Promastigote Ag</th>
<th>Amastigote Ag</th>
</tr>
</thead>
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</tr>
<tr>
<td></td>
<td><em>L. major</em></td>
<td>ND</td>
<td>72</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Uninfected</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>2</td>
<td><em>L. amazonensis</em></td>
<td>ND</td>
<td>ND</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td><em>L. major</em></td>
<td>ND</td>
<td>50</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Uninfected</td>
<td>*</td>
<td>ND</td>
<td>54</td>
</tr>
<tr>
<td>3</td>
<td><em>L. amazonensis</em></td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td><em>L. major</em></td>
<td>ND</td>
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<td>67</td>
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<td></td>
<td>Uninfected</td>
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<td>*</td>
<td>*</td>
</tr>
<tr>
<td>4</td>
<td><em>L. amazonensis</em></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td><em>L. major</em></td>
<td>ND</td>
<td>186</td>
<td>ND</td>
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<tr>
<td></td>
<td>Uninfected</td>
<td>75</td>
<td>62</td>
<td>49</td>
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Mean difference from No Stim (p values in parentheses)

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<th></th>
<th>Promastigote Ag</th>
<th>Amastigote Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. amazonensis</em></td>
<td>-2.33</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>(0.423)</td>
<td>(0.185)</td>
</tr>
<tr>
<td><em>L. major</em></td>
<td>77.50</td>
<td>35.00</td>
</tr>
<tr>
<td></td>
<td>(0.097)</td>
<td>(0.085)</td>
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<tr>
<td>Uninfected</td>
<td>13.00</td>
<td>-26.00</td>
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<tr>
<td></td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* indicates no sample
ND indicates non-detectable; for statistical analysis, ND samples were given a value of 38 because the detection limit of the ELISA was 39 pg/ml
NA indicates non-applicable because there were not enough degrees of freedom
hours with Ag were analyzed via ELISA for IL-12p40 (Table 3). Upon Ag stimulation, an increase was observed in the number of IL-12p40-producing cells compared to the unstimulated controls; however, this was not the case for either the IL-12-treated or the control *L. amazonensis*-infected groups. A substantial reduction in the number of IL-12p40-producing cells was observed in all unstimulated cultures when CD11c\(^+\) cells were depleted, consistent with a successful depletion. Unlike *L. major* CD11c-depleted cultures, no increase in the number of IL-12p40-producing cells was observed in either the IL-12-treated or the control *L. amazonensis* cultures when stimulated with Ag (Figure 4A). Although purified B cells from IL-12-treated *L. amazonensis*-infected mice stimulated with Ag produced slightly more IL-12p40 than B cells from *L. amazonensis*-infected control mice, the amount was considerably less than what was produced by Ag-stimulated B cells from *L. major*-infected mice (Figure 4B). Similar results were observed when supernatants from purified B cells

![Figure 4](image)

**Figure 4.** Administration of exogenous IL-12 at the time of *L. amazonensis* infection promotes an increase in endogenous IL-12p40 production. (A) DLN cells or CD11c-depleted cells harvested at two weeks post-infection were either left unstimulated or stimulated with amastigote Ag; the number of IL-12p40-producing cells was determined via ELISPOT. Data are from one experiment. (B) Purified B cells were cultured and assayed as in A. Data are from one experiment.
Table 3. IL-12p40 (in pg/ml) detected in total lymph node cell cultures 24 hours post-Ag stimulation at two weeks post-infection

<table>
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<th>Promastigote Ag</th>
<th>Amastigote Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>L. amazonensis</em> + IL-12</td>
<td>421</td>
<td>324</td>
<td>448</td>
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<tr>
<td></td>
<td><em>L. amazonensis</em></td>
<td>101</td>
<td>71</td>
<td>130</td>
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<tr>
<td></td>
<td><em>L. major</em></td>
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<td>338</td>
<td>437</td>
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<td><em>L. amazonensis</em> + IL-12</td>
<td>504</td>
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<td>493</td>
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<tr>
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<td><em>L. amazonensis</em></td>
<td>96</td>
<td>73</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td><em>L. major</em></td>
<td>295</td>
<td>268</td>
<td>311</td>
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Mean difference from No Stim (p values in parentheses)

