The impaired Th1 immune response of C3HeB/FeJ mice infected with

*Leishmania amazonensis*: lessons learned from immunotherapy and

vaccines

by

Yannick Frank Vanloubeeck

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Program of Study Committee:
Douglas E. Jones (Major Professor)
  Mark R. Ackermann
  Norman F. Cheville
  Jeffrey K. Beetham
  Randy E. Sacco

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Major Professor

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For the Major Program
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CHAPTER 1: General Introduction

Introduction

Leishmaniasis is a zoonotic disease endemic in tropical and sub-tropical regions of the world. It is caused by intracellular protozoan parasites of the genus *Leishmania*, which are transmitted to the host through the bite of an infected female sand fly. The resulting disease depends on the species of *Leishmania* involved and the type of immune response mounted by the host, and it is classically subdivided into three forms: visceral, cutaneous and mucocutaneous (reviewed in (109, 180, 323)). For example, *Leishmania infantum*, *Leishmania donovani* and *Leishmania chagasi* are responsible for the visceral form of the disease (also referred as Kala Azar); infection with *Leishmania major*, *Leishmania mexicana* and *Leishmania amazonensis* results in cutaneous leishmaniasis and *Leishmania braziliensis* is the main etiologic agent of mucocutaneous leishmaniasis (or espundia). However, there are some exceptions and *Leishmania* species usually responsible for visceral disease can sometimes be isolated from cutaneous lesions and vice-versa (16, 48, 320, 325, 436, 445).

During visceral leishmaniasis, *Leishmania* parasites reside within phagocytes of the spleen, lymph nodes, liver and bone marrow, subsequently resulting in splenomegaly, lymphadenopathy, hepatomegaly, pancytopenia, hypoalbuminemia and cachexia (211). This leads to multi-organ failure, which is often fatal if left untreated. Five percent of the patients recovering from visceral leishmaniasis also develop cutaneous lesions, referred to as post-Kala Azar dermal leishmaniasis (180). Cutaneous leishmaniasis causes lesions of varying severity, ranging from a self-limiting ulcer to diffuse skin lesions that may persist for months before healing and possibly leaving a permanent scar (180). Mucocutaneous leishmaniasis usually results from the metastasis of a previous cutaneous lesion that may have healed years
before (reviewed in (322)). However, it is sometimes secondary to a cutaneous lesion that extends to adjacent mucous membranes by contiguous spread. The mucosae involved during mucocutaneous leishmaniasis include those of the mouth, palate, naso-pharynx, larynx and occasionally trachea. The associated lesions are usually severely disfiguring and can be life threatening.

The widespread distribution and the increased incidence of human leishmaniasis has hampered the economic development and productivity of several regions of the world (144). The disease is currently endemic in a total of 88 countries within Africa, Asia, Europe, North America and South America (582). It affects 12 million people around the world and 350 million are at risk (144). A case of leishmaniasis was also recently described in Australia (469). Within the United States leishmaniasis is only occasionally diagnosed, usually in people coming back from endemic areas, including soldiers that have served in the Middle East (271, 320). The first autochthonous case of leishmaniasis in the U.S. has been reported in 1940, in a 26 year-old male from Chicago (50). The patient initially developed the mucocutaneous form of the disease with subsequent involvement of the bone-marrow. In addition, human cutaneous leishmaniasis due to *L. mexicana* is endemic in Texas (256, 338) and sporadic cases of canine visceral leishmaniasis have been documented throughout the United States (151, 162, 471, 518, 542).

There are approximately 1.5 to 2 million new cases of leishmaniasis every year, with cutaneous leishmaniasis representing 50 to 75% of them (582). In addition, there is an increased incidence of leishmaniasis in immunosuppressed patients infected with HIV (109, 582). Currently, there is no anti-*Leishmania* vaccine available and treatment of the disease involves aggressive, long lasting chemotherapy that is usually expensive and associated with
adverse side effects (54, 124, 582). More studies are required before efficient and safe prophylactic and/or therapeutic strategies to control leishmaniasis are available.

The susceptibility of humans to leishmaniasis has been shown to correlate with poor cell-mediated immunity. In particular, the immune response of infected patients is usually of a mixed T helper 1 (Th1) and T helper 2 (Th2) phenotype, and often characterized by the production of low to undetectable levels of IFN-γ (64, 81, 123, 432, 465). As in humans, experimental murine leishmaniasis can have different outcomes depending on the parasite strain/species and the genetic background of the host (5, 39, 60, 108, 114, 176, 237, 463). Experimental infection of mice with various *Leishmania* species has led to an understanding of some of the immune mechanisms necessary to control these intracellular protozoa. In particular, murine studies of cutaneous leishmaniasis have highlighted some of the immune factors correlating with susceptibility and resistance to the disease (reviewed in (304)). For example, the outcome of murine cutaneous leishmaniasis caused by *L. major* is determined by the phenotype of the CD4⁺ T cell response: Th1 CD4⁺ T cells producing IFN-γ promote resistance while Th2 CD4⁺ T cells producing IL-4 induce susceptibility (reviewed in (455) and (480)). Upon infection with *L. major*, BALB/c mice mount a Th2 response characterized by the production of high levels of IL-4; these mice subsequently develop progressive cutaneous lesions and succumb to infection. In contrast, C3H and C57BL/6 mice are able to control a *L. major* infection by mounting a protective Th1 immune response characterized by the production of IL-12 and IFN-γ. This resistant phenotype requires a sustained source of IL-12 and the expression of IL-12Rβ2 and T-bet, a Th1 associated transcription factor (214, 218, 234, 418, 546). This correlates with lesion resolution and very low parasite loads (reviewed in (455)). Furthermore, several studies using *L. mexicana* infection in C57BL/6
mice have illustrated how the chronic disease associated with the infection by this pathogen is a result of a balance between Th1 and Th2 immune effector functions. In this system, the inhibition of the Th1 signaling pathways through STAT-4 leads to lesion progression (80). The infection of IL-4 deficient or STAT-6 deficient mice, animals in which Th2 signaling pathways are compromised, results in lesion resolution with low parasite loads and higher amounts of IFN-γ in recall responses (502, 536). However, studies using C3H and C57BL/6 mice infected with *L. amazonensis*, a member of the *L. mexicana* complex of parasites, demonstrate that the chronic cutaneous lesions containing up to 10^8 parasites persist even with exogenous administration of the Th1-promoting cytokine IL-12 and IFN-γ (41, 236). This refractoriness to IL-12 correlates with low levels of IL-12 receptor β2 expression on the CD4+ T cell population, which remains low even in the absence of the Th2 cytokine IL-4 (236). These studies suggest that the susceptibility of these mice to *L. amazonensis* is probably multifactorial and results, in part, from an inability to develop an effective Th1 response, a phenotype that is independent of the presence or absence of IL-4. Therefore, this murine model of cutaneous leishmaniasis, in addition to visceral diseases caused by *L. donovani* and *L. chagasi* in mice, is an experimental system in which Th1 responses are limited without expansion of Th2 cells (248, 586).

Our laboratory is studying the immune response of C3HeB/FeJ mice infected with *L. amazonensis*. The main focus of our research is to characterize and overcome the immune defects associated with the impaired Th1 response of *L. amazonensis*-infected mice. The central hypothesis of my work is that a Th1 response will provide protection to a *L. amazonensis* infection. The data presented here summarize some of our studies aimed at promoting resistance to a *L. amazonensis* infection. In particular, we have attempted to
induce a Th1 response in C3HeB/FeJ mice either before or during a *L. amazonensis* infection, and we have subsequently followed the outcome of the disease. We have characterized the results and analyzed them in the context of the available *Leishmania* literature: these studies have helped us to understand some of the immune factors necessary, or not, for resistance to a *L. amazonensis* challenge and are relevant to future vaccine or immunotherapy strategies.

**Dissertation organization**

The objective of the first study was to promote a Th1 immune response in mice chronically infected with *L. amazonensis*. Dendritic cells are the main antigen-presenting cells in vitro and in vivo and their potential as prophylactic vaccine adjuvants has been exploited to promote an efficient immune response before infection with *L. major* (53, 157, 574). However, DC-based vaccines have not been tried therapeutically in mice with established cutaneous lesions. Our hypothesis was that administration of antigen-pulsed bone marrow-derived dendritic cells (BM-DC) and the Th1 promoting cytokine IL-12 would promote lesion resolution in C3HeB/FeJ mice chronically infected with *L. amazonensis*.

The objective of the second study was to test if the Th1 response induced in naïve mice vaccinated with *L. amazonensis* antigen-pulsed BM-DC and IL-12 would promote resistance to *L. amazonensis* infection. Protection to a *L. amazonensis* challenge has been achieved in mice by giving parasite Ag, DNA or by adoptively transferring a *L. amazonensis*-specific Th1 CD4^+^ T cell line before the infection (56, 82, 233, 431). This was associated with an increase in IFN-γ production within the draining lymph node (DLN), suggesting a Th1 phenotype of the local immune response. Furthermore, Ji et al. showed that CD4^+^ T cells
from the DLN of protected mice had increased expression of mRNA levels for IL-12Rβ2, further supporting that a Th1 response could provide protection to \textit{L. amazonensis} infection (233). Therefore, we hypothesized that mice vaccinated with \textit{L. amazonensis}-pulsed BM-DC and IL-12 would resist a subsequent \textit{L. amazonensis} infection.

The objective of the third study was to determine if the Th1 response of C3HeB/FeJ mice that have healed a previous \textit{L. major} infection can adequately promote protection to a subsequent \textit{L. amazonensis} infection. While the immune response to \textit{L. major} infection is used as a model in this experimental system of murine cutaneous leishmaniasis, it is not known if the Th1 response elicited by \textit{L. major} in vivo would provide protection to a subsequent \textit{L. amazonensis} infection. Therefore, it is possible that the immune response necessary to promote resistance to \textit{L. amazonensis} may not be the same as that for \textit{L. major}. Variable degrees of cross-protection between different \textit{Leishmania} species have been previously described (11, 14, 15, 42, 82, 111, 115, 161, 167, 221, 222, 280, 282, 283, 351, 395, 422, 431, 526, 571). In particular, one mouse study has reported efficient cross-protection between \textit{L. major} and \textit{L. amazonensis} after co-infection (571). However, cross-protection was achieved using an avirulent strain of \textit{L. major} and the associated immune response and parasite quantification were not determined. Therefore, we have studied the outcome of a \textit{L. amazonensis} infection in mice sequentially infected with \textit{L. major} and \textit{L. amazonensis}. We have characterized the associated immune response and parasite loads.
Literature review

1. History

There is evidence that leishmaniasis has a long history as designs of human faces reminiscent of the ones of patients with cutaneous and mucocutaneous leishmaniasis were found on pottery from 400-900 A.D (144, 279). Later, the Bedouin in the desert of Arabia described a chronic sore affecting the exposed part of their bodies, called “Al Okht” or the little sister (reviewed in (426)). It is thought that this disease corresponded to leishmaniasis. It is only in 1756 that Russell first reported a description of this chronic sore. In 1885 and 1898, Cunningham and Borovsky, respectively, described organisms in the lesions of patients with such disease. In 1903, Wright called these organisms *Helcosoma tropicum*. At the same time, Leishman and Donovan described ovoid bodies in the spleen of human patients in India. These were first called *Piroplasma donovani* by Laveran and Mesnil and then later renamed *Leishmania donovani* by Ross. In 1906, the organism described by Wright was named *Leishmania tropica* (reviewed in (426)).

2. *Leishmania* parasites

*Leishmania* are intracellular protozoa belonging to the family of Trypanosomatidae (Table 1). There are approximately 20 different species of *Leishmania* parasites and each of them is generally responsible for a specific clinical form of the disease (136). However, some species usually causing cutaneous disease have been isolated from patients with visceral leishmaniasis and vice versa (16, 48, 320, 325, 436, 445). For example, *L. amazonensis* (a member of the *L. mexicana* complex of parasites) usually causes localized cutaneous leishmaniasis, but also occasionally diffuse cutaneous leishmaniasis and mucocutaneous
leishmaniasis (38, 279, 498). In addition, it has been recovered from people with visceral leishmaniasis (37, 38).

*Leishmania* spp. are digenetic parasites, i.e. they adopt two different morphologies in their host and vector. The insect vectors harbor the promastigote forms of the parasite, which are long, slender and possess an anterior flagellum. In contrast, within the host cells, the promastigotes transform into a round, aflagellated form termed amastigotes (reviewed in (198)).

The genome of *Leishmania* spp. is subdivided into kinetoplastic (i.e. the equivalent of mammalian mitochondria) and nuclear genomes. It is composed of 36 chromosomes and the genome size is 35 Mb (588). The difference between *Leishmania* spp. at the deoxyribonucleic acid sequence level is estimated to be approximately 15% (114).

Table 1. Taxonomy of *Leishmania* parasites (adapted from Dedet et al. (136)).

<table>
<thead>
<tr>
<th>Kingdom: Protista</th>
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<tr>
<td>Subkingdom: Protozoa</td>
</tr>
<tr>
<td>Phylum: Sarcomastigophora</td>
</tr>
<tr>
<td>Subphylum: Mastigophora</td>
</tr>
<tr>
<td>Class: Zoomastigophora</td>
</tr>
<tr>
<td>Order: Kinetoplastida</td>
</tr>
<tr>
<td>Suborder: Trypanosomatina</td>
</tr>
<tr>
<td>Family: Trypanosomatidae</td>
</tr>
<tr>
<td>Section: Salivaria</td>
</tr>
<tr>
<td>Genus: <em>Leishmania</em></td>
</tr>
<tr>
<td>Sub-genus: <em>Leishmania</em> (Suprapylaria)</td>
</tr>
<tr>
<td>Vianna (Peripylaria)</td>
</tr>
<tr>
<td>Species: <em>major</em>, <em>amazonensis</em>, <em>mexicana</em>, <em>donovani</em>, <em>chagasi</em>, etc.</td>
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3. Vectors

The main vectors of leishmaniasis belong to the family of Psychodidae and subfamily of Phlebotominae (sand flies). Natural transmission of leishmaniasis generally occurs through
the bite of an infected female sand fly, either of the genus *Phlebotomus* (in the Old World) or *Lutzomyia* (in the New World), during its bloodmeal. A link between leishmaniasis and its sand fly vector was first observed in 1764 by Cosme (210). However, the first evidence of experimental transmission of leishmaniasis through the bite of a sand fly was reported in 1931 (521). There is evidence that approximately 30 different species of sand flies can act as vectors of leishmaniasis (258). In general, each species of sand fly can only transmit some specific *Leishmania* species (reviewed in (479) and (259)). This is referred to as vectorial competence and may result from different structures of the lipophosphoglycan (LPG) molecules on promastigotes between *Leishmania* (430).

*Lutzomyia flaviscutellata* is considered as the main vector of *L. amazonensis*. It is a nocturnal feeder, abundant in forests that are rarely frequented by man (279). Furthermore, there is evidence that *Lutzomyia olmeca nociva* may also be transmitting *L. amazonensis* (258). In addition, more recently, *Lutzomyia nuneztovari anglesi* has been reported as a vector for *L. amazonensis* (324).

Females sand flies require a bloodmeal every 4 to 5 days (244). The blood meal volume of a sand fly is estimated around 100-300 nl and the number of transmitted parasites evaluated between 25 and 1000 (147, 480).

Although sand flies may not always be required for the transmission of leishmaniasis (see below), passage through the sand fly vector is necessary for the parasite cycle to be completed. Indeed, the sand fly digestive tract is where the transformation of the ingested amastigotes into infective metacyclic promastigotes, takes place (reviewed in (482)). When a female sand fly takes its bloodmeal on an infected host, the ingesta containing *Leishmania* amastigotes reaches the midgut where the bloodmeal is confined within a structure called the
peritrophic membrane. It is composed of a network of chitin that may protect the parasite from digestive enzymes early after the bloodmeal. In addition, it may also later interfere with promastigote development and both *Leishmania* and sand fly derived chitinases are necessary for the parasite to escape the peritrophic membrane (reviewed in (482)). The transformation of amastigotes into promastigotes takes 12 to 18h (reviewed in (482)). However, it takes approximately 6 to 7 days for the complete differentiation of promastigotes towards their metacyclic stage, i.e. the infective form that will be inoculated into the host (487, 488). Based on the location within the gut where promastigotes develop, *Leishmania* parasites have been subdivided into suprapylarian (promastigote development restricted to the midgut and foregut) or peripylarian (development of promastigotes within the hindgut) (reviewed in (136)). In both groups however, the metacyclogenesis involves a sequential transition of promastigotes through different morphological stages, i.e. procyclic (short, ovoid, slightly motile), nectomonad (long, slender, highly motile), haptomonads (occasionally dividing) and finally metacyclic (unattached, elongated flagellum, thinner, highly motile, never seen dividing) (reviewed in (479)). During this transition, the promastigotes migrate toward the foregut and proboscis (mouth parts). Ultrastructural studies have shown that *Leishmania* spp. promastigotes, including *L. amazonensis*, intimately attach to the microvilli of the midgut epithelial cells of the sand fly and this may allow the parasites to resist gut peristaltis (261, 357). Upon feeding on the host, the infected sand fly will inoculate metacyclic promastigotes. Experimentally, the isolation of metacyclic *L. major* promastigotes is achievable as these express different cell surface carbohydrates, which results in the loss of their ability to agglutinate in the presence of peanut agglutinin (484).
4. Saliva

Many studies have characterized the composition of sand fly saliva. Most of the described pharmacologically active compounds from sand fly saliva are from *Phlebotomus papatasi* (vector of *L. major*) and *Lutzomyia longipalpis* (vector of *L. chagasi*). The saliva from these sand flies has been shown to contain an array of immunomodulatory molecules that have a wide range of actions, including vasodilation, inhibition of blood clotting, inhibition of platelet aggregation and downregulation of the innate & adaptive immunity (reviewed in (244) and Table 2). The vasodilation and inhibition of platelet aggregation inhibits the rapid formation of a blood clot and allow the parasite to be exposed to certain serum constituents such as the complement components and natural anti-leishmanial IgM, which *Leishmania* promastigotes use for their subsequent uptake by phagocytes (see complement section). The downregulation of the immune response includes the inhibition of IL-12, IFN-γ and nitric oxide, all of which are important mediators of the protective Th1 response necessary to heal *Leishmania* infection. In addition, a recent study has reported that the saliva from *Lu. longipalpis* could inhibit both the classical and alternative complement pathways (95).

Clinically, it has been suggested that qualitative differences within the sand fly saliva may have an impact on the severity of the resulting infection. Indeed, Warburg et al. have shown that *Lu. longipalpis* from Costa-Rica produce smaller amounts of maxadilan, a potent vasodilator from the saliva, than the *Lu. longipalpis* vectors from Brazil (579). Interestingly, *L. chagasi* infection has a different outcome in each of these countries: it results in the cutaneous disease in Costa-Rica while it causes the visceral form of leishmaniasis in Brazil (579). As the parasite isolates from both countries appear identical, this suggests that...
vectorial differences between the *Lu. longipalpis* from both countries influence the outcome of *L. chagasi* infection. It is also possible that host differences may have accounted for the different clinical forms of *L. chagasi* infection observed in those countries.

During experimental murine studies, the co-injection of salivary gland homogenates, extracts or sonicates (referred to as SGH below) with the parasite inoculum has been shown to exacerbate the infection, i.e. to increase lesion size and parasite numbers, in comparison to the mice injected with parasites alone (45, 57, 307, 331, 403, 499, 556). This increased severity of infection was shown to require functional lymphocytes as SGH did not exacerbate the disease in SCID mice (45). When *L. major* or *L. braziliensis* were co-injected with SGH, the exacerbated disease was associated with an increased IL-4 production in the draining lymph node (45, 307, 331). In addition, a decrease in IFN-γ, IL-12 and iNOS mRNA but not in IL-10 and TGF-β mRNA were observed in the draining lymph node of mice infected with *L. major* and the SGH (331). Moreover, the exacerbative effect of the SGH was abrogated in mice treated with neutralizing antibodies to IL-4 or in IL-4 deficient mice (45, 307).

Altogether, these data suggested that saliva could induce a Th2 response, resulting in an enhanced host susceptibility. Exacerbation of the disease by sand fly saliva was not observed in one mouse study (93). Similarly, co-injection of *Leishmania* parasites with saliva does not seem to affect the course of experimental canine leishmaniasis (416). The reasons for these discrepancies are currently unknown.

The specific effect of the saliva from *Lu. flaviscutellata*, the vector for *L. amazonensis*, on subsequent *L. amazonensis* infection remains unknown, as rearing *Lu. flaviscutellata* in the laboratory is difficult. However, it has been shown that mice co-injected with *Lu. longipalpis* SGH and *L. amazonensis* had increased lesion size in comparison to the controls
This was associated with an enhanced production of IL-4 and IL-10 in the draining lymph node; furthermore, the levels of IL-10 mRNA were increased at the lesion site (403). Although *Lu. longipalpis* is not the vector for *L. amazonensis*, it can transmit *L. amazonensis* under experimental conditions (260). In addition, the geographic distribution of *Lu. longipalpis* overlaps with the one of *Lu. flaviscutellata* (69). Therefore, it is conceivable that saliva from bites of *Lu. longipalpis* could modulate a subsequent *L. amazonensis* infection in those areas of the world.

Although the injection of SGH did exacerbate the disease, it remained to be determined if sand flies themselves inoculate a substance that would similarly affect the infection. An initial study showed that the subcutaneous injection of *L. major* promastigotes at a cutaneous location exposed to the bites of uninfected *L. longipalpis* one hour earlier resulted in increased lesion size in comparison to controls which had not been previously bitten by sand flies (553). This confirmed the available data and indicated that during their bloodmeal, sand flies also inject a material that is able to exacerbate the infection. However, more recently, Belkaid et al have shown that the exacerbation of dermal lesions due to the co-injection of *L. major* and *P. papatasi* SGH was abrogated in mice pre-exposed with SGH two weeks earlier and this correlated with the presence of anti-saliva antibodies in pre-exposed mice (45). The reasons for the discrepancies between these results, i.e. lesion exacerbation or not after saliva exposure, are unknown; however, the source of SGH was different and when saliva from *L. major* vector sand fly (*P. papatasi*) was used, it is a lack of exacerbation that was observed. Therefore, this suggested that pre-exposure to saliva may have some protective effects. This was later supported by a study demonstrating that pre-exposure of mice to the bites of uninfected *P. papatasi* resulted in an increased protection to a subsequent *L. major* challenge.
through the bite of infected sand flies (245). This was accompanied by a strong DTH response and an infiltrate of IFN-γ and IL-12 producing cells at the site of infection. Of note, the bites of infected sand flies did not induce an enhanced IL-4 production, suggesting that salivary secretions do not promote IL-4, which may be an artifact of whole salivary glands homogenate preparation used in the co-injection studies. In addition, these results are consistent with what happens in the field: indeed, people living in areas endemic for leishmaniasis are known to show attenuated infections, while tourists tend to develop more severe disease. Finally, the protection obtained after pre-exposure to saliva indicated that immunogenic salivary molecules might be used as components of anti-leishmanial vaccine. One such molecule, a 15 kD protein named SP15 has been isolated from salivary glands of *P. papatasi* and used as a vaccine to successfully confer protection to mice subsequently infected with *L. major* (567). Similarly, vaccination against the potent salivary vasodilator maxadilan was shown to provide increased resistance to *L. major* infection (362).