<table>
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<th>Infection</th>
<th>Promastigote Ag</th>
<th>Amastigote Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. amazonensis</em> + IL-12</td>
<td>-125.50</td>
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</tr>
<tr>
<td></td>
<td>(0.142)</td>
<td>(0.746)</td>
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<tr>
<td><em>L. amazonensis</em></td>
<td>-26.50</td>
<td>14.00</td>
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<tr>
<td></td>
<td>(0.084)</td>
<td>(0.522)</td>
</tr>
<tr>
<td><em>L. major</em></td>
<td>8.5</td>
<td>79.50</td>
</tr>
<tr>
<td></td>
<td>(0.850)</td>
<td>(0.429)</td>
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* indicates no sample
Table 4. IL-12p40 (in pg/ml) detected in purified CD19+ B cell cultures 24 hours post-Ag stimulation at two weeks post-infection

<table>
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<th>Experiment</th>
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<th>Promastigote Ag</th>
<th>Amastigote Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L. amazonensis + IL-12</td>
<td>69</td>
<td>*</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>L. amazonensis</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>L. major</td>
<td>ND</td>
<td>67</td>
<td>91</td>
</tr>
<tr>
<td>2</td>
<td>L. amazonensis + IL-12</td>
<td>67</td>
<td>*</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>L. amazonensis</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>L. major</td>
<td>ND</td>
<td>85</td>
<td>39</td>
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Mean difference from No Stim (p values in parentheses)

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<th>Amastigote Ag</th>
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<td>*</td>
<td>12.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.570)</td>
</tr>
<tr>
<td>L. amazonensis</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>(NA)</td>
<td>(NA)</td>
</tr>
<tr>
<td>L. major</td>
<td>38.00</td>
<td>27.00</td>
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<tr>
<td></td>
<td>(0.148)</td>
<td>(0.488)</td>
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* indicates no sample
ND indicates non-detectable; for statistical analysis, ND samples were given a value of 38 because the detection limit of the ELISA was 39 pg/ml
NA indicates non-applicable because there was no variation between the data points
stimulated for 24 hours with Ag were analyzed via ELISA for IL-12p40 (Table 4). Together, this data indicates that administration of exogenous IL-12 to <i>L. amazonensis</i>-infected mice promotes enhanced IL-12 production from predominantly non-Ag-specific CD11c<sup>+</sup> cells but does not promote Ag-specific IL-12 production from B cells at two weeks post-infection.

At ten weeks post-infection, the number of total DLN cells producing IL-12p40 from IL-12-treated <i>L. amazonensis</i>-infected mice was similar to slightly less when compared to <i>L. amazonensis</i>-infected control mice, both in the presence and absence of Ag stimulation (Figure 5A). Similar results were observed when supernatants from DLN cells stimulated for 24 hours with Ag were analyzed via ELISA for IL-12p40 (Table 5). Upon Ag stimulation of CD11c-depleted cells or purified B cells, no difference was observed in the number of IL-12p40-producing cells between the IL-12-treated and control <i>L. amazonensis</i>-infected mice (Figures 5A & B). Moreover, significantly less IL-12p40-producing B cells were observed

---

**Figure 5.** Administration of exogenous IL-12 at the time of <i>L. amazonensis</i> infection does not promote a population of Ag-specific, IL-12p40-producing B cells during <i>L. amazonensis</i> infection. DLN cells, CD11c-depleted (A) and purified B cells (B) were harvested at ten weeks post-infection and either left unstimulated or stimulated with amastigote Ag; the number of IL-12p40-producing cells was determined via ELISPOT. Data are from one experiment.
Table 5. IL-12p40 (in pg/ml) detected in total lymph node cell cultures 24 hours post-Ag stimulation at ten weeks post-infection

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<th>Experiment</th>
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<th>Amastigote Ag</th>
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<td>1</td>
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<td>92</td>
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<tr>
<td></td>
<td><em>L. major</em></td>
<td>146</td>
<td>103</td>
<td>132</td>
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<tr>
<td></td>
<td>Uninfected</td>
<td>131</td>
<td>145</td>
<td>150</td>
</tr>
<tr>
<td>2</td>
<td><em>L. amazonensis</em></td>
<td>192</td>
<td>*</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td><em>L. major</em></td>
<td>150</td>
<td>135</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>Uninfected</td>
<td>127</td>
<td>131</td>
<td>142</td>
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Mean difference from No Stim (p values in parentheses)

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<th>Promastigote Ag</th>
<th>Amastigote Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. amazonensis</em></td>
<td>-43.00</td>
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<tr>
<td></td>
<td>(NA)</td>
<td>(0.105)</td>
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<td><em>L. major</em></td>
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<td></td>
<td>(0.286)</td>
<td>(0.008)</td>
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<td>Uninfected</td>
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<td>17.00</td>
</tr>
<tr>
<td></td>
<td>(0.323)</td>
<td>(0.075)</td>
</tr>
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</table>

* indicates no sample

ND indicates non-detectable; for statistical analysis, ND samples were given a value of 38 because the detection limit of the ELISA was 39 pg/ml

NA indicates non-applicable because there were not enough degrees of freedom
in both IL-12-treated and control *L. amazonensis* cultures as compared to *L. major* cultures (Figure 5B). These data show that administering exogenous IL-12 does not promote sustained production of Ag-specific IL-12 production from B cells during chronic *L. amazonensis* infection.

**Discussion**

In the present study, we extend our understanding of IL-12 responsiveness during *L. amazonensis* infection. Our data demonstrate that CD4+ T cells from *L. amazonensis*-infected mice do indeed respond to exogenous IL-12 administered at the time of infection by developing a Th1 phenotype (Figure 1). However, these mice fail to heal their infections; a phenomenon that corresponds with an inability of these mice to maintain their Th1 CD4+ T cell response (Figure 2). Additionally, B cells are not a source of Ag-specific IL-12 during chronic *L. amazonensis* infection (Figure 3), and administration of exogenous IL-12 fails to develop a population of Ag-specific IL-12 producing B cells during *L. amazonensis* infection (Figures 4 and 5).

Our finding that B cells are a source of Ag-specific IL-12 during chronic *L. major* infection was a bit surprising, as more traditional roles for B cells have focused upon antigen presentation, promoting Th2 responses and antibody production. Although dendritic cells are considered the most potent antigen presenting cells (APCs) for capturing antigens in a non-antigen-specific manner and presenting them to T cells in a highly immunogenic form, B cells can also function as APCs (4). B cells are potent APCs for primed T cells, and are most efficient as APCs when they present Ag bound specifically to surface Ig (slg) rather than when the Ag enters the cell in an slg-independent manner (27). Although much literature
indicates that B cells likely induce Th2 responses, a recent report indicates that B cells can induce the differentiation of Th1 CD4+ T cells by producing IL-12 (33). Our data are consistent with this recent report, and suggest that B cells are an antigen-specific source of sustained IL-12 during chronic L. major infection.

Conflicting reports exist on the influence of B cells on the T cell-mediated immune response against L. major. BALB/c mice treated with anti-IgM antibody or BALB/c X-linked immunodeficient mice that lack B1 B cells either heal or display enhanced resistance to L. major, respectively (12, 29), whereas other work concludes that anti-IgM-treated BALB/c mice remain susceptible (3). Additionally, a report by Babai et al showed that depletion of peritoneal CD5+ B cells had no effect on the outcome of L. major infection in both resistance and susceptible mouse strains (2). Work by Scott et al demonstrated that resistant C3H mice treated with anti-IgM were unable to heal their L. major infections, which remained chronic but non-progressive (32). Adoptive transfer of immune T cells into IgM-depleted mice restored their ability to heal, suggesting that the T cells required for healing a L. major infection depend upon B cells for their development. This concept is supported by additional work demonstrating that B cells are required for a delayed type hypersensitivity (DTH) memory response in C57BL/6 mice (6). Our results indicate that B cells may contribute to the Th1 CD4+ T cell memory response present in L. major-infected mice by functioning as a source of antigen-specific IL-12 in resistant mouse strains.

In contrast to infection with L. major, a more exact role for B cells has been described during infection with members of the L. mexicana complex, including L. amazonensis and L. pifanoi. In the absence of circulating antibody, infection of L. mexicana complex parasites cannot be maintained because the parasites are not internalized into macrophages via the Fc
receptor (5, 18). These studies have established a critical role for antibody in the pathogenesis of cutaneous leishmaniasis caused by these parasites. Additional work has shown that B cells appear to regulate *L. donovani* infection independently of antibody as B cell-deficient mice are resistant to infection but develop destructive hepatic pathology mediated by neutrophils (34).

IL-12 was originally identified as a product of human Epstein-Barr virus-transformed B cell lines (19). For many years, murine B cells were thought incapable of producing IL-12 until recent reports demonstrated this ability in both B cells from infected mice or B cells stimulated with CpG oligodeoxynucleotides in vitro (8, 11, 23, 33). In a model originally proposed by Mamula and Janeway, B cells are important for amplifying and maintaining T cell responses initiated by dendritic cells (22). These activated T cells migrate to the outer T cell zone (21) and then to the B cell area (7) where they interact with Ag-specific B cells and a massive expansion of both T and B cells ensues (9, 20). When B cells encounter IFN-γ-producing T cells expressing CD40L, they produce IL-12, further reinforcing IFN-γ production by adjacent IL-12-responsive T cells (31). Work by Wagner et al demonstrates that IL-12 production from human B cells is also under the control of both microbial stimulation, indicating that B cells only produce IL-12 when they simultaneously encounter T cells and sense the appropriate microbial molecule, such as CpG (39). Based on this model, the failure of *L. amazonensis*-infected mice to develop a population of IL-12p40-producing B cells may correlate with the inability of these mice to sustain their Th1 response.