Table 2. Immunomodulatory molecules from sand fly saliva.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Sand fly</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apyrase</td>
<td><em>Lu. longipalpis</em></td>
<td>Hydrolyzes ATP and ADP to AMP which inhibits platelet aggregation.</td>
</tr>
<tr>
<td></td>
<td><em>P. papatasi</em></td>
<td></td>
</tr>
<tr>
<td>Maxadilan</td>
<td><em>Lu. longipalpis</em></td>
<td>Vasodilation, inhibition of T cell proliferation and delayed type hypersensitivity response.</td>
</tr>
<tr>
<td>Adenosine &amp; AMP</td>
<td><em>Lu. longipalpis</em></td>
<td>Anti-platelet aggregation, vasodilation, inhibition of IL-12, IFN-γ and TNF-α, downregulation of nitric oxide synthase gene, enhancement of IL-10.</td>
</tr>
<tr>
<td></td>
<td><em>P. papatasi</em></td>
<td></td>
</tr>
<tr>
<td>5' nucleotidase</td>
<td><em>Lu. longipalpis</em></td>
<td>Anti-platelet aggregation and vasodilation.</td>
</tr>
</tbody>
</table>
**Table 2 (Continued)**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Species</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine deaminase</td>
<td><em>Lu. longipalpis</em></td>
<td>Production of inosine which inhibit IL-12, IFN-γ, TNF-α and nitric oxide.</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td><em>Lu. longipalpis</em></td>
<td>Creates gaps in the extracellular matrix and promote diffusion.</td>
</tr>
<tr>
<td></td>
<td><em>P. papatasi</em></td>
<td></td>
</tr>
<tr>
<td>Unknown protein</td>
<td><em>Lu. longipalpis</em></td>
<td>Inhibition of the classical and alternative complement pathways.</td>
</tr>
<tr>
<td>(10000-30000 Da)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This table has been modified from Kamhawi, 2000, *Microbes and Infection*.

### 5. Atypical transmission

Although the transmission of leishmaniasis occurs mainly through the bite of an infected sand fly, other marginal ways of transmission have been reported. As parasites can be found in the blood from both asymptomatic and symptomatic patients (50, 110, 297, 326, 407), transmission through the use of contaminated syringes (125) or blood transfer is possible (183, 276). It appears that blood monocytes are the main source of *Leishmania* parasites in blood samples and these remain infective even after storage in blood banks (139, 183). In addition, congenital transmission has been described (153, 316, 406, 595). However, infection of the placenta does not always result in vertical transmission as observed in dogs infected with *L. chagasi* (19). Animal to animal propagation of the disease has also been reported through direct contacts (281, 406). The fact that *Leishmania* parasites can be detected in the nasal secretion of dogs suggests that direct contacts may be a potential, although probably unusual, way of transmission within reservoir hosts (69). Finally, mechanical transmission via the stable fly *Stomoxys calcitrans* has been achieved experimentally (284).
6. Reservoirs

Depending on the *Leishmania* species and the geographic area of interest, the main reservoirs include small rodents (e.g. rats, gerbils, squirrels), marsupials, wild canids (e.g. wolves, foxes), domestic dogs and humans (reviewed in (69) and (29)). Forest rodents and marsupials are reservoirs for *L. amazonensis*, with the rodent *Proechimys guyannensis* being the primary host reservoir (69, 279). The reservoirs for *L. mexicana* in Texas appear to be the southern plain woodrats *Neotoma micropus* and *Neotoma floridana* (256, 339). In addition, *L. mexicana* has also been cultured from *Neotoma albigula* in Arizona (257).

While dogs have been incriminated as reservoir hosts of visceral leishmaniasis (reviewed in (360)), there is only inconclusive evidence that they may have a role as reservoir in the transmission of cutaneous leishmaniasis (reviewed in (457)). Programs aimed at eradicating infected dogs have taken place in geographic areas where visceral leishmaniasis is endemic (28, 119, 145, 360, 415). However, the culling of infected dogs is controversial for ethical, emotional and economical reasons. Furthermore, the epidemiological studies that have analyzed the effect of culling on the resulting incidence of human leishmaniasis have provided conflicting results (28, 119, 145, 360, 415). There are several hypotheses as to why the culling of infected dogs did not consistently reduce the incidence of human cases. For example, all asymptomatic dogs may not have been eradicated. However, whether these dogs are infective to sand flies remains contradictory due to equivocal results (17, 360, 560). Also, other sylvatic reservoirs probably persisted in the environment, keeping the parasite cycle active.
In conclusion, it is likely that future eradicating program will require a combination of better diagnostic tools to identify infected dogs, the elimination of other potential reservoirs and strategies aimed at controlling the vector population (55, 360).

7. Virulence factors

7.1. Phosphoglycans

A variety of phosphoglycans (PGs), i.e. glycoconjugates, are expressed by *Leishmania* spp. (reviewed in (479)). Some of the PGs, such as the lipophosphoglycan (LPG) and proteophosphoglycan (PPG) are attached to the parasite surface through glycosylphosphatidylinositol (GPI) lipid anchors. In contrast, other PGs are secreted, including the secreted proteophosphoglycan (sPPG) and a secreted acid phosphatase (sAP). Phosphoglycans in general act as virulence factors: they can inhibit the production of the Th1 promoting cytokine IL-12 by macrophages and they are notably responsible for the resistance to the oxidative burst (428, 532). In particular, virulence properties have been attributed to specific PGs, and this is summarized below.

7.1.A. Lipophosphoglycans

The lipophosphoglycans (LPG) molecules of *Leishmania* spp. are mainly expressed on promastigotes and have multiple functions that are crucial for the survival and/or uptake of the parasites within both their vectors and hosts (reviewed in (563)). LPG molecules have 4 domains, i.e. a phophatidylinositol lipid anchor, a glycan core, a repeating saccharide-phosphate region and an oligosaccharide cap. It is present on the entire surface of promastigotes, including the flagellum. Although the flagellum is dispensable for
macrophage infection, it is indispensable for survival within the sand fly (126). In particular, the LPG molecules play a role in attachment to the gut epithelial cells from the sand fly (reviewed (479), (482) and (486)). Furthermore, it does promote parasite retention within the sand fly vector (486). In addition, the LPG molecules undergo structural changes during metacyclogenesis (333). These include elongation due to increased numbers of phosphorylated saccharides and changes in the nature of the terminal sugars, i.e. these are galactose and arabinopyranose on procyclic and metacyclic promastigotes, respectively. These changes explain the loss of PNA agglutinability by metacyclic promastigotes (484). Moreover, the modifications in the LPG molecules during metacyclogenesis result in a loss of binding to the sand fly gut epithelial cells (482).

In addition to its importance within the sand fly vector, LPG also acts as a virulence factor in the host (reviewed in (563)). Indeed, it can mediate the binding of both promastigotes and amastigotes to phagocytes (251, 252, 369, 551). In addition, using LPG-deficient parasites, it has been shown that LPG does confer intracellular survival (200, 341). Such enhanced viability within host cells may be mediated by the ability of LPG to protect the parasites from oxidative damage by scavenging oxygen radicals (99, 341, 531). Furthermore, LPG can downregulate macrophage functions such as pro-inflammatory cytokine production and chemotaxis (160). Similarly, LPG can inhibit signal transduction, e.g. protein kinase C activity, within macrophages (142, 143).

In support of these studies, LPG deficient-L. major parasites are highly attenuated both in vitro and in vivo (530, 531). In contrast, LPG deficient-L. mexicana are as infective and virulent as wild type control, suggesting that LPG does not act as a virulence factor for L. mexicana (226).
7.1. B. Proteophosphoglycans

The proteophosphoglycans from *L. mexicana* can activate complement via the mannan binding lectin pathway (424). Furthermore, it has been suggested that there may be a correlation between the interaction of PPG with the complement and the chronic lesions associated with *L. mexicana* infection. It is thought that PPG released at the site of infection may deplete the lesion of complement. This depletion, which is consistent with the finding that tissue-derived *L. mexicana* amastigotes do not have detectable C3 on their surface (423), would prevent complement-mediated lysis of the amastigotes.

7.2. Glycoprotein 63 (gp63)

The zinc metalloproteinase gp63, also referred as leishmanolysin, is a GPI-anchored protein present at the surface of several *Leishmania* species (68, 117, 301). Its expression increases during metacyclogenesis (239, 278). Although the development gp63-deficient parasites within sand flies is not altered (239), gp63 plays a role in the attachment and subsequent uptake of Leishmania parasites to phagocytes (311, 336, 476). In addition, gp63 promotes resistance to complement-mediated lysis (74, 239). Finally, gp63 is able to degrade components of the extra-cellular matrix and to enhance the migration of *Leishmania* parasites within the extra-cellular matrix in vitro, further supporting a role for gp63 in the pathogenesis of leishmaniasis (337).

7.3. Cysteine proteinases

Cysteine proteinases (CPs) are enzymes common to several *Leishmania* species, and in particular *L. mexicana* and *L. amazonensis* (reviewed in (371)). They are not essential for
parasite growth but act as potent virulence factors (130, 371, 372). *L. mexicana* has 3 types of CPs encoded by the cpa, cpb and cpc genes. Their expression is stage specific: it is increased on metacyclic promastigotes with maximal expression in amastigotes (121, 371, 464). In particular, the CPs are mostly localized to the megasomes, i.e. the lysosome-like organelles of *L. mexicana* and *L. amazonensis* amastigotes (227, 564). In vivo, mice infected with *L. mexicana* promastigotes deficient in one or several of the CPs could control their infection and the associated immune response was of the Th1 phenotype (13, 79, 159, 372). Furthermore, mice that had controlled a challenge of CPs deficient parasite could resist a challenge with wild-type virulent organisms (13). Similarly, immunization of mice with CPs from *L. amazonensis* provided some degree of protection towards a subsequent *L. amazonensis* infection (56). Therefore, this suggested that CPs or CPs deficient parasites could potentially be used in vaccine strategies (371). Finally, the degradation of MHC class II molecules by *L. amazonensis* parasites is likely mediated by CPs, thereby suggesting that CPs may interfere with adequate parasite-antigen presentation during the course of a *Leishmania* infection (133). This further supports a role for these proteinases as virulence factors in vivo.

7.4. LACK genes

The early production of IL-4 in susceptible BALB/c mice infected with *L. major* is mediated by a specific subset of CD4⁺ T cells recognizing the *Leishmania* homologue of mammalian receptor for activated C kinase (LACK) antigen (293). *L. major* contains four copies of the LACK gene in its genome and attempts at generating parasites lacking LACK have failed, suggesting that LACK is necessary for parasite viability (254). In addition, *L.
major containing a single LACK gene showed attenuated virulence, and this was reversed if these parasites were complemented with an additional copy of the LACK gene (254). Altogether, this suggests that LACK can be considered as a virulence factor of *Leishmania* spp.

8. Complement activation and subversion

Upon contact with host blood, promastigotes come into contact with opsonins such as natural antibodies and complement components that subsequently trigger the activation of the complement cascade (reviewed in (147)). The relationship between *Leishmania* and the complement system are paradoxical as they serve both the host and the parasites (reviewed in (365)).

Complement activation is one of the most effective host defense mechanism against infection and both the classical and alternative complement pathways can be activated by *Leishmania* parasites (148, 366, 367). The classical pathway of complement activation is the main mechanism of *Leishmania* opsonization in the serum of mammals. It is activated very rapidly, with maximum binding reached after 2 to 3 min (148). As a result, C3b is deposited on the promastigote surface and acts as the C5 convertase, which will lead to the assembly of the membrane attack complex and the subsequent lysis of the parasites. While the alternative pathway of complement activation is activated simultaneously, it is slower. However, after a few minutes, it is the only complement pathway detectable (148). As a result of complement activation, 90% of the inoculated promastigotes are killed 2.5 min after serum contact (148). Therefore, it is crucial for *Leishmania* parasites to rapidly enter host phagocytes to avoid complement lysis. To do so, *Leishmania* spp. have exploited the complement system. For
example, C3-opsonized promastigotes adhere to CR1 on erythrocytes or to platelets in
primate or non-primate mammals (including dog, rabbit, guinea-pig, hamster and mouse),
respectively (149, 150). This phenomenon has been referred to as immune adherence and its
purpose is to enhance the uptake of opsonized parasites by professional phagocytes (147,
396). In addition, the activation of complement by *Leishmania* parasites was required for
macrophage chemotaxis to occur (70). This migration of macrophages towards the products
of complement activation may enhance the killing of *Leishmania* but alternatively could
serve as a source of reservoir cells for the parasites. In addition, studies have suggested that
macrophages can produce sufficient amounts of complement components to opsonize
*Leishmania* parasites and subsequently promote their uptake by phagocytic cells (61, 589).
Therefore, *Leishmania* parasites are able to exploit the host defense mechanisms to enter host
cells.

Furthermore, *Leishmania* parasites have also evolved ways to resist complement
activation: for example, the infective metacyclic promastigotes are more resistant to
complement-mediated lysis than the non-infective log-phase promastigotes (148)(also go see
Sacks Science paper where he discovers the metacyclics). Increased resistance of metacyclic
promastigotes to complement-mediated lysis correlates with modifications of the LPG
molecules (444). In addition, a role for the zinc metalloproteinase gp63 expressed by
promastigotes was incriminated in resistance of *L. amazonensis* to complement, as gp63 is
the major binding site for C3 on promastigotes and the presence of proteolytically active
gp63 correlated with increased conversion of C3b to a form antigenically similar to its
inactive form iC3b, resulting in a decreased complement-mediated lysis (74, 474). However,
using gp63 deficient *L. amazonensis* transfected with proteolytically active or inactive gp63,
Brittingham et al. showed that gp63 was not necessary for complement fixation (74). In addition, it was recently reported that complement resistance by *L. amazonensis* was gp63 independent (405). Furthermore, resistance of *L. amazonensis* to complement-mediated lysis was shown to occur after C3b deposition and results from the inhibition of late events from the complement cascade, possibly the limitation of C9 to access the parasite surface (404). Finally, complement components may also promote parasite survival within host cells. Indeed, complement-opsonized *L. major* promastigotes displayed an increased intra-cellular survival in comparison to non-opsonized control (368). In contrast, *L. amazonensis* promastigotes appear to survive efficiently within macrophages even in the absence of complement (369).

9. Uptake by host cells

Although *Leishmania* spp. can be taken up by a variety of cell types, including dendritic cells (113, 355, 442, 573), neutrophils (291, 420, 473), eosinophils (182, 421, 429, 435) and fibroblasts (63, 101, 137, 510), macrophages are the main host cells for the parasites (reviewed in (369)). As a result, the uptake of *Leishmania* by phagocytes has mainly been studied in macrophages. Most of the data available about the uptake of either promastigotes or amastigotes have been consistent with receptor-mediated endocytosis (194, 315, 369). In addition, amastigotes have been shown to directly adhere to proteoglycans present on the surface of mamalian cells (314). Below is a summary of the several receptors that have been shown to promote the uptake of *Leishmania* parasites.
9.1. Complement receptors

As mentioned earlier, the opsonization of *Leishmania* promastigotes by components of the complement can enhance attachment and subsequently facilitate their uptake by phagocytes (149, 367, 369). Among the complement receptors (CR), CR1, CR3 (Mac-1) and CR4 have all been shown to play a role in binding and uptake of *Leishmania*.

Immune adherence, i.e. the adhesion of C3-opsonized promastigotes to CR1 on erythrocytes, is one of the earliest events that occur after infection and it may facilitate parasite uptake by phagocytes (147, 149). In addition, while *L. major* can bind CR1 on macrophages, antibodies to CR1 could not inhibit the phagocytosis of serum-opsonized promastigotes (128, 470). Finally, the binding of *L. amazonensis* to macrophages does not seem to involve CR1 as the blocking of this receptor did not affect parasite binding (369). Altogether, this data suggest that, in general, CR1 may just play a minor role in facilitating parasite entry into macrophages.

Multiple studies have reported the binding of *Leishmania* spp. promastigotes and amastigotes to CR3 on macrophages (61, 194, 250, 370, 470, 585). CR3 preferentially binds the iC3b form of the C3 molecule that may be present on the surface of opsonized parasites (369). In addition, ligands for CR3 on promastigotes include the zinc metalloproteinase gp63 and the *Leishmania* LPG (364, 475-477, 551). A role for gp63 in binding and subsequent phagocytosis by macrophages was further supported by studies using blocking antibodies (476) and after transfection of gp63-deficient parasite with gp63 (311, 336). The phagocytosis of *L. major* by Langerhans cells was also shown to involve CR3 and the uptake was blocked by anti-CR3 antibodies (62). Although *L. amazonensis* promastigotes were shown to bind CR3 (370), their enhanced binding to macrophages in the presence of
complement is minimal (369). In addition, it appears that *L. amazonensis* amastigotes do not bind CR3 (369). Finally, the uptake of both *L. amazonensis* promastigotes and amastigotes by dendritic cells has been described (442). Pre-incubation of the parasites with immune serum, but not C3, enhanced the uptake of parasites by DC, and this was more pronounced when amastigotes were used.

While both *L. major* and *L. amazonensis* promastigotes can adhere to CR4, the role of this receptor is minor in comparison to the one of CR3 (370).

### 9.2. Fc receptor (FcR)

The cell surface of phagocytes contains a variety of Fc receptors that can bind the Fc portion of immunoglobulins. Upon ligand binding, these receptors will positively or negatively activate the cells, depending on the type of signaling motifs present in their cytoplasmic tail (433). There is evidence that Fc receptors play a role in the uptake of *Leishmania* parasites (194, 209, 369, 423). Promastigotes are unlikely to interact with abundant *Leishmania*-specific antibodies. In contrast, the surface of tissue-derived amastigotes isolated from lesions of *Leishmania*-infected mice, are coated with antibodies of different isotypes (194, 246, 423). As a result, the binding and uptake through the Fc receptor may be particularly relevant for amastigotes entry into host cells. Indeed, the phagocytosis of amastigote by macrophages, dendritic cells or fibroblastoid cell lines was enhanced when previously opsonized with immunoglobulins (194, 423, 442). Although the ligation of FcR generally induces a respiratory burst, it appears that *Leishmania* parasites taken up through FcR can survive for several days intracellularly (194, 590). This suggests that *Leishmania* has evolved ways to counteract or resist the detrimental effects of the respiratory burst. For
example, it has been reported that the uptake of amastigotes by macrophages through their FcγR triggered the production of IL-10, which in turn contributed to the enhanced intracellular parasite growth (246). The subversion of the FcγR signaling pathways by Leishmania parasites to their advantage is further supported by studies showing that mice lacking FcγR develop only minimal lesions when infected with L. amazonensis and L. pifanoi (262).

9.3. Lectin-like receptors

Macrophages and/or dendritic cells express a series of lectin-like receptor on their surface, including the mannose fucose receptor (MFR), the receptor for advanced glycosylation end-products and/or dendritic cell-specific ICAM 3 grabbing nonintegrin (DC-SIGN), all of which have been incriminated in the binding and uptake of Leishmania parasites.

9.4. Mannose Fucose Receptor

Several studies have reported a role for the MFR in the binding and uptake of promastigotes from different Leishmania spp. (61, 584, 585). In contrast, the MFR does not seem to be used by L. major and L. amazonensis amastigotes, supporting the fact that promastigotes and amastigotes may use different sets of receptors to enter host cells (194, 369).
9.5. DC-SIGN

DC-SIGN is a C-type lectin expressed on the surface of dendritic cells; it has been involved in the recognition and binding of several pathogens (569). In particular, the binding and uptake of *L. infantum* and *L. pifanoi* promastigotes and amastigotes can be mediated by DC-SIGN (113, 116). In contrast, DC-SIGN does not appear to be an important receptor for *L. major* uptake (113). Finally, while *L. mexicana* LPG could interact with a DC-SIGN chimeric molecule (24), there is recent evidence that the binding of *L. infantum*, *L. pifanoi* and *L. donovani* to DC-SIGN is LPG independent (113).

9.6. Fibronectin receptor

There is evidence that the binding and uptake of *Leishmania* spp. by macrophages involve the fibronectin receptor (73, 462, 591). In particular, gp63 harbors a region antigenically similar to a region from fibronectin and thereby mediate the binding to the fibronectin receptor (73, 462). However, this adhesion was enhanced in the presence of complement, suggesting that the fibronectin receptor may cooperate with complement receptors to mediate parasite uptake (73).

10. The parasitophorous vacuole (PV)

Within host phagocytes, *Leishmania* parasites reside within vacuoles termed parasitophorous vacuoles (PV). These vacuoles have mainly been characterized in macrophages (reviewed in (23)). The PV in which parasites from the *L. mexicana* complex reside rapidly expand to form large vacuoles (22, 120, 449). There is evidence that this expansion of the PV is mediated by proteophosphoglycans (PPG) secreted by intracellular
amastigotes (425). In general, the PV have characteristics of the late endosomal-lysosomal compartments, as they contain phosphatases, hydrolases, express lysosomal-associated membrane proteins (LAMPs) and the vesicular proton-ATPase (22, 102, 120, 286, 478). As a result, the PV represent an acidic compartment characterized by a pH reaching 4.7 to 5.2 early after infection and it is likely that *Leishmania* spp. have evolved ways to resist in such acidic environment (22). In addition, there have been several reports of the localization of MHC class II molecules within the PV, suggesting that *Leishmania* parasites may interfere with antigen presentation (20, 21, 285, 286).

More recently, the characteristics of the PV from *Leishmania*-infected DC have been described. In general, the PV from *L. amazonensis*-infected DC are very similar to the ones from macrophages infected with the same parasite. Indeed, they were also acidic, expressed molecules typical of late endosomes/lysosomes, and contained both MHC calls II and H-2M molecules (442). However, in contrast to the PV of infected macrophages, the ones from DC were poor in cathepsins B, H, L & D, and lesser number of PV expressed rab7p, a protein involved in the fusion of late endosomes/lysosomes. In addition, the enlargement of the PV was less pronounced in DC than in (442).

11. Immune evasion

11.1. Evading MHC class II presentation

In vitro studies using both macrophages and dendritic cells have shown that *Leishmania* parasites, and in particular *L. mexicana* and *L. amazonensis*, can internalize MHC class II molecules and H2M, an enzyme that catalyzes the formation of MHC class II-peptide complexes (21, 133, 442). Intracellularly, these are localized within the megasomes, i.e. the
lysosome-like organelles of *Leishmania* amastigotes. In addition, there is evidence that MHC class II molecules and most likely H2M are degraded by parasitic cysteine proteinases (21, 133). Altogether, this suggests that *Leishmania* spp. may have evolved ways of evading the MHC class II pathway of antigen presentation, which could explain the poor immune response elicited by *L. mexicana* and/or *L. amazonensis*. However, the surface expression of MHC class II molecules on the surface of infected macrophages appeared normal, indicating that the sequestration of MHC class II molecules by intracellular amastigotes may not be biologically relevant (21, 285, 443). This was further supported by a recent study using *L. mexicana* showing that although H2M colocalized with the vacuoles containing parasites, the antigen presenting activity of infected macrophages was only minimally reduced (51).

### 11.2. Apoptotic mimicry

A recent study has shown that the surface membrane of *L. amazonensis* amastigotes express phosphatidylserine, a lipid typically exposed within the membrane of apoptotic cells (131). During the in vivo steady state, the elimination of apoptotic cells by macrophages does not elicit an overt inflammatory response as these cells are recognized as self. Similarly, de Freitas Balanco et al. suggest that *L. amazonensis* amastigotes use their expression of phosphatidylserine as a Trojan horse, to facilitate their entry into host cells, i.e. macrophages. Indeed, the blocking of phosphatidylserine reduced *L. amazonensis* infectivity in vitro and in vivo, and decreased the production of nitric oxide and TGF-β by macrophages (131).
12. Experimental leishmaniasis

Experimental studies of leishmaniasis have been performed in several animal species including non-human primates, dogs, hamsters and mice (reviewed in (217)). In particular, due to their small size, their affordable cost, the diversity of the available genetic backgrounds and the possibility to have animals deficient in certain specific gene/molecules, inbred strains of mice have emerged as the most commonly used animal models of leishmaniasis. The factors that influence the outcome of a *Leishmania* spp. infection in mice are multiple and notably include the genetic background of the host, of the inoculating dose of parasites used, the anatomic location of the primary infection and/or the gender.

Inbred strains of mice have helped uncover some of the immune mechanisms associated with resistance and/or susceptibility with the disease. For example, after a *L. major* infection, BALB/c mice mount a Th2 response and subsequently develop progressive, eventually fatal lesions. In contrast, C3H, C57BL/6 or C57BL/10 mice are resistant to *L. major*; this correlates with a strong Th1 immune response (reviewed in (480)). However, when these same mice are infected with *L. amazonensis*, they develop chronic cutaneous lesions (5, 236, 525). The susceptibility of these mice to *L. amazonensis* does not result from a strong Th2 response; instead, the associated immune response of the mixed Th1/Th2 phenotype. In addition, using genetically defined inbred strains of mice has allowed the identification of specific genes linked to disease control or susceptibility; these include Scl-1, Scl-2, MHC and Nramp1/Lsh/Bcg genes (reviewed in (59)).

The absolute numbers of *Leishmania* promastigotes used to experimentally inoculate mice also have an impact on the subsequent clinical outcome of the disease. The inoculating dose can be classified as to a “high dose challenge” (usually in the order of $10^5$ parasites or
more) and a "low dose challenge" (usually $10^4$ parasites or less). For example, CBA mice develop non-healing and self-healing lesions when infected with high and low doses of $L. major$, respectively (440). In addition, while BALB/c mice infected with high doses of $L. major$ display progressive lesions, they develop minimal footpad swelling when challenged with low doses of parasites (72, 122, 329). A similar correlation between the infecting dose and the resulting footpad swelling has been described in BALB/c mice infected with $L. amazonensis$ (122). However, while BALB/c mice inoculated with as low as $10 \ L. major$ can later resist a higher dose challenge, BALB/c mice immunized with $10 \ L. amazonensis$ promastigotes can not control a subsequent infection composed of $1000 \ L. amazonensis$ parasites (72, 122).

Several studies have reported that the clinical presentation of the disease varies depending on the anatomical site of infection, and this seems to be partly dependent upon the host strain (361). In general, mice are more susceptible to $L. major$ when infected at the base of the tail than when they are challenged in the ear or the footpad (33, 267, 439). In contrast, the opposite effect was observed in SWR mice infected with $L. major$, i.e. the disease was less severe when mice were infected in the footpad than at the base of the tail (392).

Finally, it has been reported that the host gender may influence the pathogenesis of leishmaniasis. In particular, the gender effect appears to be dependent upon the species and/or strain of the host. Indeed, the disease associated with the infection of hamsters with $L. panamensis$ or $L. guyanensis$ is more severe in males (i.e. larger lesion size, higher parasite load and increased spreading to distant cutaneous sites) than in females (559). In contrast, the opposite trend is observed in B10.129 mice infected with $L. major$, i.e. males are more
resistant than females (166). However, in BALB/c mice, both genders are equally susceptible to *L. major* (166).

13. The Immune Response of infected mice

13.1. The CD4\(^+\) T cell response and associated cytokines

The CD4\(^+\) T cell compartment of both mice and humans has been subdivided into two subsets, i.e. T helper 1 (Th1) and T helper 2 (Th2), based on their cytokine production profiles (reviewed in (303)). Th1 responses are characterized by the production of IFN-\(\gamma\) and IL-12 while Th2 responses are associated with the secretion of IL-4, IL-10 and/or IL-13. A critical role for each of these responses has been highlighted in murine studies of *L. major* infection, as Th1 and Th2 CD4\(^+\) T cells promote resistance and susceptibility to the disease, respectively.

13.1.A. *Leishmania major*

Among all the *Leishmania* species, *L. major* is the most studied. The immune response associated with *L. major* infection is very well characterized and has led to an understanding of some of the immune mechanisms necessary to control these intracellular protozoa (reviewed in (455) and (480). Most strains of mice (C57BL/6, C3H, CBA, 129Sv/Ev) are resistant to an infection with *L. major*; this is characterized by the development of a transient lesion that subsequently heal. In contrast, BALB/c mice are highly susceptible to a similar infection and develop progressive lesions. As mentioned above, studies from the late 1980s have shown that resistance to *L. major* infection correlated with the expansion of Th1 CD4\(^+\) T cells while Th2 CD4\(^+\) T cells promoted susceptibility (205, 515).
13.1.A.1. IFN-γ

The production of IFN-γ, characteristic of Th1 immune responses, appears critical for lesion resolution. In particular, IFN-γ produced by Th1 cells signals through STAT-1 and induces the expression of T-bet, a Th1 specific transcription factor (3). While draining lymph node cells from resistant mice produce large amounts of IFN-γ in response to antigen stimulation in vitro, the ones from susceptible mice only secrete low to undetectable levels of IFN-γ (206, 491, 513). The primary source of IFN-γ is the CD4+ T cell population (206). In addition, resistant mice deficient in IFN-γ production or treated with antibodies against IFN-γ mount a Th2 response and cannot control the disease (49, 104, 204, 292, 294, 490, 513, 543, 578). In particular, the timing of the administration of anti-IFN-γ antibodies was critical since it was only effective when given at the time of infection, suggesting that the levels of IFN-γ at the time of infection were critical to determine the phenotype of the subsequent immune response, i.e. Th1 vs Th2 (49, 513). In addition, mice will subsequently heal if the antibody treatment is abrogated (219). Furthermore, regardless of the production of IFN-γ, several studies have shown that the IFN-γ signaling pathway needed to be intact for lesion resolution to occur. First, mice lacking the IFN-γ receptor developed progressive lesions after L. major infection, despite the presence of IFN-γ-producing CD4+ T cells (269, 468, 543). Second, genetically resistant mice lacking STAT1 were unable to mount a Th1 response and subsequently became susceptible to L. major infection (468). Similarly, mice lacking T-bet developed non-healing lesions after a L. major challenge and this susceptibility correlated with the presence of CD4+ T cells producing predominantly Th2 cytokines (546).
Finally, while susceptible mice given exogenous IFN-γ or infected with *Leishmania* parasites expressing IFN-γ did initially develop smaller lesions, they still produced Th2 cytokines and display progressive disease (313, 513, 558). This suggests that IFN-γ alone is not able to promote protection and that other Th1 specific factors may be required to reverse the Th2 response of naturally susceptible mice.

13.1.A.2. IL-12

Interleukin 12 is produced by different cell types including dendritic cells, macrophages, B cells and polymorphonuclear neutrophils (reviewed in (163) and (561)); it signals through STAT-4, promotes the production of IFN-γ and favours Th1 differentiation of the CD4⁺ T cells (reviewed in (163) and (561)). The bioactive IL-12, i.e. IL-12p70, is a heterodimeric cytokine composed of 2 subunits: IL-12p40 and IL-12p35. Its receptor is also composed of 2 subunits, i.e. IL-12Rβ1 and IL-12Rβ2. While IL-12Rβ1 is mainly responsible for the binding of IL-12, IL-12Rβ2 is necessary for the signal transduction triggered by IL-12 (reviewed in (163) and (561)). In addition, IL-12Rβ2 expression is induced by T-bet and is a marker of Th1 responses as Th2 cells lose its expression (3, 373, 545). As a result, IL-12 responsiveness has been considered as a major component of protective responses towards *Leishmania* infection (214, 234).

The role of IL-12 in the protective response to *L. major* has been highlighted by studies showing that its exogenous administration to susceptible mice at the time of infection, but not later, was associated with enhanced protection, e.g. smaller lesion size and parasite burdens (207, 295, 418, 544). This was associated with an enhanced Th1 response as determined by an increased production of IFN-γ and decreased IL-10 and IL-4 expression (207, 544, 578).
Furthermore, while the protective response was IFN-γ-dependent, the suppression of IL-10 and IL-4 were IFN-γ-independent, suggesting that IL-12 can suppress Th2 responses independently of IFN-γ (207, 294, 578). Conversely, the administration of monoclonal antibodies against IL-12 to resistant mice during the acute phase of disease resulted in an inhibition of pro-inflammatory chemokine expression, increased IL-4 production, decreased secretion of IFN-γ and the development of large lesions (204, 219, 544, 599). However, if the duration of anti-IL-12 treatment was only transient, a switch for a Th2 to a Th1 response was observed and the mice subsequently healed (218, 219). This reversal of the phenotype of the immune response could be explained by the maintenance of a state of IL-12 responsiveness by the CD4⁺ T cells. Indeed, in contrast to BALB/c controls, the CD4⁺ T cells from anti-IL-12 treated resistant mice had maintained their expression of both IL-12 receptor (218). In addition, studies using mice lacking either the p40 or the p35 subunit of IL-12 have further supported the crucial role of IL-12 during *L. major* infection (329, 330, 417, 418). Furthermore, it has been demonstrated that a sustained production of IL-12 was necessary to maintain a protective Th1 immune response, notably by preventing the loss of Th1 cells (417, 418, 541).

While IL-12 is critical during the protective immune response toward *L. major*, an intact IL-12 signaling pathway is crucial as well. The relative expression of the IL-12R subunits after *L. major* infection was shown to correlate with the outcome of the disease (214, 234). In addition, STAT-4 or IL-12Rβ2 deficient mice were shown to develop large, non-healing lesions when infected with *L. major*; this susceptibility correlated with an impaired IFN-γ production in the draining lymph node (96, 537). Finally, while the expression of IL-12Rβ2 appear necessary for the control of the disease, it is not sufficient as demonstrated by the fact
that BALB/c mice bearing a transgenic IL-12Rβ2 gene were unable to control a *L. major* challenge (399).

13.1.A.3. IL-4

In contrast to resistant strains of mice, susceptible mice produce a rapid burst of IL-4 after a *L. major* infection (292, 294, 295, 513). The early production of IL-4 of susceptible mice ensures the differentiation of CD4+ T cells towards a Th2 phenotype, downregulates the expression of IL-12Rβ2 and thereby induces a state of IL-12 unresponsiveness (213, 214, 295, 545). CD4+ T cells have been shown to be the main source of IL-4 in susceptible mice (206). In particular, the early IL-4 response of susceptible mice is elicited by a population of CD4+ T cells expressing the Vβ4-Vα8 TCR chains that recognize the I-A^d^-restricted epitope LACK, i.e. the *Leishmania* homolog of mammalian receptor for activated protein kinase C (RACK1) (293). Interestingly, Vβ4-Vα8 CD4+ T cells are also the source of IL-4 in resistant mice treated with either anti-IL-12 or anti-IFN-γ antibodies (292).

The induction of a resistant phenotype in susceptible mice infected with *L. major* can be easily achieved by either giving exogenous Th1 cytokines or by blocking the early source of IL-4. For example, BALB/c mice that were either Vβ4 deficient or rendered tolerant to LACK antigen developed a stronger Th1 response that mediated disease control (213, 240). In addition, treatment of susceptible mice with anti-IL-4 at the time of infection allowed the development of a protective Th1 response and disease control (104, 105, 205, 313, 490). Similarly, the prevention of the early burst of IL-4 can be induced by exogenous administration of IL-12 or IFN-γ at the time of infection (295). It is critical that the IL-12 or the anti-IL-4 treatments are given at early timepoints after infection to effectively prevent the
early peak of IL-4 (295). However, if the infective inoculum contains only $10^3$ parasites, the anti-IL-4 treatment remains effective as long as 6 months post-infection (565).

Resistant mice have an inherent tendency to develop a Th1 response and the outcome of disease cannot be reversed by exogenous administration of IL-4 like in BALB/c mice, unless the source of IL-4 is persistent, as in IL-4 transgenic mice (105, 298, 313, 492).

To further assess the role of IL-4 during a *L. major* infection, studies have been performed in IL-4 or IL-4R deficient mice. These have provided conflicting results, as both resistance and susceptibility have been reported in these mice after *L. major* infection (269, 273, 353, 400, 402). However, it appears that these discrepancies may result from differences in the strain of *L. major* used (402). Furthermore, the definition of “resistance” is not similar among authors and it is not uncommon in experimental systems using BALB/c mice to consider resistance as the absence of progressive disease.

Paradoxically, while IL-4 has been shown to be a major cytokine driving Th2 responses, it may also promote Th1 development. First, it has been shown that IL-4 can enhance the production of bioactive IL-12 by dendritic cells (58, 216). Second, the administration of IL-4 to BALB/c mice during the initial activation of dendritic cells, i.e. before T cell activation, induced resistance to *L. major* infection (58). This protective effect was associated with a Th1 response, correlated with an enhanced production of IL-12 by dendritic cells within the draining lymph node, and was abrogated by anti-IL-12 (58). Finally, when susceptible and resistant mice, in which the IL-4 gene was linked to green fluorescent protein, were infected with *L. major*, both strains displayed similar percentages of IL-4 expressing-cells, further supporting that IL-4 could have implications in both Th1 and Th2 development (540).
In conclusion, while IL-4 may be required for the induction of both Th1 and Th2 responses, the early burst of IL-4 is only observed in susceptible, but not resistant, mice infected with *L. major*. Therefore, the modulation of the IL-4 response to promote healing has only relevancy in the BALB/c model of leishmaniasis.

13.1.A.5. IL-13

Interleukin 13 is a cytokine with similar properties to IL-4 (reviewed in (592)). It also uses the IL-4Rα chain and STAT-6 for signaling. As a result, while IL-4 deficient mice retain normal IL-13 functions, IL-4Rα deficient mice have a defective IL-4 and IL-13 signaling. The role of IL-13 during *L. major* infection has been studied in mice lacking IL-4, IL-4R or STAT6 or in mice given anti-IL13, and the results are somewhat controversial. Most studies have suggested that in susceptible mice, IL-13 has a deleterious effect on the disease outcome and that it may act synergistically with IL-4 (141, 328, 402). However, other studies have failed to attribute a detrimental role for IL-13 during *L. major* infection (274, 353). These discrepancies may result from the use of different strains of *L. major* and/or from different inoculating dose of parasites.

13.1.A.6. IL-10

Interleukin 10 is classified among the Th2 cytokines. However, only at early timepoints after *L. major* infection do susceptible mice produce significantly more IL-10 than resistant mice (103, 524). Although CD4+ T cells represent the main source IL-10 in susceptible mice (103, 206), macrophages can also produce IL-10 and in vitro studies have shown that this cytokine can enhanced the intracellular survival of *L. major* (246, 572). In addition,
transgenic mice from a resistant background, in which the IL-10 gene was under the MHC class II promoter, developed progressive lesions when infected with *L. major*, indicating a deleterious role for this cytokine (185). It is interesting to notice that when the IL-10 transgene was under the IL-2 promoter, the course of the disease was not altered (195). It is likely that IL-2 is not abundant during a chronic infection and that the levels of IL-10 secreted by these mice may not have been high enough to modulate the outcome of disease. In addition, there is further evidence that IL-10 plays an immunomodulatory role during a *L. major* infection. Indeed, susceptible mice lacking IL-10 or in which the IL-10 signaling is blocked are able to control the disease (246, 401). In contrast, one study has reported susceptible mice given anti-IL-10 before *L. major* infection still developed a Th2 response and progressive lesions (103). However, it is possible that the exogenous administration of anti-IL-10 antibodies may not have been as efficient as knocking-out the IL-10 gene, thereby explaining these conflicting results.

Altogether, these data suggest that the production of IL-10 can be detrimental for the host during a *L. major* infection.

13.1.B. *Leishmania donovani* and *Leishmania chagasi*

BALB/c mice and C3H mice are considered susceptible and resistant, respectively, to the infection with the visceralizing species *L. donovani* and *L. chagasi* (586, 587). However, spontaneous resolution is the natural outcome in both strains of mice and the only difference relies in an initially higher parasite burden within the liver of BALB/c mice while the splenic parasite load remains low during the course of infection in both strains of mice (586, 587). While the susceptibility to the visceral disease of mice does not correlate with the expansion
of Th2 cells, the induction of a Th2 response could exacerbate the course of *L. donovani* infection (248, 385, 586). In these mice however, the administration of exogenous IL-12 or IFN-γ could reverse the susceptible phenotype (385). In addition, there is evidence that the production of IFN-γ is critical for protection (247, 248, 390, 391, 533, 586). For example, IFN-γ deficient mice have an exacerbated disease, which is eventually controlled during later stages of the disease and this correlated with an enhanced production of TNF-α (552). Furthermore, the enhanced susceptibility of mice lacking IFN-γ could be reversed by exogenous administration of IL-12; this curative effect was also TNF-α mediated (552). The neutralization of IL-12 led to an increased parasite burden in the liver and spleen of susceptible but not resistant mice (154).

Several studies have reported that the limitation of the antigen-specific responses, e.g. IFN-γ production, during visceral disease was mediated by macrophages (169, 384, 586). In particular, in susceptible mice infected with *L. chagasi*, the impaired production of IFN-γ within hepatic granulomas and was mediated by TGF-β (586, 587). TGF-β is an immunomodulatory cytokine that has been shown to block Th1 differentiation by inhibiting T-bet and IL-12Rβ2 expression, although the effect of TGF-β on IL-12Rβ2 expression has been recently challenged (177, 178). As TGF-β levels are high in the hepatic granulomas from BALB/c but not from C3H mice, it suggests that this cytokine may be a strong immunomodulator in BALB/c mice while it is likely innocuous in C3H mice.

Whereas there was no evidence of an immunomodulatory role for IL-10 during *L. chagasi* infection, studies have reported such a role in mice infected with *L. donovani* (383, 386, 587). Indeed, IL-10 deficient mice or mice given anti-IL-10R were able to control the
infection faster than wild-type controls and this was associated with an enhanced Th1 response (383, 386). Finally, IL-10 transgenic mice infected with \textit{L. donovani} have been shown to develop higher, uncontrolled, parasite burdens, further supporting a detrimental role for IL-10 during a \textit{L. donovani} infection (386).

13.1.C. \textit{Leishmania mexicana}

Most inbred strains of mice are susceptible to \textit{L. mexicana} infection. In contrast to what is observed in mice susceptible to \textit{L. major}, the susceptibility to \textit{L. mexicana} is not associated with an early burst of IL-4 (188). However, experimental murine studies using \textit{L. mexicana} have illustrated how the associated cutaneous disease is also a result of a balance between Th1 and Th2 immune effector functions. In this system, the inhibition of the Th1 signaling pathways through STAT-4, IFN-\(\gamma\) or iNOS lead to lesion progression (80). In addition, mice in which Th2 signaling pathways are compromised, i.e. lacking STAT-6, IL-4, IL-4R\(\alpha\), IL-13 or both IL-4 and IL-13, did not develop lesions after \textit{L. mexicana} infection and such protection was characterized by higher amounts of IFN-\(\gamma\) produced in recall responses (12, 466, 502, 536). Although there is conflicting data about the role of IL-13 during \textit{L. mexicana} infection, it appears that while IL-13 deficient mice develop similar lesions than wild-type mice during the early stage of disease, they ultimately heal with time (12, 466). The lack of IL-10 did not modify the course of the disease, unless IL-4 was absent too (412). Finally, the role of IL-12 during \textit{L. mexicana} infection has also been controversial. While it was suggested that the susceptibility to \textit{L. mexicana} resulted from an inability to produce IL-12, the course of disease was similar between IL-12p40 deficient mice and wild-type controls (80, 466). Altogether, this suggested a new IL-12p40 independent but STAT-4 dependent
pathway of controlling *L. mexicana* infection (80). However, another study has reported that although IL-12p40 is not required for the early control of a *L. mexicana* infection, it was critical for controlling the late stages of infection (7). The discrepancies between those studies may result from differences strains of parasite strains or inoculum doses used.

13.1.D. *Leishmania amazonensis*

*L. amazonensis* induces non-healing lesions in mice that are both susceptible (e.g. BALB/c) and resistant (e.g. C3H, C57BL/6 or C57BL/10) to *L. major*. Susceptibility does not correlate with the expansion of Th2 cells but rather with a mixed Th1/Th2 immune response, characterized by the production of low to undetectable levels of both IFN-γ and IL-4 (5, 232, 236). While BALB/c mice infected with *L. amazonensis* develop uncontrolled disease, C3H, C57BL/6 and C57BL/10 mice develop a single chronic cutaneous lesion (5, 233, 235, 413, 446). Furthermore, it is important to notice that although C3H, C57BL/6 and C57BL/10 mice are susceptible to *L. amazonensis* infection, the resulting lesions are not progressive but rather persistent over time. Indeed, their lesions reach a plateau at approximately 8 to 10 weeks post-infection and persist as such over time. This suggests that despite their overall susceptible phenotype, these mice are able to partially control the infection. This was further supported by a study in which C57BL/10 mice had their draining lymph node surgically removed before infection with *L. amazonensis* (454). These mice developed progressive lesions with occasional spreading to other cutaneous locations and/or death, in comparison to the sham-operated control mice.