In summary, CD4⁺ T cells are able to respond to IL-12 in vivo by developing a Th1 response early during *L. amazonensis* infection. However, these mice do not maintain that Th1 response over time and subsequently fail to heal their infections. Additionally, B cells
are not a source of antigen-specific IL-12 during chronic *L. amazonensis* infection, a defect not overcome by the administration of exogenous IL-12. Together, these data implicate a B cell population defective in IL-12 production as a potential mechanism contributing to the chronicity of *L. amazonensis* infection.

**Acknowledgements**

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**References**


Chapter 5

Conclusions

Summary

The work presented in this dissertation demonstrates that infection with *L. amazonensis* creates a population of antigen-responsive, CD44^hi^CD4^+^ T cells that proliferate and produce IL-2 but do not polarize to an effector phenotype. While these T cells are able to enhance IFN-γ production in response to IL-12, the amount of IFN-γ produced is only 10-15% of that produced in *L. major* CD4^+^ T cell cultures. These data demonstrate that the failure of CD4^+^ T cells from *L. amazonensis*-infected mice to become efficient Th1 effector cells is due to factors beyond the presence or absence of IL-12.

Additional analysis of the IL-12 responsiveness of this T cell population indicates that IL-2 may be one of the factors involved in limiting the immune response during *L. amazonensis* infection as it limits enhanced proliferation of CD44^hi^CD4^+^ T cells in response to IL-12. Providing multiple Ag stimulations in the presence of IL-12 yields equivalent percentages of IFN-γ-producing CD44^hi^CD4^+^ T cells in cultures of T cells from both *L. amazonensis-* and *L. major-*infected mice. However, the total amount of IFN-γ produced by T cells isolated from *L. amazonensis*-infected mice is 40% less than that produced by CD4^+^ T cells from *L. major*-infected mice; a deficit resulting, at least in part, from a failure of CD44^hi^CD4^+^ T cells from *L. amazonensis*-infected mice to accumulate in culture. In contrast to CD44^hi^CD4^+^ T cells from *L. major*-infected mice, CD44^hi^CD4^+^ T cells from *L. amazonensis*-infected mice are less responsive to both IL-2 and IL-12 for enhancing proliferation. However, neutralization of IL-2 allows enhanced proliferation of CD44^hi^CD4^+^
T cells from *L. amazonensis*-infected mice in response to IL-12. Following multiple Ag stimulations, CD44\(^{hi}\) CD4\(^{+}\) T cells from *L. amazonensis*-infected mice sustain a higher expression of CD25 than do CD44\(^{hi}\) CD4\(^{+}\) T cells from *L. major*-infected mice, a phenotype regulated by IL-2 and consistent with that of regulatory T (T\(_{reg}\)) cells. Together, these results indicate that IL-2 suppresses enhanced proliferation in response to IL-12 for CD4\(^{+}\) T cells from *L. amazonensis*-infected mice.

To recapitulate continual culture of CD4\(^{+}\) T cells with IL-12 in vivo, multiple doses of IL-12 were administered to mice during the first two weeks of *L. amazonensis* infection. Although CD4\(^{+}\) T cells respond to exogenous IL-12 administered during *L. amazonensis* infection by developing a Th1 phenotype at two weeks post-infection, that response is not maintained and the mice fail to heal their infections. IL-12 treatment also fails to promote a sustained source of antigen-specific IL-12 from B cells in these mice. These data indicate that the failure of IL-12-treated *L. amazonensis*-infected mice to heal their infection is not due to an inability to mount a Th1 response, but rather from a lack of sustained, antigen-specific IL-12 production from B cells.