Attempts at modulating the immune response before or during the course of *L. amazonensis* infection have generally been unsuccessful. In BALB/c mice, which are
generally very sensitive to the modulation of the Th1/Th2 cytokine balance, as mentioned earlier, the injection of anti-IL-4 or anti-TGF-β induced a Th1 response and subsequent control of the disease (5, 40). Despite this apparent healing response, the infected feet of these mice were still swollen and/or contained high parasite loads (5, 40). In addition, the lack of IL-10 or the exogenous administration of IFN-γ to BALB/c mice did not alter the course of disease (41, 412). In C3H, C57BL/6 and C57BL/10 mice, the manipulation of the T helper response has not been successful either. Indeed, such mice lacking IL-10, IL-4 or given anti-IL-4 still developed chronic cutaneous lesions after a *L. amazonensis* challenge (5, 233, 235, 236). In addition, mice remained susceptible after exogenous administration of IL-12 (236). This refractoriness to IL-12 correlated with low levels of IL-12Rβ2 expression on the CD4+ T cell population, which remained low even in the absence of IL-4 (233, 236). Furthermore, it has been suggested that IL-12 production may be limited during *L. amazonensis* infection (236, 446). Finally, while TGF-β seems to limit the production of IFN-γ during the infection of C3H mice with *L. chagasi*, this does not seem to play a major role in *L. amazonensis*-infected C3H, as anti-TGF-β did not affect the percentage of IFN-γ producing cells in these mice (236). Altogether, these studies suggest that the susceptibility of these mice to *L. amazonensis* is probably multifactorial and results, in part, from an inability to develop an effective Th1 response. Therefore, the murine model of cutaneous leishmaniasis caused by *L. amazonensis* is, in addition to visceral diseases caused by *L. donovani* and *L. chagasi* in mice, an experimental system in which Th1 responses are limited without expansion of Th2 cells (248, 586).
13.2. CD4+ CD25+ regulatory T cells

CD4+ CD25+ regulatory T cells (Treg), which represent 5 to 10% of the peripheral CD4+ T cells, have the ability to suppress immune responses, i.e. T cell proliferation and/or activation (reviewed in (453) and (519)). These cells can inhibit both Th1 and Th2 responses and it has been suggested that they can do so either through the production of IL-10 and/or TGF-β or by cell to cell contact (186, 238, 393, 554). It has been shown that their suppressive effect on cell proliferation results from the inhibition of IL-2 production by the other lymphocytes (554).

Recent studies have suggested that Treg cells play a role in both susceptible and resistant mice during *L. major* infection. For example, the depletion of Treg cells before the infection of BALB/c mice with *L. major* resulted in an enhanced early burst of IL-4 and exacerbated disease (27). In addition, adoptive transfer studies in SCID mice have further supported a protective role for Treg cells during *L. major* infection (593). Finally, Treg cells have also been shown to play a role in C57BL/6 with cutaneous leishmaniasis. Indeed, the lack of sterile cure observed in C57BL/6 mice infected with *L. major* was shown to correlate with the accumulation of Treg cells within the dermis (46). Consequently, these suppressor cells inhibit the dermal effector CD4+ T cells necessary to eliminate the persistent parasites (46). This suppression of T cell responses by the Treg cells was partially mediated by IL-10 (46). Finally, the persistence of parasites at the infection site was shown to be responsible for the maintenance of an immunological memory (46).
13.3. CD8⁺ T cells

CD8⁺ T cells represent an important arm of the adaptive immune response. They recognize pathogen-derived antigens expressed by antigen-presenting cells in the context of MHC class I. Activated CD8⁺ T cells can produce cytokines such as IFN-γ and TNF-α that stimulate anti-microbial effector functions; they can also induce the cytolysis of their target cells by producing perforin (reviewed in (201)). In experimental studies of leishmaniasis, most of the interest has focused on the CD4⁺ T cells, as the phenotype of the CD4⁺ T cell response determines disease outcome. However, there has been evidence that CD8⁺ T cells also have a protective role during both visceral and cutaneous *Leishmania* infections (378, 539).

The priming of mice following *L. major* infection or vaccination leads to the development of parasite-specific CD8⁺ T cells that are subsequently important for inducing resistance upon a secondary challenge (127, 155, 192, 193, 374, 451, 458). The protective role was associated with an expansion and concomitant production of IFN-γ (193, 376, 377).

The role of CD8⁺ T cells during the course of a primary *L. major* infection, using mice that lack CD8 or β2-microglobulin or that have been depleted from CD8⁺ T cells, has been less clear and controversial results have been reported (155, 212, 225, 411, 555, 577). For example, it appears that in the absence of CD8⁺ T cells, the outcome of a *L. major* infection depends on the number of promastigotes inoculated. Indeed, when mice lacking CD8⁺ T lymphocytes were infected with a high dose challenge, i.e. 10⁶-10⁷ parasites, they were able to control the infection as well as wild-type mice (225, 411, 555, 577). However, CD8⁺ T cells were required for the control of a primary *L. major* infection when the inoculum was composed of a low dose of parasites, i.e. 100 metacyclic promastigotes (47). This was further
supported by the fact that protection of *Leishmania*-infected RAG deficient mice, which lack T and B cells, was optimally achieved when both CD4+ and CD8+ T cells were adoptively transferred (47). This data would suggest that vaccine strategies should be designed to activate both CD4+ and CD8+ T cells, as the the sand fly vector generally inoculate low numbers of promastigotes to the host.

After infection with *L. mexicana*, CD8+ T cells deficient mice are as susceptible as wild type mice, suggesting that CD8+ T cells may not have an important role in this experimental system (80, 411).

The role of CD8+ T cells *L. amazonensis* infection has also been investigated. While CD8+ T cell-deficient and perforin-deficient mice are as susceptible as wild-type controls, there is evidence of a protective role for CD8+ T cells after vaccination ((115), and our unpublished observations). Protection following vaccination involved both IFN-γ and perforin-mediated processes (115). In addition, the low levels of IFN-γ detected in the in vitro recall response of mice infected with *L. amazonensis* are significantly reduced if CD8+ T cells are depleted (100). However, this production of IFN-γ by CD8+ T cells was CD4+ T cells-dependent (100). This is consistent with recent data showing that although CD8+ T lymphocytes can expand and become cytotoxic in the absence of CD4+ T cells, their expansion upon antigen re-encounter is CD4+ T lymphocytes-dependent (230).

In conclusion, although CD8+ T cells may not be essential for protection to leishmaniasis, their role after re-infection and/or vaccination suggest that the goal of future vaccine strategies should include the induction of parasite-specific CD8+ T lymphocytes.
13.4. NK cells

A role for NK cells during cutaneous and visceral leishmaniasis has been clearly demonstrated (266, 289, 505). After a *L. major* infection, NK cells are rapidly recruited at the inoculation site (379). In addition, there is evidence that these cells participate in the control of the infection as the cytotoxic activity of NK cells from inbred strains of mice early after *L. major* infection positively correlates with the ability of those strains to control parasite replication (505). Activation of NK cells can occur directly through the stimulation of their TLR2 by *Leishmania* LPG molecules (43). However, there is also evidence of indirect pathways of activation as IL-12 and CD4+ T cells are required for NK cell activation during *L. major* infection (505, 506). The main role of NK cells is to provide an early source of IFN-γ that in turn will limit parasite spreading (288, 505). In mice depleted from NK cells before infection, there is in an enhanced pathology at early timepoints, as determined by an increased lesion size and parasite load (289, 296); however, in NK cell-deficient mice this is not observed (503). Nevertheless, the production of IFN-γ by NK cells is not necessary for CD4+ T cells to promote resistance to *L. major* and mice either depleted or deficient in NK cells are both able to control a *L. major* infection (289, 503, 575).

Altogether, this suggests that while NK cells may be important early on, their presence is not necessary for the control of a *L. major* challenge.

13.5. Polymorphonuclear neutrophils

Polymorphonuclear neutrophils (PMNs) are part of the first line of immune defense against pathogens and as a result interact with *Leishmania* parasites soon after their inoculation (reviewed in (290)). The migration of PMNs can be triggered by different
Leishmania species in vitro (570) and, in vivo, they are recruited very rapidly at the site of infection (306, 379, 429, 435, 547). In addition, PMNs have been shown to efficiently take up and kill Leishmania promastigotes (291, 420, 473). However, it also appears that some of the Leishmania parasites internalized by PMNs can resist killing and increase the survival of PMNs (6, 291). In particular, live L. major promastigotes have been shown to delay the apoptosis of PMNs by reducing the activity of the pro-apoptotic caspase-3 within those cells (6). Based on these data, Laskay et al. have elaborated the Trojan horse hypothesis in which infected neutrophils serve as carriers for Leishmania to enter macrophages (290). They proposed that the delayed apoptosis observed in infected PMNs would give time to macrophages to be recruited at the lesion site. Subsequently, these macrophages will clear the tissue of apoptotic cells, a process that does not lead to macrophage activation. As a result, Leishmania parasites end up within their preferred host cell without any activation of the antimicrobial functions of macrophages (290). Interestingly, a recent study has shown that co-incubation of inflammatory neutrophils and infected macrophages from resistant or susceptible strains of mice promoted parasite killing or replication, respectively (459). Finally, depletion studies in mice have also tried to address the role of PMNS in vivo. In mice infected with L. infantum, the early, but not late, depletion of neutrophils was associated with increased parasite burdens (473)). However, studies in which BALB/c mice were depleted from their neutrophils soon before infection with L. major have provided conflicting results. In one study, neutrophil depletion was shown to result in increased parasite burden (306). However, in another study, it was reported to be protective, characterized by a decreased Th2 response, and a maintained IL-12 responsiveness from the CD4+ T cells (547).
The discrepancies between these two studies may be accounted for by the use of different strains of parasites or depleting antibodies.

13.6. Eosinophils

Recruitment and migration of eosinophils can be triggered by *Leishmania* parasites in vitro and in vivo (429, 435, 495). Although their exact role during a *Leishmania* infection remains undetermined and is likely not crucial, eosinophils can engulf the parasites and subsequently release their granule content (421, 429, 435). Furthermore, it appears that their production of nitric oxide may be involved in the anti-leishmanial immune response (408).

13.7. B cells

Resistance to *Leishmania* infection is essentially induced by cell-mediated immunity and although it is associated with an antibody response, the role of humoral immunity during leishmaniasis appears to be minimal (304, 409). Nevertheless, the exact role of antibodies and/or B cells has been investigated. Initial studies using C3H mice treated with anti-IgM antibodies and infected with *L. major* provided controversial results; indeed both resistance and enhanced susceptibility were reported in these mice (489, 516). In contrast, in BALB/c mice, the administration of anti-IgM or the lack of B cells is associated with disease control after *Leishmania* spp. infection, suggesting a detrimental role for B cells in this strain of mice (112, 489). However, more recently, studies using B cell-deficient mice or mice depleted from B cells failed to support a role for B cells during *Leishmania* spp. infections (31, 77, 138, 523). In addition, B cell-deficient mice were resistant to a secondary *L. major* challenge despite lower production of IFN-γ and reduced DTH response (138).
The role of antibodies during leishmaniasis has been controversial as well. Although antibodies are not necessary for healing a *Leishmania* infection, lines of mice producing high levels of serum antibodies have been shown to be more susceptible to *L. major* than the ones producing low antibodies titers (197). In addition, the exogenous administration of either IgG from naïve mice or from mice chronically infected mice with *L. mexicana* induced different disease progression after a subsequent *L. mexicana* infection in B cell-deficient mice (423). Indeed, the mice that received the *Leishmania*-specific IgG developed lesions faster and these contained higher parasite loads (423). Finally, a role for antibodies in disease progression was suggested in a study in which mice lacking the FcyR only developed minimal lesions after *L. amazonensis* or *L. pifanoi* infection (262). This would be consistent with a report indicating that the uptake of IgG-opsonized parasites through the Fcy receptor on the surface of macrophages triggered the production of IL-10, which subsequently enhanced parasite survival (246). However, in FcyR-deficient mice infected with *L. pifanoi*, the CD4+ T cells from the draining lymph node produced similar levels of IL-10 as the wild-type controls, suggesting that in this experimental system IL-10 may not be sufficient to maintain the infection (Colmenares 2002 IAI). Rather, the absence of lesion in these mice has been linked to the inability of infected macrophages to activate CD4+ T cells (112).

13.8. Macrophages

The two main roles attributed to macrophages during leishmaniasis are antigen presentation and parasite killing (263, 410, 535). Macrophages were considered, for a long time, as the main APC. As a result, macrophages were initially thought to be the main host cells initiating a *Leishmania* infection and studies have reported the ability of *Leishmania*
parasites to limit antigen-processing and presentation by macrophages (21, 133, 264, 312, 345, 410, 443). However, the importance of macrophages as antigen presenting cells during murine experimental leishmaniasis has been challenged. Indeed, recent data has indicated that while the main function of macrophages is to kill intra-cellular amastigotes, DC likely represent the host cells priming the parasite-specific immune response (573). In addition, more recently, a study reported that mice in which the expression of MHC class II was restricted to DC were able to control a *L. major* infection (300). This demonstrates that DC are sufficient to prime a protective response towards *L. major*. In addition, it indicates that macrophages are likely to kill the parasites in an MHC II-TCR independent fashion. Altogether, this suggests that the role of macrophages in presenting *Leishmania* antigens may be of minimal importance for the outcome of the disease.

Regardless of the relevance of their ability to present antigen, the role of macrophages in the killing of engulfed parasites remains undisputed. One of the most potent microbicidal molecules produced by macrophages is nitric oxide (NO) (reviewed in (318)). It is synthesized by the cytokine-inducible NO synthase (iNOS), which can be triggered by cytokines such as IFN-γ and TNF-α or inhibited by IL-4, IL-10 and TGF-β (reviewed in (304)). iNOS catalyzes L-arginine with oxygen to produce NO, and mice lacking iNOS or given NO inhibitors are highly susceptible to most *Leishmania* spp. (80, 387, 538, 580). Furthermore, a continuous iNOS activity is necessary to maintain a healed phenotype, as the administration of iNOS inhibitor to mice that had previously cured their *L. major* infection lead to the reactivation of the disease (538). Although the leishmanicidal effects of NO have been extensively studied, how NO mediates parasite killing is still unclear (reviewed in (304) and (305). For example, a recent study using *L. amazonensis* suggested that NO may only be
cytostatic but that peroxynitrites, resulting from the association of NO with superoxide, are the molecules with cytotoxic activity (309). Finally, although NO is mainly secreted by macrophages, it can also be produced by DC. However, while NO has microbicidal activities in macrophages, it mediates apoptosis in DC and subsequently inhibits T cell (65). This further supports the distinct roles that DC and macrophages have during leishmaniasis, i.e. antigen presentation and parasite killing, respectively.

Although *Leishmania*-infected macrophages can become activated and subsequently kill intracellular amastigotes, *Leishmania* spp. have also evolved ways to inhibit several macrophages effector functions, including microbicidal activity (517) and IL-12 production (86, 335, 428, 456). In particular, *L. amazonensis* can resist the microbicidal effects of NO (170). In addition, *L. amazonensis* was also shown to inhibit the production of NO in vitro, but not in vivo (34, 308).

Altogether, this suggests that parasite persistence result from a fine balance between the host microbicidal defense mechanisms and the parasites intrinsic ability to evade the host immune response.

13.9. Dendritic cells

Dendritic cells (DC) are the main antigen-presenting cells (APC) in vivo, inducing both tolerance and immunity. DC act as a link between the innate and adaptive immune systems by recognizing specific molecular patterns on the surface of pathogens and subsequently priming antigen-specific naïve T cells (reviewed in (36) and (35)). Their pivotal role in the induction of cellular immune responses has focused attention on DC as a powerful tool for eliciting anti-pathogen and/or anti-tumor immunity.
Dendritic cells are found in primary and secondary lymphoid organs and peripheral tissues such as the skin. Several subsets of mouse and human DC have been characterized, i.e. myeloid, lymphoid, follicular and plasmacytoid DC (reviewed in (520). These characterizations are based on their differential expression of an array of surface markers, location, function and/or morphology. The direct lineage relationships between those subsets are not fully understood.

DC arise from bone-marrow progenitor cells that give rise to circulating precursor DC in the blood, which can enter tissues as immature DC (e.g. Langerhans cells in the skin). The main function of immature DC is antigen capture through pinocytosis, phagocytosis and receptor-mediated endocytosis (reviewed in (187)). Upon antigen uptake, DC undergo a maturation process triggering their transition from an antigen-capturing cell to an antigen-presenting cell. This is characterized by the upregulation of their surface expression of MHC (both class I and class II) and costimulatory (e.g. CD80/B7.1 & CD86/B7.2) molecules. Furthermore, mature DC produce an array of pro-inflammatory cytokines and chemokines. Maturation is also associated with the DC leaving the tissues and migrating, via the afferent lymphatics, to the T cell-rich area of the draining lymphoid organ. There, DC can present peptide antigens to T cells (reviewed in (35). While exogenous antigens acquired by phagocytic cells are usually presented on MHC class II molecules, DC also have the ability to present such antigens in the context of MHC class I molecules, a process termed cross-priming (reviewed in (187). This allows DC loaded with exogenous antigens to initiate both CD4⁺ and CD8⁺ T cell responses. In addition, DC can also present lipid antigens to T cells through CD1 molecules, which have a MHC class I like structure (reviewed in (438)). Therefore, through their unique ability to prime antigen-specific naïve T cells, DC play a
crucial role in determining the phenotype (Th1 vs Th2) of a developing immune response (reviewed in (363)). Finally, because of their strategic role in the induction and outcome of cell-mediated immune responses, DC represent a tool for eliciting or modulating anti-pathogen and/or anti-tumor immune responses.

Dendritic cells express a variety of Pattern Recognition Receptors (PRR), such as Toll-like Receptors (TLR), that bind to specific, highly conserved, pathogen-associated molecular patterns (PAMP), i.e. lipopolysaccharide (LPS) and peptidoglycans, on the pathogens surface (reviewed in (229), (549) and (344)). The interaction of the PRR with a PAMP induces an intracellular signaling cascade leading to the maturation of the DC. The specific characteristics of the DC stimulation, in terms of what subset of genes is expressed, depends on the type of pathogen encountered and the resulting PRR signaling pathway that is activated (106, 224). Once stimulated, the DC can activate antigen-specific naïve and/or memory T cells, turning on the adaptive arm of the immune system. Immature DC express high levels of most of the ten TLR that have been described to date. TLR2 has been shown to bind to a wide variety of pathogen-derived molecules such as the LPS from *Leptospira interrogans* (581), lipoprotein/lipopeptide from *Borrelia burgdorferi*, *Treponema pallidum* and *Mycoplasma fermentans* (302) and glycosylphosphatidylinositol anchors from *Trypanosoma cruzi* (84). There is also evidence that *Mycobacterium avium* and *Staphylococcus aureus* signal through TLR2 (302, 597). While *Leishmania* LPG molecules have been shown to activate NK cells and transfected cell lines through TLR2, this has not been confirmed in dendritic cells (43, 134). TLR4 can bind to LPS from gram-negative bacteria such as *E. coli* (220, 448) and to other molecules such as lipoteichoic acid (550) and the fusion protein of respiratory syncitial virus (277). Several mycobacterial species can also
activate cells through TLR4 (343, 562). Recently, mice deficient in TLR4 were reported to be more susceptible to L. major, suggesting a role for TLR4 in the control of this disease (272). TLR5 recognizes bacterial flagellin (202). To date, the only ligand for TLR9 has been shown to be unmethylated CpG (cytidine linked to a guanosine by a phosphate bond) motifs characteristic of bacterial DNA (208).

In addition to the TLR, DC express a variety of C-lectin type receptors including the mannose receptor, DC-SIGN, DEC-205, and Langerin (reviewed in (156)). These receptors can bind carbohydrates, lipids and/or peptides and may therefore also act as PRR for the specific recognition of pathogens. DC-SIGN, for example, binds ICAM 3 on resting T cells, mediates DC-T cell adhesion and is essential for DC-induced T cell proliferation (165). At the same time, DC-SIGN has been shown to bind human immunodeficiency virus-1 (HIV-1), simian immunodeficiency virus (SIV), cytomegalovirus and Ebola virus (18, 164, 196, 434, 598) and it plays a role in the transmission of those viruses to permissive cells. DC-SIGN is also a receptor for Leishmania amastigotes (116) and Mycobacterium tuberculosis (548). Furthermore, DC-SIGN is able to discriminate between different mycobacterial species (319). Therefore, DC-SIGN and TLR serve as recognition receptors for the binding and/or transmission of many different pathogens.

There is increasing evidence that DC play a crucial role during the protective immune response towards Leishmania parasites (300, 354, 460). Although, macrophages were initially considered as the main APC, this role is now attributed to DC and recent evidence suggest that the major role of macrophages during Leishmania infection is to kill intracellular parasites (573). The uptake of Leishmania spp. by DC has been widely reported (52, 62, 175, 355, 356, 446, 573, 583). In addition, DC from the skin and draining lymph node harbor the
parasites in vivo (62, 355, 356). Furthermore, DC are the main APC in the in vitro recall response during both acute and chronic *L. major* infection (355, 356). Finally, *Leishmania* parasites have been shown to modulate DC responses (reviewed in (481) and (514)). Indeed, these parasites influence DC migration (30, 231, 437), surface expression of co-stimulatory molecules, and IL-12 production to varying degrees, depending on the species and strain of *Leishmania* (52, 334, 446, 573). Below is a summary of the different DC functions that *Leishmania* parasites are known to modulate.

**13.9.A. Antigen presentation**

As mentioned earlier, in vitro studies using macrophages have suggested that *Leishmania* parasites, and in particular *L. amazonensis*, may evade the MHC class II pathway of antigen presentation, as both MHC II molecules and H2M colocalized within the engulfed (21, 133). Similar findings have recently been reported in dendritic cells infected with *L. mexicana* or *L. amazonensis* (51, 442). However, despite the colocalization of MHC II molecules in intracellular parasites, *L. amazonensis* did not seem to inhibit DC activation, especially when the parasites were incubated with immune serum before infection (442). Indeed, the uptake of opsonized *L. amazonensis* induced the upregulation of the surface expression of MHC class II and co-stimulatory molecules. In contrast, such activation was much less pronounced when parasites were not coated with antibodies prior to infection (442). Altogether, this suggests that DC taking up *L. amazonensis* parasites in the absence of antibodies may remain immature, and subsequently do not prime efficient T cell responses.
Finally, whether or not the sub-cellular localization of MHC class II and H2M molecules to the PV does impair antigen presentation is unclear. In fact, a recent study indicated that *L. mexicana*-infected DC were not compromised in their ability to present antigen (51).

13.9.B. Co-stimulation
13.9.B.1. CD28-B7

The interaction between CD28 and B7 molecules provides an essential second signal at the time of TCR and antigen-MHC complexes interactions (reviewed in (97)). Both B7.1 and B7.2 are expressed at low levels on resting DC and are upregulated on activated DC (97, 228, 253, 287). The specific roles of B7.1 and B7.2 during Th1 and Th2 responses have been controversial and seem to be dependent upon the efficiency of T cell priming used experimentally (511). In addition, the effect of B7 blockade on T lymphocyte functions will vary whether it is a naïve or a primed T cell (512).