**Discussion**

**The immune response to *L. major***

A protective immune response against *L. major* consists of a CD4\(^{+}\) T cell population that produces IFN-\(\gamma\) and responds to IL-12, thereby promoting a Th1 response. Early after infection, dendritic cells are the primary source of IL-12 (7, 8, 11, 16). *Leishmania*-infected macrophages are inhibited in their ability to make IL-12 in response to additional stimuli, and therefore contribute very little to IL-12 production during *L. major* infection (2). IFN-\(\gamma\)-
producing, T-bet$^+$ CD4$^+$ Th1 cells are able to effectively activate macrophages at the site of lesion development to kill the intracellular amastigotes in a nitric oxide-dependent manner (reviewed in (12)). Not only is the development of this Th1 response dependent upon IL-12, its maintenance is as well (10, 14). Our data extend the knowledge of cellular sources of IL-12 by demonstrating that Ag-specific B cells produce IL-12 during the later stage of *L. major* infection. Ultimately, the productive Th1 response generated during *L. major* infection leads to lesion resolution and a very small parasite load (less than 100 parasites per infected footpad). Additionally, a population of CD4$^+$ CD25$^+$ T_{reg} cells develops during *L. major* infection, and is necessary for the establishment of chronic *L. major* infection and the accompanying low parasite load. Furthermore, the functions of this T_{reg} population are dependent upon IL-10 (1). A productive memory CD4$^+$ T cell response has also been shown to develop following *L. major* infection (17).

The model presented in Figure 1 demonstrates the maturation of the productive immune response during *L. major* infection described above. During *L. major* infection, dendritic cells are appropriately activated in the presence of a sufficient amount of inflammatory mediators including IL-12. In turn, these DCs efficiently prime a population of Th1 CD4$^+$ effector/memory T cells that promote the resolution of antigen load by activating macrophages to kill the parasite. This decrease in parasite load subsequently facilitates a reduction in the inflammatory response associated with infection. At the beginning of the immune response, early-activated CD4$^+$ T cells are likely sources of IL-2. As inflammation increases, the predominant cytokine produced becomes IL-12, which promotes the early-activated CD4$^+$ T cell population to differentiate into more mature effector T cells.
Figure 1. Summary of the maturation of the immune response to *L. major*. The healing immune response to *L. major* is characterized by a CD4$^+$ T cell population that can resolve antigen load in the context of an appropriate amount of inflammation. Ultimately, a chronic host-parasite relationship develops with low amounts of antigen and inflammation, as do populations of regulatory T cells and memory T cells. The maintenance of the Th1 response to *L. major* requires a sustained source of IL-12.
These mature CD4\(^+\) T cells are able to reduce antigen load, which perpetuates a decrease in inflammation including IL-12 production. However, low amounts of IL-12 continue to be important for memory Th1 cell maintenance during the later stage of the immune response.

**The immune response to *L. amazonensis***

In contrast to the productive immune response to *L. major*, the host immune response to *L. amazonensis* does not facilitate healing and instead promotes a state of chronic disease characterized by a high parasite burden. Work from our laboratory indicates that the immune mechanisms required to kill *L. amazonensis* amastigotes are more rigorous than those for *L. major* amastigotes, and that appropriate B cell and CD4\(^+\) T cell responses are also necessary for effective clearance of *L. amazonensis* (9). The data presented in this thesis describe numerous defects in the CD4\(^+\) T cell population during *L. amazonensis* in terms of accumulation and IL-12 responsiveness. This work also reports a B cell population defective in IL-12 production during *L. amazonensis* infection. We believe that these cellular defects cause inefficient parasite killing during *L. amazonensis* infection thus creating a persistent antigen environment.

*L. amazonensis* infection may also create a semi-mature dendritic cell population in vivo. Our laboratory has shown that in vitro infection of bone-marrow derived dendritic cells (BMDCs) with *L. amazonensis* amastigotes enhances surface expression of MHC II and the costimulatory molecules CD80 and CD86 but not CD40; these DCs also produce less IL-12 than those infected with *L. major* (5). Indeed, others have described decreased IL-12 production during *L. amazonensis* infection as well as decreased mRNA expression of other multiple inflammatory mediators (4, 6, 15). All these factors likely contribute to the
inappropriate maturation and priming of DCs during *L. amazonensis* infection. Recent work has shown that inappropriately primed dendritic cells in vivo can support CD4\(^+\) T cell clonal expansion but cannot prime an effector response (13); the data presented in this dissertation describes a similar CD4\(^+\) T cell response during *L. amazonensis* infection. Based on our data, we hypothesize that the high antigen load present during *L. amazonensis* infection results in continual antigen stimulation of CD4\(^+\) T cells by inappropriately primed DCs. These early-activated CD4\(^+\) T cells produce high amounts of IL-2, which in turn limits the maturation and differentiation of the cellular immune response by limiting expansion of IL-12-responsive CD4\(^+\) T cells. Although administration of exogenous IL-12 during *L. amazonensis* infection promotes the development of an effector CD4\(^+\) T cell response, this population of cells fails to eliminate parasites and perpetuate healing; upon removal of IL-12, the Th1 response is not maintained and the CD4\(^+\) T cell response returns to an unpolarized one. Our in vitro results indicate that IL-2 limits the survival and accumulation of the CD4\(^+\) Th1 cell population, and in the absence of a sustained source of IL-12, the Th1 response is not maintained. Indeed, we believe IL-2 may promote the development of an antigen-specific regulatory T cell population that prevents the subsequent differentiation of neighboring CD4\(^+\) T cells to effector cells.

Administration of exogenous IL-12 at the time of *L. amazonensis* infection also fails to promote a population of antigen-specific IL-12-producing B cells. We propose that the B cells present during *L. amazonensis* infection fail to mature to a population that produces IL-12 and secretes a predominance of IgG2a antibodies, the isotype of antibody associated with a Th1 response. Should a productive B cell response be present during *L. amazonensis* infection, we hypothesize that IgG2a antibodies would bind Fc\(\gamma\) receptor II on infected
macrophages and activate the intracellular signaling cascades necessary for superoxide production. Superoxide would then combine with nitric oxide to produce peroxynitrite, a potent cytotoxic factor, to mediate killing of *L. amazonensis*. Together, defective CD4⁺ T cell and B cell responses preclude the cellular immune response from maturing and resolving a *L. amazonensis* infection (Figure 2).

We believe the immune response associated with *L. amazonensis* infection is consistent with the theoretical model of cellular self-renewal and differentiation proposed by Grossman and colleagues (3). In this model, self-renewal and differentiation are competing cellular events regulated by both antigen/APC interactions and cytokines. Antigen load influences whether or not a cell is activated or resting; if antigen is presented by APCs in the context of an inflammatory milieu, then a cell becomes activated. Cytokines can subsequently influence whether or not the cell differentiates or self-renews. Presumably, in a resolving antigen environment and the presence of polarizing cytokines, including IL-4 and IFN-γ, the CD4⁺ T cell differentiates towards a Th1 or Th2 effector phenotype. If cytokines such as IL-10 or TGF-β are present, a cell may differentiate towards a regulatory phenotype. In contrast, persistent antigen causes T cells to adapt to a higher threshold for activation; these cells will self-renew rather than differentiate. We propose that repeated antigen stimulation and IL-2 are preventing the CD4⁺ T cells present during *L. amazonensis* infection from differentiating into a more mature effector cell population and is instead holding them in a pattern of self-renewal (Figure 3). This immature CD4⁺ T cell phenotype continues to perpetuate the persistent antigen environment by inefficiently activating macrophages to kill the parasites, which ultimately creates a cycle of inadequate activation and high antigen load.
Figure 2. Summary of the immune response to *L. amazonensis*. The dysfunctional immune response to *L. amazonensis* is characterized by early-activated CD4<sup>+</sup> T cells and immature B cells that cannot resolve antigen load. Low amounts of inflammatory signals, including IL-12, are present during infection. The CD4<sup>+</sup> T cells fail to differentiate into an efficient Th1 population, receive repeated antigen stimulations due to the high antigen load, and subsequently produce IL-2. When exogenous IL-12 is present, a Th1 response develops but is not maintained, presumably because IL-2 limits the accumulation and survival of Th1 CD4<sup>+</sup> T cells.
Figure 3. Model for self-renewal versus differentiation applied to *Leishmania* infection. Teal circle, arrows and text denote phenotype of CD4^+^ T cells during *L. major* infection. Pink circle, arrows and text denote phenotype of CD4^+^ T cells during *L. amazonensis* infection. CD4^+^ T cells from both *L. amazonensis*- and *L. major*-infected mice transition between a resting and activated phenotype upon antigen stimulation. The probability of whether or not a CD4^+^ T cell will self-renew or differentiate is influenced by persistent or resolving antigen load and the presence of inflammatory signals, including IL-12. See page 25 for original figure legend. Modified and reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Immunology*, Grossman, Z. et al. 4(5): 387-95, copyright 2004.
Recommendations for future studies