Several studies have investigated the role of B7 molecules during *Leishmania* infection. The surface expression of B7.1 has been shown to be down-regulated on the surface of *L. donovani*-infected macrophages (249). In addition, *L. major*-infected Langerhans cells from BALB/c mice have a decreased surface expression of B7.1 in comparison to the ones from C3H mice (332). Similarly, BMDC infected with *L. mexicana* do not upregulate their surface expression of co-stimulatory molecules, thereby keeping the infection presumably silent (52). Furthermore, the blockade of B7.1 and B7.2 in vitro resulted in a decreased production of IFN-γ, supporting a role for these co-stimulatory molecules for adequate T cell activation (75, 332). In vivo, although the absence of CD28 does not alter the course of infection, the blockade of B7 molecules using CTLA-4 Ig at the time of *L. major* infection...
conferred protection to BALB/c mice (118, 414). The critical role of B7-mediated co-stimulation during the early stages of lymphocyte response, i.e. cytokine production and proliferation, was further supported recently in both BALB/c and C3H mice infected with *L. major* (152). In particular, the blockade of B7.2, but not B7.1, in BALB/c mice infected with *L. major* was associated with a decreased Th2 response and lower parasite burden (78). This was further supported by in vitro data reporting that in the absence of B7.2 signaling, Langerhans cells were unable to promote IL-4 secretion by CD4⁺ T cells (332). In C57BL/6 mice infected with *L. major*, the administration of anti-B7.2 similarly led to a decreased parasite load without alteration of the cytokine profile (78). Similarly, B7.2 blockade in mice infected with *L. donovani* resulted in decreased parasite load and enhanced IFN-γ production (382). Altogether, these experiments suggested that B7.2 signaling may play a role in Th2 responses. However, this was recently challenged by a study reporting that when B7.1 or B7.2 are expressed at similar levels, they can equally induce Th2 responses (152). Similarly, the fact that the course of *L. major* infection in CD28-deficient BALB/c mice was unaltered suggested that these mice may have developed alternative pathways of Th2 responses induction (76).

Finally, although not all these studies have specifically addressed the role of co-stimulatory molecules on DC, it is likely that the blockade of B7-mediated co-stimulation has affected DC-T cells interactions, as DC play a crucial role during T cell priming.

13.9.2. CTLA-4

In order to avoid uncontrolled T cell activation and proliferation, the immune system has evolved mechanisms to maintain T cell homeostasis. In particular, activated T cells
upregulate CTLA-4, which binds to both B7.1 and B7.2 with an affinity that is 10 times higher than CD28 (310, 568, 576). As a result, CTLA-4 signaling mediates an inhibitory co-stimulation that is essential for the maintenance of T cell homeostasis (275, 576). Indeed, mice lacking CTLA-4 display massive T cell proliferation, resulting in lymphadenopathy and splenomegaly, and die at 3 to 4 weeks of age (557).

The role of CTLA-4 during experimental cutaneous and visceral leishmaniasis has been recently investigated (reviewed in (172)). During cutaneous disease due to *L. major*, the blocking of CTLA-4 results in an enhanced Th2 response and disease exacerbation (203, 327, 494). This suggests that CTLA-4 signaling may be crucial for the development of a protective Th1 response. However, in murine studies of visceral leishmaniasis, the blocking of CTLA-4 restored T cell responsiveness, increased IFN-γ production and enhanced parasite clearance both in vitro and in vivo (171, 381). In particular, in this experimental system, CTLA-4 engagement was shown to promote CD4+ T cells to secrete TGF-β, which enhances parasite growth (173).

In conclusion, it appears that the role of CTLA-4 engagement may have opposite effects during cutaneous and visceral diseases. In particular, blocking CTLA-4 binding to its receptor may be useful in immunotherapeutic strategies to control visceral leishmaniasis.

**13.9.B.4. CD40-CD40L**

The interaction between CD40, expressed on the surface of dendritic cells, and its ligand CD40L, expressed on T cells is an important secondary signal that enhances the production of IL-12 by the DC and the subsequent Th1 response (reviewed in (181)). In particular, signaling through CD40 appears important for the production of IL-12p35 the limiting
subunit of IL-12 in DC (509). In addition, in the absence of CD40L, Langerhans cells fail to migrate to the draining lymph node, further supporting the crucial role of CD40L signaling pathways for adequate T cell priming (358).

The importance of CD40-CD40L interactions during experimental murine leishmaniasis has been investigated both in vitro and in vivo. The blockade of CD40-CD40L interactions in in vitro co-cultures of *L. major*-infected macrophages and PBMC induced an inhibition of effector cytokine production (75). In addition, CD40 or CD40L-deficient C57BL/6 mice are susceptible to a high dose *L. major* challenge (83, 243, 414). However, the outcome of the disease depends on the size of the inoculum, as CD40L deficient C57BL/6 mice can control a low dose *L. major* challenge, suggesting that in the presence of only few numbers of parasites the immune system can be skewed toward a Th1 phenotype, even in the absence of CD40-CD40L interactions. (413). When CD40L-deficient C57BL/6 mice were infected with *L. amazonensis*, they displayed an exacerbated disease in comparison to wild-type controls, indicating that some CD40-CD40L engagement has some protective role in infected control mice (527). Finally, vaccination of mice using transfected cells expressing CD40L conferred enhanced protection to both *L. major* and *L. amazonensis* (107).

Altogether, this suggests that *Leishmania* parasites may interfere with CD40-CD40L signaling and that modulation of this pathway may be useful to promote resistance or healing.

### 13.9.C. IL-12 production

As mentioned earlier, IL-12 is an important component of Th1 responses and DC have been shown to produce IL-12 following *Leishmania* spp. infection (175, 268, 447, 573, 574).
In addition, the production of bioactive IL-12p70 by infected DC has been reported to be CD40-CD40L dependent (321). However, the amplitude of the CD40L-mediated IL-12 protection varies depending on the genetic background of the host and on the strain or species of *Leishmania* spp. involved (334, 446). For example, BMDC infected with *L. mexicana* do not acquire an activated phenotype and their production of IL-12 is not upregulated after infection with amastigotes (52). Similarly, there is increasing evidence that *L. amazonensis* may be limiting the production of IL-12 by DC. Indeed, the draining lymph nodes from *L. amazonensis*-infected mice contain low numbers of IL-12 producing cells (236). Furthermore, in vitro, *L. amazonensis*-infected DC from BALB/c mice do not upregulate their production of IL-12 when stimulated with anti-CD40 (446). This impaired IL-12 production occurred despite the upregulation of surface MHC II and co-stimulatory molecules (446). These data indicate that the maturation stage of *L. amazonensis*-infected DC may lie between the one of an immature and of a mature cell. In fact, a new differentiation stage of DC maturation, i.e. semi-mature DC, has been recently identified (317). While semi-mature DC have a surface phenotype reminiscent of mature DC, they lack the production of pro-inflammatory cytokines such as IL-12. Altogether, these data suggest that DC infected with *L. amazonensis* remain in a semi-mature state.

13.9.D. Migration

The migration of DC from the peripheral site of antigen uptake to the draining lymphoid organ is crucial for the priming of subsequent immune responses. This migration is orchestrated by an array of adhesion molecules (441, 594), cytokines (26, 265), and chemokines (reviewed in (94)and (529)). In particular, chemokines are small secreted
molecules that are involved in leukocyte activation and migration (reviewed in (472)). Four classes or families of chemokines have been described so far, namely the alpha (or CXC), beta (or CC), gamma and delta families (reviewed in (600)). The biologic effect of chemokines is mediated through their binding to 7 transmembrane domain G protein-coupled receptors expressed by leukocytes, including DC (reviewed in (346) and (472)). The recruitment of immature DC to peripheral tissues or of mature DC to lymphoid organs is under the control of various specific chemokines and chemokine receptors (146, 528). For example, the expression of CCR2 or CCR7 is important for DC migration to secondary lymphoid organs (158, 500). In particular, mice that are deficient for CCR7 or its ligands display DC migration defects and fail to mount primary T cell responses (158, 189). While expressed at very low levels on immature DC, CCR7 is rapidly upregulated upon DC maturation (419, 493, 497, 528, 596). As a result, mature DC display an enhanced migratory response to CCR7 ligands (493, 497, 528). CCR7 ligands include CCL21 (SLC/6Ckine) and CCL19 (ELC/MIP-3β). SLC is expressed in the T cell zones of lymphoid organs, by lymphatic endothelial cells and in the high endothelial venules of lymph nodes and Peyer’s patches (190). Blocking of the activity of SLC by neutralizing antibodies has been shown to inhibit the migration of skin derived DCs to the draining lymph node (493). Similarly, ELC is expressed in high endothelial venules, but also by dendritic cells within the T cell areas of secondary lymphoid organs (32, 398, 600).

During experimental leishmaniasis, the importance of chemokine-chemokine receptor interactions on the subsequent disease progression has been highlighted in studies using knockout mice. For example, CCR2-deficient mice on a resistant background cannot control a L. major infection (500). Similarly, when these mice are infected with L. donovani, the
resulting granulomas are poorly organized in comparison to the ones from wild type control mice (501). In addition, protozoan parasites including *Leishmania* spp. have evolved strategies to exploit or subvert chemokine-mediated responses (reviewed in (71)). For example, while *L. major* infection triggers the overt expression of certain chemokines, it appears that the production of chemokines is impaired in *L. amazonensis*-infected mice (233, 450). Furthermore, DC from *L. donovani*-infected mice failed to migrate to the T cell rich areas of the spleen and this correlated with a decreased expression of CCR7 (30). Finally, *L. major* parasites themselves have been shown to inhibit DC migration in vitro (231).

13.9.E. Signal transduction through MyD88

Myeloid differentiation factor 88 (MyD88) is an adaptor protein that acts as a signaling molecule downstream of different Toll-like receptors (TLR), including TLR2 and TLR4, leading to the activation of the NF-κB family of transcription factors (reviewed in (242)). DC maturation is partly dependent on MyD88 expression and MyD88 has been shown to be important for the production of IL-12p40 (223, 242, 504). As a result, it was likely that MyD88 would play a role in the control of *Leishmaniasis*. Indeed, when infected with *L. major*, C57BL/6 mice deficient for MyD88 develop an exacerbated clinical disease that is associated with the development of a Th2 response (134, 135, 380). This also correlated with undetectable levels of IL-12p40 in the serum of MyD88-deficient mice (380). However, the susceptible phenotype observed in the absence of MyD88 could be reversed by the exogenous administration of either IL-12 or anti-IL4, suggesting that MyD88 acts as a critical switch of Th1 responses (135, 380). Although these studies have not specifically
addressed the role of MyD88 in DC, it is likely that DC responses from MyD88-deficient mice were impaired.

14. The immune response of infected humans

As in mice, the cellular immune response of humans has been subdivided into two major phenotypes, i.e. Th1 and Th2 (reviewed in (303)). In addition, resistance or susceptibility to leishmaniasis in humans has also been shown to correlate with the phenotype of the immune response. Indeed, control of the disease has been associated with a potent Th1 cell mediated immunity, and the enhanced expression or production of IFN-γ usually correlates with a healing phenotype (64, 89, 90, 389, 496). Typically, patients with leishmaniasis have a defective cellular immunity, especially when during the visceral or diffuse cutaneous form of leishmaniasis. These individuals do not develop a DTH reaction in response to the Montenegro test, suggesting the presence of an anergic immune response (215, 427). In patients with cutaneous leishmaniasis, peripheral blood monocytes and cells at the lesion site have a mixed Th1/Th2 cytokine profile and/or produce low levels of IFN-γ (64, 81, 123, 432, 465). In addition, peripheral blood monocytes usually display reduced proliferative response to stimulation in vitro, a phenomenon also observed in patients with active visceral leishmaniasis (87, 88, 90-92, 215, 389, 427, 485). During or after successful treatment however, the immune response of patients is characterized by an increased lymphoproliferative response in vitro and the cytokine profile of their immune response is of the Th1 phenotype, i.e. characterized by an increased production of IFN-γ (64, 89, 91, 215, 350, 389, 496). A decrease in the expression of IFN-γ mRNA has also been shown during clinical relapse (64). Finally, the susceptibility of some patients with active cutaneous
leishmaniasis correlated with a state of IL-12 unresponsiveness (66, 67). This was characterized by a decreased production of IFN-γ in response to IL-12 in vitro and by a decreased expression of IL12Rβ2, a marker of human Th1 cells, in biopsy samples and PBMC (66, 67, 467). Furthermore, these studies suggested that the production of the Th2 cytokine IL-13 was responsible for inhibiting IL-12 responsiveness in patients with active cutaneous leishmaniasis (66, 67).

15. Treatment and prevention

15.1. Chemotherapy

As vaccines are currently not available for the prevention and/or treatment of leishmaniasis, chemotherapy remains the only approach to cure the disease. Systemic, oral and topical drugs have been used with variable degrees of success (reviewed in (54) and (124)). The efficacy of a drug regimen may vary according to the Leishmania species involved, the potential development of drug resistance, and the immune status of the host as some drugs have been shown to be less efficient in immunodeficient individuals (124, 388). Also, the pharmacokinetic requirements of the drugs to be used to treat cutaneous or visceral leishmaniasis are different (124). The current drugs that are recommended include the pentavalent antimonials, and different formulations of amphotericin B, a drug also used in the treatment of fungal diseases (124). The mechanism of action of antimonial drugs is unknown. Amphotericin B creates pores in the leishmanial membranes, resulting in osmotic lysis of the parasites (452).

Systemic injections of pentavalent antimonials (i.e. sodium stibogluconate and meglumine antimoniate) were used soon after Leishmania parasites were recognized. The
typical drug regimen is composed of a daily injection of the drug for 20 to 28 days. The clinical response can be rapid but normalization of the clinical parameters can take months. In case of treatment failure with those agents, the use of alternative agents is recommended as increasing the dose or duration of the treatment with pentavalent antimonials could become toxic. Other systemic agents that have been described in the literature include pentamidine, paromomycin (aminosidine), cytokines (i.e. IFN-γ) and amphotericin B, which can also become toxic to the patient. Although amphotericin B has been successfully used in patients developing resistance to antimonial drugs, its parenteral administration needs to be done for 4 hours and it is also rather toxic (124). However, lipid associated formulations of amphotericin B are now available: these have reduced toxicity and increased plasma half-life (54, 124). Successful combinations of various doses of these systemic agents have been reported in the literature (reviewed in (54)). Oral agents that have been used in the treatment of leishmaniasis include the anti-fungal imidazoles (i.e. ketoconazole) and triazoles (i.e. itraconazole); however, their efficacy was equivocal. In addition, in the mid-1980s, the anticancer drug Miltefosine was shown to have anti-leishmanial activity after oral administration. It has been used successfully to treat patients with visceral leishmaniasis (124). Finally, the use of topical agents has also been investigated, especially in patients with cutaneous disease, as this type of treatment would allow the drug to act directly at the level of the lesion and would decrease the likelihood of systemic toxicity. Miconazole, clotrimazole or a combination of paromycin and methylbenzethonium chloride have all been tested clinically with however equivocal results (reviewed in (54)). It is possible that the local application of these agents does not allow the drug to reach the Leishmania parasites present in macrophages within the deeper lesion.
Alternatively, studies have suggested the use of thermotherapy for the treatment of cutaneous leishmaniasis. This method has been successfully used in humans infected with *L. tropica, L. amazonensis, L. mexicana* and *L. braziliensis* (241, 394, 397). In addition, in both humans and mice, thermotherapy was shown to be as effective as antimonial drugs, or better (25, 132, 394). However, as different species of *Leishmania* have different thermosensitivity, the efficiency of thermotherapy may vary accordingly (397, 483).

Regarding *L. amazonensis* infection, a recent mouse study has shown that an enhanced oral absorption of low doses of pentavalent antimonials could be achieved using β-cyclodextrin. This drug regimen was as efficient as parenteral administration of twice as much pentavalent antimonials alone (140). In addition, new anti-fungal compounds have proven to be equivalent or better than amphotericin B, with decreased toxicity in mice infected with *L. amazonensis* (9, 10). Similarly, the topical application of paromomycin and gentamycin was recently shown to promote the healing of mice infected with *L. amazonensis* while also limiting the size of the scar (184).

In conclusion, the diversified panel of drugs currently available for the treatment of leishmaniasis gives the practitioners the ability to combine or switch therapies when patients show signs of toxicity or resistance to some of the drugs. However, the development of new, more efficient and less toxic drugs is needed, especially since there is an increased rate of resistance in certain endemic areas. Advancement in genomics, i.e. completion of the *Leishmania* genome, and proteomics will hopefully help identify new drug targets and design new therapeutic strategies (129).
15.2. Sterile cure

Although clinical cure can be achieved, there is evidence that *Leishmania* parasites can persist in the scar and/or the blood of healed patients (139, 349, 507, 508). Persistence of parasites is also observed in mice that have healed a cutaneous *Leishmania* infection (132, 299). In particular, in mice that have healed a *L. major* infection, parasites are found within macrophages, dendritic cells, fibroblasts and necrotic areas of the draining lymph node (63, 355, 538). It is tempting to suggest that fibroblasts may be a major host cell in the scar tissue of healed patients. *Leishmania* parasites, including *L. amazonensis*, can infect fibroblasts in vitro but do not multiply within those cells (63, 101, 137, 510). Furthermore, although some amastigotes are degraded within fibroblasts, these cells produce less nitric oxide than macrophages and therefore may represent a less hostile environment where parasites can persist during the latent phase of infection (63, 137, 510). The lack of clinical disease in the presence of parasites may suggest that the persistent *Leishmania* have an attenuated virulence. However, the persistent parasites from mice that had healed a *L. major* infection appeared genetically identical to the parent strain and remained virulent (1). In addition, in clinically cured mice, the maintenance of the resistant phenotype was dependent upon CD4⁺ T cells, CD8⁺ T cells, IFN-γ, IL-12 and/or iNOS as the inhibition or absence of one of those immune factors reactivated the infection (44, 375, 418, 538). Similarly, immunosuppression of clinically cured mice caused lesion to reappear, raising some concerns with regards to the increased incidence of *Leishmania*-HIV co-infected patients (132). Therefore, this has implications in human patients co-infected with *Leishmania* and HIV.

Finally, it has been demonstrated that IL-10 is required for the establishment of persistent parasites: indeed, sterile cure could be achieved in IL-10 deficient mice or in mice treated
with blocking antibodies against IL-10 (44). While there is a risk that persistent parasites from clinically cured patients or animals may be able to initiate subsequent relapses, they may also be important for maintaining an immunological memory in the host (355). Indeed, T cell memory has been shown to be short-lived in the absence of antigen (179). Furthermore, after sterile cure, IL-10 deficient mice or mice treated with anti-IL-10R are no longer immune to reinfection (46).

15.3. Vaccines

Leishmanization, i.e. the inoculation of virulent *Leishmania* promastigotes as a vaccine, was initially used with success to protect humans against leishmaniasis in different endemic regions of the world (reviewed in (352)). However, due to the development of severe lesions in some patients, leishmanization was later discontinued and to date, there is no vaccine available for the prevention of leishmaniasis. However, experimental studies of leishmaniasis are still actively working on the elaboration of new vaccination strategies (reviewed in (304) and in (199)). In particular, these studies have tested the efficacy of vaccines composed of killed parasites, attenuated live parasites, recombinant protein, and DNA with or without additional adjuvants such as IL-12 (4), Bacillus Calmette Guérin (BCG)(2), Freund incomplete or complete adjuvant (56, 98), alum (255), CpG-ODN (348, 458, 534) and/or *Propionibacterium acnes* (formerly *Corynebacterium parvum*) (526).

Studies using killed *Leishmania* parasites as a vaccine have resulted in variable degrees of protection. For example, in BALB/c mice, irradiated *L. major* or *L. donovani* promastigotes were able to confer protection against a subsequent *L. major* challenge (221, 222). The route of administration was critical for the subsequent protection; indeed, in
contrast to the intravenous route, the subcutaneous and intra-peritoneal immunizations were ineffective at promoting protection (222). Similarly, radioattenuated *L. major* increased the resistance of CBA mice to a subsequent *L. mexicana* infection (11). In addition, the vaccination of rhesus macaques with heat-killed *L. amazonensis*, IL-12 and Alum provided protection against a subsequent *L. amazonensis* challenge and the protective effect positively correlated with the dose of IL-12 used in the vaccine (255). In contrast, when autoclaved *L. major* were administered in combination with IL-12 to vervet monkeys, a protective effect could not be observed (168). More recently, the oral administration of freeze-thawed *L. amazonensis* promastigotes to mice allowed them to better control a subsequent infectious challenge (431).

The use of attenuated live parasites in a vaccine is controversial, as it is inconvenient and cost-effective to maintain doses of live organisms. In addition, there is always the possibility that attenuated pathogens revert to a virulent phenotype. However, these organisms likely better mimic a real infection and they are a source of more antigens as a whole. Experimentally, avirulent *L. major* promastigotes lacking DHFR-TS (i.e. a gene encoding for a dihydrofolate reductase-thymidylate synthase) have been shown to promote the subsequent control of a heterologous challenge with virulent *L. amazonensis* (571). Similarly, mice that had controlled a challenge with cysteine protease-deficient parasites could resist a challenge with wild-type virulent organisms (13). Finally, phosphoglycan-deficient *L. major* parasites, which can persist indefinitely in the host without causing pathology, are able to protect against a secondary virulent challenge (532, 566). As a result, such parasites may become candidates for vaccine strategies involving attenuated live parasites.
Vaccines composed of recombinant proteins/antigens have the advantage of being easily produced, distributed and stored on a large scale. Different degrees of protection toward *L. amazonensis* infection have been shown after vaccination with purified proteins and adjuvants (56, 98, 115, 526). However, recombinant protein/antigen-based vaccines may not elicit a T cell response that is as diverse as the one induced by whole parasites. For example, a mixture of several antigens is usually more protective than each single antigen used individually, and different antigenic cocktails also have different effectiveness depending on the antigens included in the vaccine (2, 359). This suggests that specific antigens may be better at specifically expanding certain effector cells. Indeed, for example, the *Leishmania* elongation initiation factor (LeIF) has been shown to preferentially induce the production of IL-12 and IFN-γ, suggesting that this antigen may be used in vaccine suspension to promote Th1 responses (522). In all of these vaccination studies, the protective effect was critically dependent upon the presence of an adjuvant within the vaccine. Furthermore, the level of protection may depend on the site of vaccination relative of that of the infectious challenge (461).

The expression of certain immunogenic proteins within attenuated bacteria or viruses represents an alternative to vaccines composed of recombinant proteins/antigens only. For example, *Leishmania* antigens expressed into vaccinia viruses or Salmonella have induced protection in different murine models (174, 340, 342). The role of the carrier organisms is to serve as an adjuvants and likely to target the antigen of interest to specific subcellular location, e.g. the phagolysosomal compartment of dendritic cells.

Recently, DNA vaccines have emerged as powerful tools to elicit effective immune responses against multiple pathogens. The concept implies that the injected plasmid that
encodes the antigen(s) of choice will reach the nucleus of host cells and subsequently transcribed into a messenger RNA. This mRNA will then be translated and the resulting antigen be presented to the immune system, which will hopefully mount an effective response against this antigen. DNA vaccines encoding for one or several \textit{Leishmania} antigens have reported promising results (8, 82, 85, 191, 192, 347). Finally, due to the crucial role of DC in priming immune responses, their potential as prophylactic vaccine adjuvants has been exploited to promote an efficient immune response before infection with \textit{L. major} (53, 157, 574). Similarly, the direct in vivo expansion of the DC compartment on the outcome of a \textit{Leishmania} infection has been investigated using Flt3-L (270). Pretreatment of BALB/c mice with this cytokine promoted resistance to a subsequent \textit{L. major} infection in 40% of the mice.