The long-term goal of the research presented in this dissertation is to understand how an adaptive tolerance environment develops and is regulated during *L. amazonensis* infection. Additional experiments are needed to more clearly demonstrate that the immune response present during chronic *L. amazonensis* infection is consistent with mechanisms of adaptive tolerance. Continued use of the mini-double culture system is recommended to determine if the CD4<sup>+</sup> CD25<sup>+</sup> T cell population present in cultures of T cells from *L. amazonensis*-infected mice does indeed regulate the Ag-responsiveness of CD4<sup>+</sup> CD25<sup>-</sup> T cells. The CD25<sup>+</sup> CD44<sup>hi</sup> CD4<sup>+</sup> T cells from *L. amazonensis*-infected mice should also be evaluated for expression of additional markers associated with adaptively tolerant phenotypes such as PD-1 and OX40. Additional studies should also focus upon assessing IL-2 production during *L. amazonensis* infection, as well as determining if the CD25<sup>+</sup> CD44<sup>hi</sup> CD4<sup>+</sup> activated effector T cells observed following multiple in vitro Ag stimulations exist in vivo during infection. Studies in which *L. amazonensis*-infected mice are treated with anti-IL-2 plus IL-12 would be beneficial to understanding if our observations of IL-2 limiting IL-12 responsiveness in vitro are true in vivo. To more thoroughly establish the role of persistent Ag as promoting the dysfunctional CD4<sup>+</sup> T cell response present during *L. amazonensis* infection, studies in which mice are treated with anti-leishmanial drugs at the time of infection are warranted. In turn, the effector phenotype of the CD4<sup>+</sup> T cells isolated from these mice could then be evaluated using the mini-double culture system to determine if effector functions are restored. By better understanding the dysfunctional immune response associated with *L. amazonensis* infection, the potential exists to develop new therapeutic
strategies and novel vaccines that will aid in the fight not only against leishmaniasis but also against the many other chronic diseases that burden our world.

References


Appendix

Use of Staphylococcal enterotoxin B to study the interactions of antigen presenting cells and CD4⁺ T cells during Leishmania amazonensis infection

Introduction

Dendritic cells (DCs) are the most efficient antigen presenting cells (APCs) in vivo. DCs act as a link between the innate and adaptive branches of the immune system by recognizing specific molecular patterns on the surface of pathogens and subsequently priming antigen-specific naïve T cells (1). During this priming process, DCs also influence the phenotype of the responding T cell population. For instance, DCs that are producing IL-12 promote a Th1 phenotype in the T cells they prime (6). DCs that are indirectly activated by inflammatory mediators and do not come in contact with pathogen components prime a T cell population that proliferates but does not differentiate to either a Th1 or Th2 phenotype (9). We believe that the dysfunctional CD4⁺ T cell response observed during L. amazonensis infection results, in part, from priming by an improperly matured DC population (7). To further understand the interactions between DCs and T cells during L. amazonensis infection, we activated naïve T cells in the presence of an APC population derived from either L. amazonensis- or L. major-infected mice using Staphylococcal enterotoxin B (SEB) and assessed T cell effector functions. SEB is a powerful superantigen; the ability of T cells to respond to SEB requires that the toxin first bind to the MHC II molecule on the surface of an APC (5). Furthermore, only T cells bearing a certain Vβ structure in their TCR (primarily the Vβ8 chain of the TCR) are able to respond to the combination of toxin and MHC II. Additionally, only naïve T cells respond to stimulation with SEB (4).
**Results and discussion**

Previous work indicates that Vβ8-bearing T cells are not present in certain inbred mouse strains due either to a gene deletion or the presence of an endogenous retrovirus (2, 3). To determine if C3H mice possess T cells bearing the Vβ8 TCR, draining lymph node (DLN) cells were harvested from mice infected with either *L. amazonensis* or *L. major* for four weeks or from age-matched uninfected control mice and stained with fluorescent antibodies for CD4, Vβ8.1/8.2 (clone MR5-2) and Vβ8.3 (clone 1B3.3) and analyzed via flow cytometry. Similar percentages of CD4⁺ T cells bearing the Vβ8.1/8.2 isoforms of the TCR were observed among *L. amazonensis*- and *L. major*-infected and uninfected mice, indicating that C3H mice do indeed possess CD4⁺ T cells bearing the Vβ8 TCR, and that *Leishmania* infection does not promote selection or deletion of this particular subset of T cells (Figure 1). Similar results were observed for the Vβ8.3 isoform of the TCR.