In conclusion, multiple vaccination strategies aimed at controlling leishmaniasis are currently under investigation. The goal of future studies will be to design safe vaccines with cross-protecting and long-lasting efficacy.

16. Concluding remarks

Experimental murine leishmaniasis shares many similarities with the human disease and mouse models of \textit{Leishmania} spp. infection have helped to uncover some of the immune mechanisms associated with resistance and susceptibility to the disease. For example, the mouse model of \textit{L. major} infection has been extensively studied and has been very useful to study Th1 and Th2 immune responses, which mediate resistance and susceptibility to leishmaniasis. In particular, resistance to a \textit{L. major} infection correlates with the production of high levels of IL-12 by antigen-presenting cells, the subsequent priming of Th1 CD4$^+$ T
cells producing IFN-γ, and the induction of parasite killing in a NO-dependent manner. In contrast, susceptibility to *L. major* is mediated by Th2 CD4+ T cells producing high levels of IL-4 and/or IL-10. In addition, resistance and susceptibility to *L. major* can usually be easily induced or reversed manipulated by exogenous administration of Th1 or Th2 cytokines. As a result of these studies, it is generally accepted that resistance and/or susceptibility to other *Leishmania* spp. infections follow the same rules. However, there is recent evidence that the factors mediating resistance to *L. major* may not be applicable to parasites belonging to the *L. mexicana* complex, including *L. amazonensis*. Indeed, mouse strains that are resistant to *L. major* are susceptible to *L. amazonensis* and this susceptibility does not correlate with a Th2 phenotype of the host immune response. Instead, the associated immune response has been characterized by a mixed Th1/Th2 cytokine pattern. Some of the immune defects associated with a *L. amazonensis* infection have been characterized and include defective pro-inflammatory cytokine and chemokine production, decreased IL-12 production and lower IL-12 responsiveness, decreased production of IFN-γ and enhanced resistance to macrophage microbicidal activity (e.g. NO-mediated killing). In addition, the susceptibility to *L. amazonensis* infection could not be reversed by exogenous administration of IL-12 or IFN-γ. Similarly, mice deficient in IL-4 or IL-10 remained susceptible to a *L. amazonensis* challenge.

In conclusion, the immune response mounted against *L. amazonensis* appears very robust to any exogenous immunomodulatory factors. Therefore, it would be of interest to successfully modulate this mixed immune response towards an effective Th1 phenotype. This would have implication for both immunotherapeutic and vaccine strategies aimed at curing the disease caused by *L. amazonensis*. Finally, although the Th1 immune response of
resistant mice infected with *L. major* is commonly used as a model to characterize the immune defects of *L. amazonensis*-infected mice, it is not known if such promote resistance to a *L. amazonensis* challenge. Indeed, this would confirm that comparisons between the immune responses of mice infected with *L. major* or *L. amazonensis* is appropriate. The present manuscript has attempted to answer some of these questions.

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CHAPTER 2: CD4⁺ Th1 cells induced by a dendritic cell–based immunotherapy in mice chronically infected with *L. amazonensis* do not promote healing

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Yannick F. Vanloubbeeck¹, Amanda E. Ramer², Fei Jie² and Douglas E. Jones ¹,²

Department of Veterinary Pathology¹, College of Veterinary Medicine and Immunobiology Program², Iowa State University, Ames IA 50011-1250.

Abstract

The susceptibility of mice to *Leishmania amazonensis* infection is thought to result from an inability to develop a Th1 response. Our data show that the low levels of IFN-γ produced by the draining lymph node (DLN) cells of chronically infected mice could be enhanced *in vitro* and *in vivo* with *L. amazonensis* Ag-pulsed bone marrow-derived dendritic cells (BM-DC) and the Th1 promoting cytokine IL-12. Given intralesionally to chronically infected mice, this treatment induced the up-regulation of mRNA levels for IFN-γ, T-bet, and IL-12Rβ2 in CD4⁺ T cells from the DLN and an increase in parasite-specific IgG2a in the serum. However, this Th1 response was not associated with healing and the Ag-specific enhancement of IFN-γ production remained impaired in the DLN. However, addition of IL-12 to the *in vitro* recall response was able to recover this defect, suggesting that APC derived IL-12 production may be limited in infected mice. This was supported by the fact that *L. amazonensis* amastigotes limited the production of IL-12p40 from BM-DC *in vitro*. Taken together, our data indicate that the immune response of mice chronically infected with *L.
*Amazonensis* can be enhanced towards a Th1 phenotype, but that the presence of Th1 CD4+ T cells does not promote healing. This suggests that the phenotype of the CD4+ T cells may not always be indicative of protection to *L. amazonensis* infection. Furthermore, our data support growing evidence that APC function, such as IL-12 production, may limit the immune response in *L. amazonensis* infected mice.

**Introduction**

Leishmaniasis is a zoonotic disease caused by intracellular protozoa of the genus *Leishmania*. These parasites are transmitted to the host by a sandfly vector and the resulting clinical disease can be categorized into three forms: cutaneous, mucocutaneous and visceral. Infection of mice with various *Leishmania* species has led to an understanding of some of the immune mechanisms necessary to control these intracellular protozoa. As in humans, murine cutaneous leishmaniasis can have different outcomes depending on the parasite strain/species and the genetic background of the host (1, 4, 10, 11, 21, 37). For example, the outcome of murine cutaneous leishmaniasis caused by *L. major* is determined by the phenotype of the CD4+ T cell response: Th1 CD4+ T cells producing IFN-γ promote resistance while Th2 CD4+ T cells producing IL-4 induce susceptibility (reviewed in (36, 38)).

C3HeB/FeJ and C57BL/6 mice are able to control a *L. major* infection by mounting a Th1 immune response. This resistant phenotype requires a sustained source of IL-12 and the expression of IL-12Rβ2 and T-bet, a Th1 associated transcription factor (14, 15, 18, 31, 41). The associated cutaneous lesion subsequently resolves and contains very low parasite numbers (reviewed in (36, 38)). In contrast, when infected with *L. amazonensis*, C3HeB/FeJ and C57BL/6 mice develop chronic cutaneous lesions containing from 10^5 to 10^8 parasites.
This is associated with low levels of both IFN-γ and IL-4 produced in the \textit{in vitro} recall responses from the draining lymph node (DLN)\textsuperscript{3} (1, 20, 39), which suggests that an antigen (Ag)-specific memory/effector cell population may not develop or persist after infection. In addition, there is decreased IL-12Rβ2 expression in the CD4\textsuperscript{+} T cells from the DLN and this persists even in the absence of IL-4 (17, 20). Also, mice given the Th1 promoting cytokines IL-12 or IFN-γ and mice deficient for the Th2 cytokines IL-4 or IL-10 remain susceptible to \textit{L. amazonensis} (5, 17, 19, 20). Therefore, in addition to the mouse models of visceral leishmaniasis caused by \textit{L. donovani} or \textit{L. chagasi}, the murine model of cutaneous leishmaniasis caused by \textit{L. amazonensis} is an experimental system in which the Th1 response is limited without the expansion of Th2 cells, (23, 45).

Dendritic cells (DC) are the main Ag-presenting cells (APC) \textit{in vivo} and \textit{in vitro} (3). They play an important role in priming naïve T cells toward a Th1 or Th2 phenotype (reviewed in (30)). Their potential as prophylactic vaccine adjuvants has been exploited to promote an efficient immune response before infection with \textit{L. major} (8, 12, 44). However, DC-based immunotherapies have not been tried in mice with established cutaneous lesions. Protection to a \textit{L. amazonensis} challenge has been achieved in mice by giving parasite Ag, DNA or by adoptively transferring a \textit{L. amazonensis}-specific Th1 CD4\textsuperscript{+} T cell line before the infection (9, 17, 32, 34, 40). This was associated with an increase in IFN-γ production within the DLN, suggesting a Th1 phenotype of the local immune response. Furthermore, Ji \textit{et al.} showed that CD4\textsuperscript{+} T cells from the DLN of protected mice had increased expression of mRNA levels for IL-12Rβ2, further supporting that a Th1 response could provide protection to \textit{L. amazonensis} infection (17).
Our data show that upon *L. amazonensis* infection the phenotype of the CD4⁺ T cells is not indicative of disease outcome as the induction of Th1 CD4⁺ T cells expressing T-bet and IL-12Rβ2 in chronically infected mice did not promote healing. This was associated with an impaired production of IFN-γ in the DLN, which could be restored by addition of IL-12 in the DLN recall response. This suggests that IL-12 may have been a limiting factor in the DLN and is further supported by the ability of *L. amazonensis* amastigotes to limit IL-12p40 production by BM-DC.

**Materials and methods**

**Mice.** C3HeB/FeJ mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and bred and maintained in a specific-pathogen-free (SPF) facility. Prkdc.Scid (C3H SCID) mice were purchased from the same source and maintained in our SPF facility. The Committee on Animal Care at Iowa State University approved all protocols involving animals. Five to eight week old C3HeB/FeJ females were inoculated with 5 x 10⁶ stationary-phase promastigotes in 50 μl of PBS in the left hind footpad. Lesion size was monitored weekly with a dial micrometer (L.S. starrett Co., Athol, MA), and the results were expressed as the difference between the footpad thickness for the infected foot and the footpad thickness for the uninfected foot. C3H SCID mice were inoculated subcutaneously with 10 to 20 x 10⁶ stationary-phase promastigotes in 50 μl of PBS in the left hindfoot and later sacrificed to harvest tissue-derived amastigotes.

**Parasites.** Culture of *L. amazonensis* (MHOM/BR/00/LTB0016) or *L. major* (MHOM/IL/80/Friedlin) parasites and the preparation of parasite Ag were performed as
previously described (19). For the parasite quantification from cutaneous lesions, the infected feet were disinfected with 70% ethanol and the skin was dissected away. The remaining subcutaneous lesion was homogenized using a Tenbrock tissue homogenizer, washed twice in PBS and resuspended in Grace's insect cell culture medium (Life Technologies, Grand Island, NY) supplemented with 20% heat-inactivated FBS, 2 mM glutamine, 100U/ml penicillin, and 100 µg/ml streptomycin. A ten-fold serial dilution of the parasite suspension was then performed and incubated at 24°C for 8 days before parasite quantification was assessed. Amastigotes were directly recovered from the lesion of C3H SCID mice. The number of live amastigotes recovered from the footpad tissue was determined by fluorescence microscopy using fluorescein diacetate (Acros Organics, Morris Plains, NJ) and propidium iodide (Sigma, St Louis, MO).

**Antigen challenge.** At 10 weeks post infection, mice were injected with 20 µg of Ag in 50 µl of PBS in the right hind footpad. Footpad swelling was measured daily for 72 hours with a dial micrometer and the results were expressed as the difference between the footpad thickness of the Ag-challenged foot and its thickness before the Ag challenge.

**Histology.** Feet from challenged mice were fixed in 10% buffered formalin and routinely processed for paraffin embedded sectioning. Five µm thick sections were cut, applied to poly-L-lysine-coated slides and stained with H&E. Toluidine blue stains were performed to assess the tissue infiltration by basophils and/or mast cells.
Isolation and culture of BM-DC. BM-DC were cultured in vitro in the presence of 10 ng/ml of murine GM-CSF (PeproTech Inc, Rocky Hill, NJ) according to a method from Lutz et al. (26). At day 10 of culture, approximately 90% of the BM-DC were positive for the DC marker CD11c. For in vitro studies, 10 day old BM-DC were incubated 24h at 37°C with fresh, tissue-derived, amastigotes at a cell: parasite ratio of 1:3.

BM-DC immunotherapy. Mice infected with L. amazonensis for 10 weeks or naïve control mice were injected every other day for 12 days in their left hindfoot. Each injection consisted of 20 μg L. amazonensis Ag and 0.2 μg murine IL-12 (PeproTech Inc, Rocky Hill, NJ) with 1x10⁶ BM-DC resuspended in a total of 25μl of PBS. Control groups were injected with 25μl of PBS. The mice were sacrificed three days after the last injection. As a control for the in vivo efficacy of murine IL-12, BALB/c mice were infected with L. major and simultaneously injected with IL-12, as previously described (31).

Recall responses. Lymph node cell recall responses (1 x 10⁶ lymph node cells per well) were performed as previously described (19). Spleen cells were incubated with a lysing buffer (0.15M ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM ethylene diamine tetra-acetic acid) to lyse the red blood cells and plated as the lymph node cells. Supernatants were harvested after 72 h or 96h (in experiments in which BM-DC were added to the cultures), and IFN-γ and IL-4 levels were determined by ELISA. Recombinant IFN-γ (Pharmingen, San Diego, CA) and IL-4 (PeproTech Inc, Rocky Hill, NJ) were used on each ELISA plate to set up a standard curve. The limit of detection of the IFN-γ ELISA ranged between 39 and 78 pg/ml; the limit of detection of the IL-4 ELISA was 78 pg/ml. When BM-DC were added to
the cultures, a total of 1 or $2 \times 10^5$ BM-DC were co-cultured with 4 times more LN cells in 200 $\mu$l of complete tissue culture medium $\pm$ *L. amazonensis* Ag (50 $\mu$g/ml). The concentration of murine IL-12 (PeproTech Inc, Rocky Hill, NJ) and anti-murine IL-12p70 (Pharmingen, San Diego, CA) used were 1ng/ml and 10$\mu$g/ml, respectively. Endotoxin levels in culture supernatants were undetectable as determined by the LAL assay (Sigma, St Louis, MO).

**Purification of CD4$^+$ T cells.** Lymph node cells were incubated with anti-CD4-coupled microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol, followed by one passage through an autoMACS. Purity of the cell preparation was higher than 95%.

**Flow cytometry.** Intracellular staining for IFN-γ was performed as previously described (20). Live events were gated based on forward scatter versus side scatter.

**Amastigote infection of BM-DC.** *L. amazonensis* and *L. major* amastigotes were harvested from C3H SCID mice as indicated above. BM-DC ($2 \times 10^6$) were incubated 24h with $6 \times 10^6$ live amastigotes (3:1 parasite to BM-DC ratio). The infection rate was determined by microscopic examination of Hema 3-stained (Fisher Scientific, Middletown, VA) cytospin preparations. Infection rates ranged from 56 to 65%. There were no significant differences in the infection rate between the two organisms.
**IL-12p40 ELISA.** Supernatants from BM-DC 24h post-amastigote infection were harvested and the IL-12p40 ELISA was performed using commercially available antibodies (Pharmingen, San Diego, CA), peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) and ABTS microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

**IgG2a ELISA.** ELISA plates were coated with 10μg/ml of *L. amazonensis* Ag. The serum samples were serially diluted from 1/100 to 1/12800 and the ELISA was performed as previously described (19).

**RT-PCR.** Total RNA was extracted from CD4+ T cells using the Rapid Total RNA Purification System (Marligen Biosciences, Ijamsville, MD) according to the manufacturer's protocol. RNA concentration was measured spectrophotometrically; RNA samples were then treated with 1.5 U of DNase I (Pierce, Milwaukee, WI) per 1 μg of RNA for 45 min at 37°C. Two μg of each DNase I-treated RNA sample was reverse transcribed in 80 μl reaction volumes using 1X M-MLV reaction buffer (Promega, Madison, WI), 200 U M-MLV reverse transcriptase (Promega, Madison, WI), 40 U RNase inhibitor (Applied Biosystems, Foster City, CA), 2.5 μM random hexamers (Amersham Biosciences Corp, Piscataway, NJ) and 0.5 mM of each dNTP (USB Corporation, Cleveland, OH). RNA samples and random hexamers were incubated together at 70°C for 3 min and room temperature for 10 min. The reverse transcription reaction was performed at 42°C for 1 hr; samples were then heated at 95°C for 5 min and incubated on ice for 10 min. Real-time PCR experiments were run on the ABI PRISM 5700 Sequence Detection System (Applied
Each 50 μl reaction contained 1 μl of cDNA and 1X ABsolute QPCR Mix (Abgene, Surrey, UK). Forward and reverse primers were used at either 300 or 350 nM (IDT, Coralville, IA) and TaqMan probes (labeled with the reporter dye FAM and the quencher dye TAMRA, Applied Biosystems, Foster City, CA) were used at either 75 or 100 nM. Primer pairs and probes were designed using Primer Express 1.5 software (Applied Biosystems, Foster City, CA) and are listed in Table 1. For quantitative analysis of gene expression, normalized to an endogeneous control, we used the standard curve method, which was detailed previously (13). Briefly, control cDNA samples were serially diluted to obtain standard curves for GAPDH cDNA and each target cDNA. For each assay, the resultant cycle threshold (Ct) values were plotted against the log ng input of RNA. For all experimental samples, the cDNA quantity for the target of interest was then determined from its standard curve and then normalized to the GAPDH control.

Statistical procedure. Statistical analysis was performed using Statview (SAS, Cary, NC). When multiple treatment groups were compared, the data were entered into an ANOVA and analyzed with the Fisher's PLSD post-hoc test. When a comparison between 2 groups was done, a t-test was performed. Differences were considered significant when p<0.05.

Results

Low levels of IFN-γ production in the DLN and spleen from mice chronically infected with *L. amazonensis*. While C3HeB/FeJ mice are able to resolve a *L. major* infection, they develop chronic cutaneous lesions when infected with *L. amazonensis* (Fig. 1). The susceptibility to *L. amazonensis* is thought to result from an inability to develop a Th1
response. Within DLN and splenic recall responses from *L. amazonensis*-infected mice, the absolute amounts of IFN-γ detected were low (0.31 ± 0.11 and 1.75 ± 0.37 ng/ml, respectively), and typically 10 to 20 times lower than the ones detected in the recall responses from the DLN and spleen from *L. major*-infected animals (6.79 ± 2.59 ng/ml and 21.57 ± 4.45 ng/ml, respectively). There was no IL-4 detected in the recall responses at 10 weeks post-infection. However, the low levels of IFN-γ detected *in vitro*, together with the absence of IL-4, do suggest that the immune response of mice chronically infected with *L. amazonensis* has some component of a Th1 response.

**Antigen-specific footpad swelling upon subcutaneous antigen challenge indicates a functional memory response *in vivo* during the chronic *L. amazonensis* infection.** The low levels of effector cytokines within the recall response of mice chronically infected with *L. amazonensis* may reflect an absence of effector/memory response *in vivo*. To test this, mice infected for 10 weeks with *L. amazonensis* in their left hindfoot were challenged with 20μg of *L. amazonensis* Ag in the contralateral foot, and the associated footpad swelling was measured over a 3-day period. Naïve mice challenged with *Leishmania* Ag were used as negative controls. In parallel, as a positive control for the kinetics and histological changes of a DTH response, similar experiments were done using *L. major*-infected mice challenged with 20μg of *L. major* Ag. As shown in Figure 2A, mice chronically infected with *L. amazonensis* developed an Ag-specific footpad swelling, indicating the presence of a memory/effector response *in vivo*. The observed footpad swelling was not as pronounced as in *L. major* infected mice challenged with *L. major* Ag, but it was significantly higher than in uninfected control mice challenged with *Leishmania* Ag (Fig. 2A). Histologically, the
footpad swelling observed in the Ag-challenged feet of mice infected with *L. amazonensis* or *L. major* was similar and characterized by an infiltrate of lymphocytes with scattered neutrophils and rare basophils and/or mast cells (Fig. 2B and data not shown), which is consistent with a DTH response. In mice infected with *L. amazonensis*, the cellular infiltrate was found multifocally throughout the dermis and subcutaneous tissue, while it was diffusely present in the dermis and subcutis of mice infected with *L. major*. A cellular infiltrate was not present in naïve mice challenged with *Leishmania* Ag (Fig. 2C). However, a focus of neutrophils and few mononuclear cells was occasionally observed in the feet of these mice, likely resulting from traumatic injury at the injection site (data not shown).

**Impaired IFN-γ production in the lymph node draining the site of Ag challenge.** Since Ag-specific memory cells were able to elicit a DTH *in vivo* we determined if this response was associated with increased effector cytokine production, i.e. IFN-γ and IL-4. *L. major*-infected mice demonstrated a typical memory response when challenged with *L. major* Ag in the contralateral footpad. The cells from the lymph node draining the site of Ag challenge produced two times more IFN-γ than their uninfected controls during the recall response (Fig. 3). In contrast, *L. amazonensis*-infected mice had an inhibition of effector cytokine production when challenged with *L. amazonensis* Ag in the contralateral footpad. The cells of the lymph node draining the site of Ag challenge had a 75% reduction in IFN-γ production in comparison to the uninfected mice challenged with *L. amazonensis* Ag (Fig. 3). The IFN-γ production was dependent on the presence of leishmanial Ag in all assays, and there was no IL-4 detected in any of the recall responses (data not shown). This demonstrates that in mice chronically infected with *L. amazonensis*, the production of IFN-γ is also impaired in
lymph nodes distant from the site of infection, i.e. the lymph node draining the site of a subcutaneous Ag-challenge.