Our laboratory has shown that *L. amazonensis*-infected mice possess a population of antigen-responsive CD44⁺ CD4⁺ T cells that produce IL-2 and proliferate upon antigen (Ag) stimulation but are limited in their ability to produce effector cytokines, including IFN-γ (7). We wished to determine if naïve T cells stimulated in vitro with SEB recapitulate the phenotype observed for Ag-responsive T cells. DLN cells were harvested from mice infected with either *L. amazonensis* or *L. major* for four weeks or from age-matched uninfected control mice and labeled with CFSE as previously described (7). One million DLN cells were plated per well of a 96 well plate and stimulated with 1 μg/ml SEB (Toxin Technology, Sarasota, FL) for four days. Cells were harvested, stained with fluorescent antibodies for CD4 and CD44 and analyzed via flow cytometry as previously described (7). As observed
Figure 1. Similar percentages of CD4\(^+\) T cells bearing the V\(\beta\)8 TCR among *L. amazonensis*- and *L. major*-infected and uninfected C3H mice. DLN cells were harvested at four weeks post-infection and stained with fluorescent antibodies against CD4, V\(\beta\)8.1/8.2 and V\(\beta\)8.3 and analyzed via flow cytometry. Histograms are based on live CD4\(^+\) T cell gates and are from one experiment.
in the Ag stimulation experiments, similar percentages of the total CD44\textsuperscript{hi} CD4\textsuperscript{+} T cells present in culture from the \textit{L. amazonensis}- and the \textit{L. major}-infected mice proliferated in response to SEB stimulation (Figure 2). Although not statistically significantly, a trend for enhanced proliferation was observed for CD44\textsuperscript{hi} CD4\textsuperscript{+} T cells from both \textit{L. amazonensis}- and \textit{L. major}-infected mice over CD44\textsuperscript{hi} CD4\textsuperscript{+} T cells from uninfected mice (p = 0.063 and p = 0.062, respectively).

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**Figure 2.** CD44\textsuperscript{hi} CD4\textsuperscript{+} T cells from \textit{L. amazonensis}-infected mice proliferate similarly to CD44\textsuperscript{hi} CD4\textsuperscript{+} T cells from \textit{L. major}-infected mice in response to SEB stimulation. DLN cells were harvested from mice infected with either \textit{L. amazonensis} or \textit{L. major} for four weeks or from age-matched uninfected control mice, labeled with CFSE, and cultured in the presence or absence of SEB for four days. Cells were harvested, stained with fluorescent antibodies for CD4 and CD44 and analyzed via flow cytometry. Data are represented as the mean ± the SEM of two separate experiments. Means for SEB stimulation were compared among groups using Scheffe’s post-hoc test.
To determine if naïve CD4⁺ T cells stimulated with SEB in the presence of APCs from *L. amazonensis*-infected mice demonstrate defects in IFN-γ production, DLN cells were harvested from mice infected with either *L. amazonensis* or *L. major* for four weeks or from age-matched uninfected control mice and stimulated with 1 µg/ml SEB for three days. Culture supernatants were harvested and assayed for IFN-γ via ELISA as previously described (7). The IFN-γ produced by DLN cells from *L. amazonensis*-infected mice upon SEB stimulation was found to be only 39% of that produced by cells from *L. major*-infected mice (p = 0.133, Figure 3). Although not statistically significant, a trend for increased IFN-γ

![Figure 3. Less IFN-γ produced by CD4⁺ T cells from *L. amazonensis*-infected mice following SEB stimulation as compared to CD4⁺ T cells from *L. major*-infected mice. DLN cells were harvested from mice infected with either *L. amazonensis* or *L. major* for four weeks or from age-matched uninfected control mice and cultured in the presence or absence of SEB for three days. Culture supernatants were harvested and assayed for IFN-γ via ELISA. Data are represented as the mean ± the SEM of three separate experiments. Means for SEB stimulation were compared among groups using Scheffe’s post-hoc test.](image)
production from DLN cells from *L. major*-infected over uninfected mice was observed (*p* = .059); however, no difference in IFN-γ production was observed between DLN cells from *L. amazonensis*-infected and uninfected mice (*p* = 0.698). These preliminary observations indicate that naïve CD4⁺ T cells stimulated in the presence of SEB and APCs from the DLN of *L. amazonensis*-infected mice recapitulate the defect in IFN-γ production that is observed in Ag-responsive CD4⁺ T cells from *L. amazonensis*-infected mice. More studies looking specifically at both IFN-γ production and proliferation specifically from the Vβ8 CD4⁺ T cell population following SEB stimulation are warranted.

These preliminary experiments suggest that the DLN environment present during *L. amazonensis* infection is not conducive for APCs to effectively prime a naïve T cell population towards a Th1 phenotype. Instead, the APCs present in the DLN during *L. amazonensis* infection give rise to a T cell population limited in its effector functions—a scenario reminiscent of adaptive tolerance (8), which suggests that the DLN environment during *L. amazonensis*-infection is a tolerizing one. In summary, the culture system described here will be a useful tool for studying the transition of CD4⁺ T cells from a naïve to effector phenotype in an environment that promotes T cell proliferation but not differentiation.

References


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