Ag-pulsed BM-DC enhance IFN-\(\gamma\) production from DLN cells in an IL-12 independent manner. Dendritic cells have previously been shown to enhance the \textit{in vitro} production of IFN-\(\gamma\) by T cells from the DLN of \textit{Leishmania}-infected mice (6). Therefore, we wanted to test the hypothesis that the addition of BM-DC as Ag presenting cells in the \textit{in vitro} recall response would enhance the secretion of IFN-\(\gamma\) of DLN cells. Cells from the DLN of mice chronically infected with \textit{L. amazonensis} were co-cultured for 4 days with either BM-DC alone (non-pulsed) or \textit{L. amazonensis} Ag-pulsed BM-DC (see materials and methods) at a 4:1 ratio. As shown in Figure 4A, \textit{L. amazonensis} Ag-pulsed BM-DC increased IFN-\(\gamma\) levels up to 3 to 7 times the level of IFN-\(\gamma\) detected in the standard recall response. In addition, non-pulsed BM-DC were unable to promote IFN-\(\gamma\) production by the lymph node cells, demonstrating the Ag-specificity of the response. In our system, IFN-\(\gamma\) was not detected from cultures containing Ag-pulsed BM-DC alone and IL-4 was not detected in any culture condition (data not shown). We then hypothesized that this Ag-specific enhancement of the IFN-\(\gamma\) production could result from an enhanced IL-12p70 production by the BM-DC, skewing the results towards a Th1 response. Blocking the bioactive IL-12p70 in the co-cultures did not result in a significant decrease in the levels of IFN-\(\gamma\) recovered in the supernatants, consistent with a Th1 bias of the Ag responsive cells (Fig. 4B). Addition of IL-12 in the co-cultures significantly enhanced the levels of IFN-\(\gamma\) detected, further suggesting that the lymphocyte response is skewed towards a Th1 response (Fig. 4C).
Immunotherapy with Ag-pulsed BM-DC and IL-12 promotes the polarization of CD4+ T cells from the DLN towards a Th1 phenotype. Since Ag-pulsed BM-DC and IL-12 were able to enhance the Th1 phenotype of the recall response in vitro, we tested if such treatment would promote a Th1 response and subsequent healing in vivo. In particular, we determined the phenotype of the CD4+ T cells from the DLN. Th1 CD4+ T cells have been shown to play a major role in resistance to *Leishmania major* infection (36, 38) and to promote protection to a *L. amazonensis* challenge, after adoptive transfer (17, 34). Therefore, mice chronically infected with *L. amazonensis* were injected in their infected foot every other day for 12 days with either PBS or *L. amazonensis* Ag-pulsed BM-DC + IL-12 (see Material and Methods). Naïve mice were included in each treatment group as negative controls. Three days after the last injection, the CD4+ T cells from the DLN were purified and their mRNA levels for T-bet, GATA-3 (a Th2 associated transcription factor (46)), IFN-γ, IL-12Rβ1 and IL-12Rβ2 were determined by RT-PCR. In comparison to the PBS control, the administration of Ag-pulsed BM-DC and IL-12 led to the up-regulation of T-bet, IFN-γ and IL-12Rβ2 mRNA levels in both naïve and infected mice (Fig. 5A and data not shown). Furthermore, mRNA levels for IL-12Rβ1 and GATA-3 remained similar between treated and control mice (Fig. 5A), indicating that the administration of Ag-pulsed BM-DC and IL-12 led to a specific enhancement of a Th1 phenotype of the CD4+ T cell population from the DLN. Finally, as another indication that the BM-DC immunotherapy induced a Th1 response, treated mice had increased levels of *L. amazonensis*-specific IgG2a levels in their serum in comparison to PBS control mice (Fig. 5B).
Specific limitation of the Ag-specific IFN-γ production in the DLN of mice chronically infected with *L. amazonensis*. As Ag-pulsed BM-DC and IL-12 induced a Th1 response *in vivo*, we hypothesized that it would be accompanied by an enhancement of the IFN-γ production in the *in vitro* recall response from the DLN. In the absence of Ag in the recall response, DLN cells from treated infected mice produced significantly more IFN-γ than DLN cells from PBS control mice (6.8 ± 1.6 ng/ml vs 0.5 ± 0.1 ng/ml, respectively). This enhanced IFN-γ production induced after immunotherapy recapitulated the one previously observed *in vitro* when Ag-pulsed BM-DC and IL-12 were added to the cell cultures (Fig. 4C). However, the increased levels of IFN-γ produced by DLN cells from chronically infected mice treated with Ag-pulsed BM-DC +IL-12 were not further enhanced in the presence of Ag *in vitro*, in contrast to what is seen in treated-naïve mice (Fig. 6A). In fact, the amount of IFN-γ recovered in the recall response of treated naïve mice was significantly greater than that of treated infected mice (Fig. 6A). These discrepancies occurred despite the presence of similar percentages of IFN-γ producing cells within the DLN of both groups of mice (Fig. 6B). However, the addition of IL-12 to the cell cultures led to similar levels of IFN-γ produced by the DLN cells from treated naïve and treated infected mice (59.35 ± 15.94 ng/ml and 47.41 ± 10.96 ng/ml, respectively; Fig. 6A). This demonstrates that the defective Ag-specific IFN-γ production of infected mice could be overcome by IL-12 and supports a Th1 phenotype of the DLN cells. Finally, a limitation of IFN-γ production was not observed in the spleen, as after immunotherapy with Ag-pulsed BM-DC and IL-12, splenocytes from both naïve and infected animals produced comparable levels of Ag-specific IFN-γ in the recall response (Fig. 6C). Altogether, this indicates that the limitation of Ag-specific IFN-γ production from the lymphocyte population is confined to the DLN, but not the spleen, of
mice chronically infected with *L. amazonensis* and that it can be overcome by IL-12.

Consistent with the limitation of the lymphocyte response in the DLN, the immunotherapy did not induce a reduction in lesion size 2 weeks after the first BM-DC injection: the mean lesion size ± SEM of PBS control and treated mice was 0.99 ± 0.11 mm and 0.95 ± 0.11 mm, respectively. This was further supported by the absence of a significant decrease in the parasite numbers in the infected feet at this timepoint (Fig. 6D). Furthermore, to determine if a healing response would require more time, in one experiment, lesion size and parasite quantification were measured 5 weeks after the first BM-DC injection. At this timepoint, lesion size and parasite loads were still similar between treated mice and PBS control (data not shown). Taken together, these data demonstrate that the administration of Ag-pulsed BM-DC +IL-12 to mice chronically infected with *L. amazonensis* induced a Th1 response *in vivo*; however, this was not associated with lesion resolution.

*L. amazonensis* amastigotes limit IL-12p40 production by BM-DC. As the impaired production of IFN-γ in the DLN of infected mice after treatment was restored by the addition of IL-12 in the in vitro recall response, it suggested that the infection with *L. amazonensis* may be limiting the production of IL-12 by APC, i.e. dendritic cells. Therefore, we determined the ability of BM-DC to produce IL-12p40 *in vitro*, after infection with *L. amazonensis* amastigotes. BM-DC were infected, or not, with *L. amazonensis* amastigotes at a 1:3 ratio. As a positive control, BM-DC were infected with *L. major* amastigotes at a similar cell:parasite ratio. The levels of IL-12p40 were determined 24h post-infection by ELISA. As shown in Fig. 7, BM-DC infected with *L. amazonensis* amastigotes produced similar levels of IL-12p40 as non-infected BM-DC. In contrast, BM-DC infected with *L.*
major amastigotes produced significantly higher amounts of IL-12p40. Altogether, this demonstrates that L. amazonensis amastigotes can limit the production of IL-12p40 by BM-DC.

Discussion

Our data demonstrate that the poor lymphocyte response of mice chronically infected with L. amazonensis is not the result of an absence of a memory cell population that can recognize parasite Ag, as these mice developed a DTH response after a subcutaneous Ag challenge (Fig. 2A and B). However, the elicited DTH reaction is attenuated in comparison to that of L. major-infected animals, which have developed a strong Th1 response (Fig. 2A). In addition, the undetectable levels of IL-4 and the impaired IFN-γ production from the lymph node draining the site of Ag challenge indicates that the defective Th1 response of mice chronically infected with L. amazonensis is present at peripheral sites distant from the primary infection (Fig. 3).

It has been recently suggested that defective priming of T cells by DC may be responsible for the poor lymphocyte response to L. amazonensis Ag (17). Dendritic cells play a crucial role in priming T cells; in particular, IL-12 produced by DC promotes the polarization of the T cells towards a Th1 phenotype (reviewed in (30) and (25)). During both acute and chronic L. major infection, DC are the main APC in the in vitro recall response (28, 29). Our data show that BM-DC could enhance the Ag-specific production of IFN-γ from DLN cells in vitro in an IL-12 independent manner (Fig. 4A & B). This enhanced lymphocyte response could result from a more efficient DC to T cell ratio, Ag presentation and/or co-stimulation. In addition, the levels of IFN-γ detected in the DLN recall response were further increased
when IL-12 was added to the co-cultures (Fig. 4C). This and the fact that IFN-γ, but not IL-4, were detected in the DLN in vitro recall response indicate a Th1 bias of the lymphocyte response in mice infected with *L. amazonensis*. Furthermore, the enhanced production of IFN-γ in response to IL-12 suggests that cells fully capable of producing IFN-γ are present within the DLN. Therefore, in addition to the CD4+ T cell defects present during *L. amazonensis* infection (17, 20), it is probable that APC from the DLN are limited qualitatively and/or quantitatively, thereby contributing to low effector cytokine production in the recall response. This is supported by the low numbers of IL-12 producing cells associated with the DLN of *L. amazonensis*-infected mice (20) and by studies showing that *Leishmania* parasites can modulate the phenotype and functions of APC, i.e. macrophages and dendritic cells (2, 7, 22, 24, 27, 33, 35, 43).

Based on our in vitro results, we hypothesized that giving *L. amazonensis* Ag-pulsed BM-DC +IL-12 as an immunotherapy to mice chronically infected with *L. amazonensis* would promote a Th1 response in vivo. Variable degrees of protection towards *L. amazonensis* infection have been achieved in mice by prophylactic administration of Ag (32, 40), DNA (9), attenuated *L. major* promastigotes (16, 42) or *L. amazonensis* derived Th1 CD4+ T cell line (17, 34). DC-based vaccines have successfully been used prophylactically in experimental studies of cutaneous leishmaniasis caused by *L. major* (8, 12, 44) and protection has been linked to the ability of the DC to produce IL-12 (8). However, to our knowledge, the present study is the first to use a therapeutic DC-based strategy, in mice with established cutaneous lesions. The repeated administration of this treatment led to the development of a Th1 response in vivo as measured by the upregulation of T-bet, IFN-γ, and IL-12Rβ2 mRNA levels on the CD4+ T cell population from the DLN (Fig. 5A). This is
different from what was previously observed in BALB/c mice where administration of IL-12 alone 48h after infection with *L. major* did not induce a Th1 phenotype of the CD4+ T cells from the DLN (14). In our study, the presence of a Th1 response was further demonstrated by the presence of increased levels of *L. amazonensis*-specific IgG2a in the serum (Fig. 5B). As adoptive transfer of Th1 CD4+ T cells was recently shown to promote protection to a subsequent *L. amazonensis* infection, a correlation between healing and the vaccine-induced Th1 CD4+ T cell response was expected (17, 34). This was also supported by a recent study of murine visceral leishmaniasis caused by *L. donovani* in which the administration of BM-DC alone was associated with decreased parasite burden 7 days later (2). However in the present study, despite the Th1 response, there was no significant decrease in parasite numbers in treated mice, as measured at 2 weeks (Fig. 6D) and 5 weeks after the first BM-DC injection (data not shown). In addition, although the percentage of IFN-γ producing cells was similar between treated infected and treated naïve mice, there was no enhancement of the IFN-γ production when Ag was added to the DLN recall response of infected mice (Fig. 6A and B). This indicates that the impaired Ag-specific IFN-γ production in the DLN of mice chronically infected with *L. amazonensis* does not result from quantitative defects from the IFN-γ producing cell population, but rather from qualitative defects of these cells, i.e. efficiency to produce IFN-γ. In addition, it is possible that the qualitative defect affects cells other than CD4+ T cells, possibly APC. This is supported by the fact that addition of IL-12 in the recall response from the DLN overcomes the defective production of IFN-γ by DLN cells (Fig. 6A). Furthermore, we tested the hypothesis that *L. amazonensis* amastigotes, the only parasite stage present during chronic infection, could limit IL-12p40 production by BM-DC in vitro. Our data provide evidence *L. amazonensis* amastigotes-infected BMDC do not have
enhanced production of IL-12p40 in comparison to non-infected BMDC (Fig. 7), supporting previous studies showing that *Leishmania* parasites can modulate the production of IL-12 by DC (7, 27, 35). Therefore, our data suggest that in chronically infected mice, and even in the presence of Th1 CD4$^+$ T cells, the production of IFN-γ may be limited by insufficient IL-12 production. This is consistent with studies showing that sustained production of IL-12 is critical to promote and maintain resistance to *L. major* (31). However, we can not rule out that the IFN-γ production from the DLN cells is actively suppressed by immunomodulatory factors produced in the DLN. Finally, our results show that the limitation of the IFN-γ production in response to Ag was localized to the DLN, as it was not observed in the spleen, suggesting a compartmentalization of the immune response (Fig. 6C). As *L. amazonensis* parasites are present in the DLN, but not the spleen of infected animals, it is tempting to suggest that such limitation could be specifically mediated by *L. amazonensis* amastigotes. This would be consistent with a very recent study showing that the transfer of a Th1 cell line prior to infection did confer resistance to a subsequent challenge with *L. amazonensis* promastigotes, but not amastigotes (34).

Taken together, our data indicate a local impairment of the Ag-specific IFN-γ production in the DLN of mice chronically infected with *L. amazonensis*. This defective response persists even after the induction of a Th1 CD4$^+$ T cells within the DLN, suggesting that the phenotype of the CD4$^+$ T cells may not always be indicative of protection to *L. amazonensis*, as it is the case with *L. major*. Furthermore, our data support growing evidence that in addition to CD4$^+$ T cell defects, APC functions such as IL-12 production may be impaired in *L. amazonensis*-infected mice (17).
Acknowledgments

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References


Figure 1. Upon infection with *L. amazonensis*, C3HeB/FeJ mice develop a chronic cutaneous lesion. 5 to 8 week-old C3HeB/FeJ mice were infected in the left hind footpad with $5 \times 10^6$ stationary phase *L. amazonensis* (open squares) or *L. major* (closed circles) promastigotes. Lesion size was monitored weekly as described in Materials and Methods. The values shown are the mean ± standard deviation (12 mice per group).
Figure 2. Ag-specific footpad swelling consistent with a DTH indicates the presence of a memory/effector response *in vivo*. (A) C3HeB/FeJ mice were infected in the left hind footpad with *L. amazonensis* or *L. major* as described in Figure 1. Ten weeks post-infection, the *L. amazonensis*-infected mice were challenged subcutaneously with *L. amazonensis* Ag (open squares) and the *L. major*-infected mice were challenged subcutaneously with *L. major* Ag (closed triangles) in the right hindfoot as described in the Materials and methods. Uninfected mice challenged subcutaneously with either *L. amazonensis* or *L. major* Ag (closed circles) were used as negative controls. The footpad thickness was monitored daily over 3 days and was expressed as the difference between the footpad thickness of the challenged foot at the time of measurement and its thickness before the Ag challenge. The values shown are the mean ± SEM based on 3 separate experiments. (B & C): Two days post antigen-challenge, the mice were sacrificed and the antigen-challenged feet were fixed and processed for histology (H&E staining). (B) Foot from a mouse infected with *L. amazonensis* and challenged with *L. amazonensis* Ag: the cellular infiltrate (arrows) within the dermis is composed of lymphocytes, scattered neutrophils and rare basophils and/or mast cells. (C) Foot from an uninfected mouse challenged with *L. amazonensis* Ag: there is no inflammatory infiltrate within the dermis.
Figure 3. Impaired IFN-γ production during the memory response of mice infected with *L. amazonensis*. Mice were infected and challenged with Ag as in Figure 2. Three days post-Ag challenge, the mice were sacrificed and cells from the lymph node draining the Ag-challenged feet were plated *in vitro* for 3 days with *L. amazonensis* or *L. major* Ag. IFN-γ levels were detected by ELISA. Results are expressed as a fold increase ± SEM over their respective negative controls, that is uninfected mice challenged with *L. amazonensis* or *L. major* Ag. In parentheses are the mean amounts of IFN-γ detected by ELISA for each group. Results shown are from 3 separate experiments with 3 mice per group. *Indicates a statistically significant difference (p<0.05).
Figure 4. Ag-pulsed BM-DC enhance the production of IFN-γ by lymph node cells from mice chronically infected with *L. amazonensis*. Ten weeks post-infection with *L. amazonensis*, mice were sacrificed and lymph node cells from the lymph node draining the infected foot were plated either (A), in medium alone (no Ag), with *L. amazonensis* Ag (+ La Ag), with BM-DC (+DC), or with *L. amazonensis* Ag-pulsed BM-DC (+La Ag +DC); (B) with *L. amazonensis* Ag-pulsed BM-DC and an IL-12p70 blocking antibody (+La Ag +DC +anti-IL12) or (C) with *L. amazonensis* Ag-pulsed BM-DC and recombinant murine IL-12 (+La Ag +DC +IL12). The DLN cells to DC ratio was 4:1. After 4 days of *in vitro* culture, IFN-γ levels in the culture supernatants were detected by ELISA. The values shown are the mean ± SEM based on 3 (C) or 4 (A and B) separate experiments. *Indicates a statistically significant difference in comparison to the other group(s) (p<0.05).
Figure 5. Immunotherapy using Ag-pulsed BM-DC +IL-12 induced a Th1 response in chronically infected mice. Mice were infected with *L. amazonensis* as in Fig. 1. Ten weeks post-infection, the mice were injected every other day for 12 days in their infected foot with *L. amazonensis* Ag-pulsed BM-DC and IL-12. Negative control mice were injected with PBS. Three days after the last injection, the mice were sacrificed. (A) CD4\(^+\) T cells from the DLN were purified and the expression of GAPDH, T-bet, IFN-\(\gamma\), IL-12R\(\beta2\), IL-12R\(\beta1\) and GATA-3 determined by RT-PCR as described in Materials and Methods. In each experiment, the amount of target cDNA in CD4\(^+\) T cells from PBS control mice was given a value of 1 (open histogram) and the amount of target cDNA in CD4\(^+\) T cells from treated mice was expressed as a fold increase (closed histogram). The values shown are the mean fold increase \(\pm\) SEM based on 3 separate experiments. *Indicates a statistically significant
difference with the PBS control mice (p<0.05). (B) Sera from treated (closed squares) and PBS control (open circles) mice were collected and the levels of *L. amazonensis*-specific IgG2a were determined by serial dilution as described in Materials and Methods. The data shown is the mean O.D. value ± SEM based on 4 separate experiments.

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Figure 6. BM-DC-based immunotherapy does not promote decreased parasite numbers and is still associated with impaired Ag-specific production of IFN-γ within the DLN. Naïve and *L. amazonensis* (*La*)-infected mice were injected every other day with PBS (PBS control) or *La* Ag-pulsed BM-DC +IL-12 (treated) as in Fig. 5. Three days after the last injection, the mice were sacrificed. (A) DLN cells from each treatment group were plated for 3 days in vitro in medium alone (no Ag) or in medium containing *La*Ag (+*La Ag*) or *La* Ag and IL-12 (+*La*Ag +IL-12). The levels of IFN-γ were determined in the supernatants by ELISA. The values shown are the mean ± SEM based on 5 separate experiments (3 experiments with exogenous IL-12 in the cell culture). *Indicates a statistically significant difference between the groups (p<0.05). (B) DLN cells from naïve- and infected- treated mice were plated in vitro with *La* Ag for 24h and the percentage of CD4⁺ IFN-γ producing cells was determined by flow cytometry. The data shown is representative of 2 separate experiments. (C) Splenocytes from each treatment group were plated for 3 days in vitro in medium alone (no Ag) or in medium containing *La* Ag (+*La Ag*). The levels of IFN-γ were determined in the supernatants by ELISA. The values shown are the mean ± SEM based on 5 separate experiments. *Indicates a statistically significant difference between the groups (p<0.05). (D) Infected feet from PBS control- and treated- infected mice were collected and parasite quantification was determined. The values shown are the mean ± SD based on 5 separate experiments.
Figure 7. *L. amazonensis* amastigotes limit IL-12p40 production from BM-DC. Ten day old BM-DC were infected or not with *L. amazonensis* or *L. major* amastigotes at a parasite:cell ratio of 3:1. Culture supernatants were harvested 24h later and the levels of IL-12p40 were determined by ELISA. The values shown are the mean ± SEM based on 3 separate experiments. *Indicates a statistically significant difference (p<0.05).
CHAPTER 3: Th1 CD4+ T cells induced by a DC-based vaccine do not protect against a subsequent *L. amazonensis* infection

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Yannick Vanloubbeeck a, Amanda E. Ramer b, Douglas E. Jones a,b

a Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames IA 50011-1250

b Immunobiology Program, Iowa State University, Ames IA 50011-1250

Abstract

One of the goals of vaccine strategies against leishmaniasis is to induce Th1 CD4+ T cells, which mediate resistance to the disease. However, in the present study, we show that although vaccination of naïve C3HeB/FeJ mice with *L. amazonensis* antigen-pulsed dendritic cells and IL-12 induced Th1 CD4+ T cells producing IFN-γ and expressing high levels of IL-12Rβ2 mRNA, vaccinated mice were not protected against a subsequent *L. amazonensis* challenge. Indeed, 10 weeks post-infection, vaccinated mice had similar lesion size and parasite loads compared to non-vaccinated control mice. In addition, this correlated with decreased production of IFN-γ in the in vitro recall response from the draining lymph node (DLN) and with reduced expression of IL-12Rβ2 mRNA in the CD4+ T cells from the DLN of vaccinated mice in comparison to non-vaccinated control mice. Altogether, this data indicates that the induction of Th1 CD4+ T cells by a vaccine may not be sufficient and/or indicative of subsequent protection towards a *L. amazonensis* infection.
Introduction

Leishmaniasis is a zoonotic disease caused by intracellular protozoan parasites of the genus *Leishmania* spp. Infection occurs through the bite of an infected sand fly vector that transmits flagellated *Leishmania* spp. promastigotes during its blood meal. Within host cells, promastigotes transform into non-flagellated amastigotes, which are the only parasite stage subsequently found during chronic infection (reviewed in (13)). The phenotype of the host immune response after infection is critical for the outcome of the disease and murine studies of leishmaniasis have helped determine some of the immune factors necessary for disease control. For example, while Th1 CD4+ T cells producing IFN-γ mediate resistance, Th2 CD4+ T cells producing IL-4 and/or IL-10 promote susceptibility (reviewed in (25) and (36)).

*L. amazonensis* is prevalent in the New World and the infection with this parasite usually results in localized cutaneous leishmaniasis; however, it can also cause diffuse cutaneous and mucocutaneous leishmaniasis (4, 23, 37). Furthermore it has been sporadically recovered from patients with visceral leishmaniasis (3, 4). Most inbred strains of mice are susceptible to a *L. amazonensis* infection; for example, when C3H and C57BL/6 mice are subcutaneously challenged with *L. amazonensis*, they develop chronic cutaneous lesions containing up to 10^8 parasites (1, 22, 38). The susceptibility of these mice to *L. amazonensis* is thought to result from an inability to mount an effective Th1 response, rather than from a Th2 response. Indeed, susceptibility is associated with low to undetectable levels of IFN-γ in the in vitro recall response from the draining lymph node (DLN) (1, 22). In addition, the susceptibility to *L. amazonensis* has been shown to correlate with CD4+ T cell defects, i.e. decreased expression of pro-inflammatory cytokines and chemokines (19). Furthermore, CD4+ T cells
from *L. amazonensis*-infected mice have a defective expression of IL-12Rβ2, a marker of Th1 responses (19, 22).

Although there is no vaccine available against leishmaniasis, the availability of a vaccine would represent an effective tool in the control of the disease. As a result, the design of vaccination strategies that promote safe, effective, long-lasting protection to leishmaniasis has been and still is currently investigated (reviewed (14)). In particular, variable degrees of protection against a *L. amazonensis* challenge have been achieved after vaccination with *Leishmania* spp. antigens (Ag) or DNA (6-8, 10, 31, 39). When determined, the immune response associated with protection was characterized by an increased IFN-γ production (6, 7, 10, 31, 39). In addition, more recently, the adoptive transfer of Th1 CD4+ T cells to naïve mice completely protected mice against a subsequent *L. amazonensis* promastigote challenge (19, 32). Altogether, these studies suggested that vaccine strategies inducing Th1 CD4+ T cells should likely promote protection against a subsequent infectious challenge.

Dendritic cells (DC) are the most potent antigen-presenting cells in vitro and in vivo (2). Their critical role in the priming of anti-*Leishmania* immune responses has been clearly demonstrated (24, 28, 29, 35). In addition, in experimental studies of leishmaniasis, DC have been successfully used as vaccine adjuvants to promote protective Th1 responses (5, 11, 34, 42). However, in mice chronically infected with *L. amazonensis*, we have recently shown that an immunotherapy composed of *L. amazonensis* antigen-pulsed dendritic cells and IL-12 was ineffective at promoting healing, despite the induction Th1 CD4+ T cells (41). As *L. amazonensis* amastigotes have been shown to limit Th1 responses, it is possible that the high numbers of amastigotes present during chronic *L. amazonensis* infection may have been partly responsible for the failure of the DC-based immunotherapy to promote healing (33,
Therefore, we tested the hypothesis that the Th1 response induced by the administration of *L. amazonensis* Ag-pulsed DC and IL-12 to naïve mice as a vaccine would promote protection against a subsequent *L. amazonensis* promastigote challenge.

Our data indicate that while the vaccination of naïve mice with *L. amazonensis* Ag pulsed DC and IL-12 induced Th1 CD4+ T cells in vivo, it did not promote resistance to a subsequent *L. amazonensis* promastigote infection. This lack of protection correlated with decreased production of IFN-γ in the recall response from the DLN and with a reduced expression of IL-12Rβ2 mRNA in CD4+ T cells from the DLN. Altogether, this suggests that the presence of Th1 CD4+ T cells does not necessarily correlate with protection to a *L. amazonensis* infection.

**Material and Methods**

**Mice.** C3HeB/FeJ mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and bred and maintained in a specific-pathogen-free facility. The Committee on Animal Care at Iowa State University approved all protocols involving animals. Five to eight week old C3HeB/FeJ females were inoculated with 5 x 10⁶ stationary-phase promastigotes in the left hind footpad (3 to 5 mice per group). Lesion size was monitored weekly with a dial micrometer (L.S. Starrett Co., Athol, MA), and the results were expressed as the difference between the footpad thickness for the infected foot and the footpad thickness for the uninfected foot.

**Parasites.** Culture of *L. amazonensis* promastigotes (MHOM/BR/00/LTB0016) and the preparation of parasite Ag were performed as previously described (21). For the parasite
quantification from cutaneous lesions, the infected feet were disinfected with 70% ethanol and the skin was dissected away. The remaining subcutaneous lesion was homogenized using a Tenbrock tissue homogenizer, washed twice in phosphate buffered saline (PBS) and resuspended in Grace's insect cell culture medium (Life Technologies, Grand Island, NY) supplemented with 20% heat-inactivated FBS, 2 mM glutamine, 100U/ml penicillin, and 100 µg/ml streptomycin. A ten-fold serial dilution of the parasite suspension was then performed and incubated at 24°C for 8 days before parasite quantification was assessed.

**Vaccine.** Bone marrow-derived dendritic cells (BM-DC) were cultured for 10 days *in vitro* in the presence of 10 ng/ml of murine GM-CSF (PeproTech Inc, Rocky Hill, NJ) according to a method from Lutz et al. (26). In each experiment, naïve mice were injected every other day for 12 days in their left hindfoot. Each injection consisted of 20µg *L. amazonensis* Ag, 0.2µg murine IL-12 (PeproTech Inc, Rocky Hill, NJ) and 1x10^6 BM-DC resuspended in a total of 25µl of PBS. Control mice were injected with 25µl of PBS. Three days after the last vaccine injection, some vaccinated mice were killed to determine the phenotype of the immune response induced by the vaccine and the remaining mice were infected with *L. amazonensis* as described above.

**Recall responses.** Lymph node cell recall responses were performed as previously described (21). Supernatants were harvested after 72 h, and IFN-γ and IL-4 levels were determined by ELISA. Recombinant IFN-γ (Pharmingen, San Diego, CA) and IL-4 (PeproTech Inc, Rocky Hill, NJ) were used on each ELISA plate to set up a standard curve. The lower detection limit
of the IFN-γ ELISA ranged between 39 and 78 pg/ml; the lower detection limit of the IL-4 ELISA was 39 pg/ml.

**Purification of CD4+ T cells.** Lymph node cells were incubated with anti-CD4-coupled microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol, followed by one passage through an autoMACS. The average purity of the cell preparation was 92%.

**Flow cytometry.** Surface staining for CD4 and CD44, and intracellular staining for IFN-γ were performed using commercially available antibodies (Pharmingen, San Diego, CA) as previously described (22). Live events were gated based on forward scatter versus side scatter, and the CD4+ T cells from this live gate were further analyzed for their expression of CD44 and production of IFN-γ.

**RT-PCR.** Total RNA was extracted from CD4+ T cells using the Rapid Total RNA Purification System (Marligen Biosciences, Ijamsville, MD) according to the manufacturer's protocol. RNA concentration was measured spectrophotometrically; RNA samples were then treated with 1.5 U of DNase I (Pierce, Milwaukee, WI) per 1 μg of RNA for 45 min at 37°C. Two μg of each DNase I-treated RNA sample was reverse transcribed in 80 μl reaction volumes using 1X M-MLV reaction buffer (Promega, Madison, WI), 200 U M-MLV reverse transcriptase (Promega, Madison, WI), 40 U RNase inhibitor (Applied Biosystems, Foster City, CA), 2.5 μM random hexamers (Amersham Biosciences Corp, Piscataway, NJ) and 0.5 mM of each dNTP (USB Corporation, Cleveland, OH). RNA
samples and random hexamers were incubated together at 70°C for 3 min and room
temperature for 10 min. The reverse transcription reaction was performed at 42°C for 1 hr;
samples were then heated at 95°C for 5 min and incubated on ice for 10 min. Real-time PCR
experiments were run on the ABI PRISM 5700 Sequence Detection System (Applied
Biosystems, Foster City, CA). Each 50 μl reaction contained 1 μl of cDNA and 1X
ABsolute QPCR Mix (Abgene, Surrey, UK). Forward and reverse primers were used at
either 300 or 350 nM (IDT, Coralville, IA) and TaqMan probes (labeled with the reporter dye
FAM and the quencher dye TAMRA, Applied Biosystems, Foster City, CA) were used at
either 75 or 100 nM. Primer pairs and probes were designed using Primer Express 1.5
software (Applied Biosystems, Foster City, CA). The sequences of the Forward (F) and
Reverse (R) primers for IL-12Rβ2 (Genbank accession # U64199) were as follows: F:
5’CTTCACCTCCGCATACGTTCAC3’ and R: 5’GCTCCAGAAGCATTTAGAAAGTT3’.
The sequence of the IL-12Rβ2 probe was 5’TCCCTCTTTCTCCGTGGGAC ATC A
3’. The
sequence of the GAPDH primers (Genbank accession # M32599) were F:
5’TGTGTCCGTCGTGGATCTGA3’ and R: 5’CCTGCCCAAGGCTTTGCAATG3’.
The
GAPDH probe sequence was 5’CCGCCCTGGGAGAAACCTGCCAAGTATG3’. For
quantitative analysis of gene expression, normalized to an endogenous control, we used the
standard curve method, which was detailed previously (15). Briefly, control cDNA samples
were serially diluted to obtain standard curves for GAPDH cDNA and each target cDNA.
For each assay, the resultant cycle threshold (Ct) values were plotted against the log ng input
of RNA. For all experimental samples, the cDNA quantity for the target of interest was then
determined from its standard curve and then normalized to the GAPDH control.
Statistical analysis. The statistical significance between different groups was determined by using a t-test. Differences were considered significant when p<0.05.

Results

Vaccination of naïve mice with *L. amazonensis* antigen-pulsed dendritic cells and IL-12 induces a Th1 response. Naïve female C3HeB/FeJ mice were subcutaneously injected in their left hind footpad every other day for 12 days with PBS or a vaccine composed of *L. amazonensis* Ag-pulsed BM-DC and IL-12 as described in Material and Methods. Three days after the last injection, some of the vaccinated mice were sacrificed and the cells from the popliteal DLN were plated in vitro for 72h with or without *L. amazonensis* Ag. As shown in Figure 1A, there was an antigen-specific production of IFN-γ by DLN cells in response to *L. amazonensis* Ag, consistent with a Th1 response. Furthermore, as the phenotype of the CD4+ T cells has been shown to correlate with the outcome of murine cutaneous leishmaniasis, we determined the effect of vaccination on the CD4+ T cell compartment. Although naïve and vaccinated mice both had similar percentages of CD4+ T cells in the lymph node draining the vaccinated foot, vaccinated mice had an increased percentage of CD4+ T cells expressing the memory/effector marker CD44 (Fig 1B and data not shown). In addition, vaccinated mice had higher percentages of CD4+CD44+ T cells producing IFN-γ in comparison to naïve mice (Fig 1C). Furthermore, these CD4+CD44+ T cells were the only CD4+ T cells producing IFN-γ during the in vitro re-stimulation with *L. amazonensis* Ag, further supporting their role as effector cells (Fig 1C). Finally, as another marker of Th1 response, we determined by RT-PCR the expression of IL-12Rβ2 mRNA on the CD4+ T cells from the DLN of vaccinated mice in comparison to naïve mice. As shown in Figure 1D, the vaccination with *L.
Amazonensis Ag pulsed BM-DC and IL-12 induced an increased expression of mRNA levels for IL-12Rβ2 on the CD4+ T cells from the DLN in comparison to naïve mice. Altogether, this indicates the vaccine induced a Th1 response in vivo.

The Th1 response of vaccinated mice does not confer protection to a L. amazonensis challenge. Protection to L. amazonensis in mice has been shown to correlate with increased level of IFN-γ produced in recall responses in vitro (6, 7, 10, 31, 39). In addition, the adoptive transfer of Th1 CD4+ T cells was recently shown to induce resistance to a subsequent L. amazonensis promastigote challenge (19, 32). Therefore, we hypothesized that the Th1 response induced by L. amazonensis Ag-pulsed DC and IL-12 in naïve mice would promote protection against a L. amazonensis promastigotes infection. As indicated above, naïve mice were subcutaneously vaccinated in their left hind footpad every other day for 12 days with L. amazonensis Ag-pulsed BM-DC and IL-12 or PBS as a control. Three days after the last injection, the mice were infected with 5x10^6 L. amazonensis stationary phase promastigotes in their left hind footpad and the associated lesion size was measured weekly. As shown in Figure 2A, the size of the infected foot from vaccinated mice remained stable and significantly smaller than the one from control mice for 4 to 9 weeks. However, between week 5 and week 8 post-infection, depending on the experiment, the lesion size of the infected feet from vaccinated mice slowly increased to reach the size of infected feet from control mice (Fig 2A). In addition, we determined the parasite load of the L. amazonensis-infected feet 10 weeks post-infection. As shown in Figure 2B, the infected feet of vaccinated and control mice contained similar numbers of parasites 10 weeks post-infection. Altogether,
this data indicates a failure of the vaccine to protect against a subsequent *L. amazonensis* challenge.

**Vaccine failure correlates with decreased production of IFN-γ by DLN cells and decreased expression IL-12Rβ2 by CD4*+ T cells.** Ten weeks post-*L. amazonensis* infection, the mice were sacrificed and the cells from the lymph node draining the infected feet were re-stimulated in vitro with or without *La* Ag. While the levels of IL-4 secreted in the in vitro recall response from vaccinated and control mice were low to undetectable, DLN cells from vaccinated mice produced higher levels of IFN-γ than control mice (Figure 3A). However, the absolute amounts of IFN-γ secreted by the DLN cells of vaccinated mice 10 weeks post-infection were drastically reduced in comparison to the levels produced before infection (see Fig 1A). In addition, the susceptible phenotype of vaccinated mice correlated with decreased levels of IL-12Rβ2 mRNA expression in CD4*+ T cells. Indeed, CD4*+ T cells from the DLN of vaccinated mice expressed levels of IL-12Rβ2 mRNA that were comparable to the ones of purified CD4*+ T cells from either naive or *L. amazonensis*-infected control mice, as determined by RT-PCR (Figure 3B).

**Discussion**

Our data shows that the subcutaneous vaccination of naïve mice with *L. amazonensis* Ag-pulsed dendritic cells and IL-12 does induce a Th1 response in vivo, as evidenced by the Ag-specific production of IFN-γ in the draining lymph node. In addition, within the lymph node draining the vaccinated feet, there was an expansion of effector CD4*+ T cells producing IFN-γ and expressing IL-12Rβ2 mRNA, a phenotype consistent with Th1 CD4*+ T cells. However,
the vaccine did not mediate resistance to a subsequent *L. amazonensis* promastigote infection and both vaccinated and non-vaccinated control mice had similar lesion size and parasite load 10 weeks post-infection. This lack of protection is in contrast to recent data indicating that the intravenous adoptive transfer of *L. amazonensis*-specific Th1 CD4\(^+\) T cells provided protection against a *L. amazonensis* promastigote challenge (19, 32). These discrepancies may be explained by different numbers of effector CD4\(^+\) T cells or by qualitative differences in the Th1 CD4\(^+\) T cells present before infection. In addition, the route of immunization may have had an impact on the efficiency of the protection. In particular, intravenous immunization has usually resulted in better resistance to a subsequent *Leishmania* spp. challenge (18, 27). However, resistance has also been described after subcutaneous immunization (6, 8, 12) and, in this study, the administration of the vaccine subcutaneously did skew the profile of the CD4\(^+\) T cells compartment towards a Th1 phenotype, indicating that the vaccine could influence the immune response locally in the DLN and therefore suggesting that protection may have been achieved.

The susceptibility of mice to *L. amazonensis* infection is characterized by the production of low levels of IFN-\(\gamma\) in the in vitro recall response from the DLN (1, 22) and the control of a *L. amazonensis* infection after vaccination with parasite Ag or DNA was previously shown to correlate with an increased production of IFN-\(\gamma\) by DLN cells (6, 7, 31). This suggested that enhanced IFN-\(\gamma\) levels in the recall response could be indicative of protection to a *L. amazonensis* challenge. In the present study, vaccinated mice failed to resist a *L. amazonensis* challenge despite an increased Th1 response in the DLN 10 weeks post-infection, as determined by the ratio of IFN-\(\gamma\) and IL-4 secreted in the in vitro recall response. However, the absolute amounts of IFN-\(\gamma\) detected in the in vitro re-stimulation of
DLN from vaccinated mice were significantly decreased in comparison to the ones produced before the infection. This indicates that a Th1 response, as determined by the ratio of IFN-γ to IL-4 secreted, may not be indicative of protection during a *L. amazonensis* infection.

Finally, the lack of protection after vaccination correlated with a decreased expression of the Th1 marker IL-12Rβ2 by the CD4+ T cells from the DLN. Indeed, although the vaccine initially up-regulated IL-12Rβ2 on the CD4+ T cells from the DLN, this expression decreased over the 10 week course of infection to reach basal levels. The production of IL-12 and the maintenance of IL-12 responsiveness through the expression of IL-12Rβ2 are crucial components of the immune response mediating protection to *L. major* (16, 17, 20, 30). The sustained production of IL-12 is necessary to maintain effector/memory Th1 cells (40) and the importance of a persistent source of IL-12 for a successful protection induced by previous DC-based vaccination has also been reported (34). Interestingly, there is evidence that *L. amazonensis* can limit the production of IL-12 by dendritic cells in vitro and ex vivo (22, 33, 41). Therefore, it is possible that the loss of IL-12Rβ2 by the CD4+ T cells and the ultimate failure of our vaccine to promote resistance to *L. amazonensis* infection resulted from a suboptimal source of IL-12.

In conclusion, our study shows that the Th1 response induced by a DC-based vaccine was not effective at promoting resistance to a *L. amazonensis* infection. Furthermore, it indicates that the presence of Th1 CD4+ T cells cannot be considered as a reliable indicator of protection to a subsequent *L. amazonensis* challenge. It reinforces the thought that the pathogenesis caused by members of the *L. mexicana* complex, including *L. amazonensis*, is different from the one induced by *L. major* and subsequently, that the immune factors necessary to promote resistance to one *Leishmania* sp. may not be applicable to other
Leishmania spp. (9). In addition, it is possible, as previously reported, that effector cell types other than CD4+ T cells, i.e. CD8+ T cells, are required to induce successful protection to L. amazonensis infection (10). This should be taken into consideration when designing future vaccine strategies.

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Figure 1. Vaccination with *L. amazonensis* antigen-pulsed BM-DC and IL-12 induces Th1 CD4+ T cells in vivo. Naïve C3H mice were subcutaneously vaccinated with *L. amazonensis* antigen (*La Ag*)-pulsed BM-DC and IL-12 in their left hindfoot as described in the Materials and Methods. Three days after the last vaccination, the mice were sacrificed and the popliteal draining lymph nodes (DLN) were harvested. (A): 1 x 10^6 DLN cells from vaccinated mice were plated in vitro with or without *La Ag* for 72h, and the levels of IFN-γ from the culture supernatants were determined by ELISA. The data shown is the mean ± SEM from 6 independent experiments. (B) The percentages of CD4+ T cells expressing the memory/effectort marker CD44 in the DLN from both naïve and vaccinated mice were
determined ex vivo by flow cytometry. The data shown is the mean ± SEM from 4 independent experiments. (C) DLN cells were plated with La Ag as in (A) for 24h and then surface stained for CD4 and CD44. The cells were subsequently stained for intracellular IFN-γ and analyzed by flow cytometry. Live events were gated based on forward scatter versus side scatter and the percentages of CD4+ T cells producing IFN-γ were determined (see inset quadrant). The data shown is from 1 experiment representative of 2 independent experiments. (D) The levels of IL-12Rβ2 mRNA expressed in purified CD4+ T cells from the DLN of both naïve and vaccinated mice were determined by RT-PCR as described in the Materials and Methods. The values shown represent the ratio between of IL-12Rβ2 mRNA and the housekeeping gene mRNA control GAPDH. The data shown is the mean ± SEM from 4 independent experiments. * Indicates a statistically significant difference between the groups (p<0.05).
Naïve C3H mice were subcutaneously vaccinated with La Ag-pulsed BM-DC and IL-12 in their left hindfoot as in Figure 1. (A) Three days after the last vaccination, the mice were subcutaneously infected with $5 \times 10^6$ stationary phase *L. amazonensis* promastigotes in their left hindfoot (open circles). At the time of infection, naïve C3H mice were also infected with $5 \times 10^6$ stationary phase *L. amazonensis* promastigotes as controls (closed triangles). Lesion size was monitored weekly as described in the Materials and Methods. The data presented is
from one experiment representative of 4 independent experiments; the values shown are the mean ± standard deviation. (B) Ten weeks post-infection, vaccinated and naïve mice were killed and the parasite loads from the infected feet were determined as described in the Materials and Methods. The data shown is the mean ± SEM from 4 independent experiments.
Figure 3. Vaccine failure occurred despite a Th1 cytokine profile in the DLN but decreased IL-12Rβ2 mRNA expression in purified CD4+ T cells from the DLN. C3H mice were vaccinated or not and infected with *L. amazonensis* as in Figure 2. Ten weeks post-infection, the mice were killed and the DLN harvested. (A) 1 x 10^6 DLN cells from vaccinated and
control mice were plated in vitro with *L. amazonensis* antigen for 72h, and the levels of IFN-\(\gamma\) and IL-4 within the culture supernatants were determined by ELISA. The data shown is the mean ± SEM from 4 independent experiments. * Indicates a statistically significant difference (\(p<0.05\)). (B) The levels of IL-12R\(\beta2\) mRNA expressed in purified CD4\(^+\) T cells from the DLN of naïve (naïve mice), vaccinated-infected (vaccinated mice) and *L. amazonensis*-infected control (control mice) mice were determined by RT-PCR as described in the Materials and Methods. The values shown represent the ratio between of IL-12R\(\beta2\) mRNA and the housekeeping gene mRNA control GAPDH for each group of mice. The data shown is the mean ± SEM from 3 independent experiments.
CHAPTER 4: Infection of C3HeB/FeJ mice with *Leishmania major* protects against subsequent challenge with *Leishmania amazonensis*

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Yannick Vanloubbeeck and Douglas E. Jones

Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames Iowa

Abstract

The Th1 response elicited in mice infected with *L. major* has been used as a model to characterize cellular immune defects associated with *L. amazonensis* infection. However, it is not known if the immune response associated with the infection by virulent *L. major* parasites can promote resistance to a subsequent *L. amazonensis* infection. Our data demonstrate that C3HeB/FeJ mice infected subcutaneously with virulent *L. major* are resistant to a *L. amazonensis* challenge. The healing phenotype is characterized by a Th1 response as measured by increased IFN-γ production and low levels of IL-4 in the draining lymph node. Altogether, this indicates that the Th1 response associated with *L. major* infection can promote resistance to *L. amazonensis* infection and that it can be used as a tool to study the immune defects associated with *L. amazonensis* infection.

Introduction

Leishmaniasis is a zoonotic disease caused by protozoa of the genus *Leishmania*, which are transmitted to the host through the bite of an infected sandfly. The disease is characterized by a variety of clinical manifestations, depending on the *Leishmania* species
involved and the type of immune response mounted by the host.\textsuperscript{1,2} *Leishmania major* and *Leishmania amazonensis* are both associated with the cutaneous form of the disease: the lesions induced by *L. major* are typically localized and self-healing while the ones caused by *L. amazonensis* can become diffuse and chronic, sometimes affecting mucous membranes.\textsuperscript{1,3} Mouse models of leishmaniasis have helped to uncover some of the immune factors involved with resistance and susceptibility to the disease. For example, while C3H and C57BL/6 mice are resistant to *L. major*, they are susceptible to *L. amazonensis*. Resistance and susceptibility to *L. major* are mediated by Th1 and Th2 immune responses, respectively.\textsuperscript{4} The susceptibility to *L. amazonensis* is thought to result from an inability to mount a Th1 response.\textsuperscript{5–9} In particular, it has been shown that the CD4\textsuperscript{+} T cells from *L. amazonensis*-infected mice have defective expression of several cytokine and chemokine receptors in comparison to the CD4\textsuperscript{+} T cells from *L. major*-infected mice.\textsuperscript{6,9} However, while the immune response to *L. major* infection is used as a model in this experimental system of murine cutaneous leishmaniasis, it is not known if the Th1 response elicited by *L. major in vivo* would provide protection to a subsequent *L. amazonensis* infection. In fact, it has been reported that even after exogenous administration of IL-12 or in the absence IL-10 or IL-4, mice still develop chronic cutaneous lesions upon *L. amazonensis* infection.\textsuperscript{6,7,9,10} However, in mouse studies using *L. major*, the administration of IL-12 or anti-IL-4 or the absence of IL-10 was associated with disease control.\textsuperscript{11–14} Therefore, it is possible that the immune response necessary to promote resistance to *L. amazonensis* may not be the same as that for *L. major*.

Cross-protection between different *Leishmania* species using either *Leishmania* antigens, attenuated parasites or live *Leishmania* promastigotes have been previously described in mice,\textsuperscript{15–30} monkeys,\textsuperscript{31–33} and humans.\textsuperscript{32,34} However, the effectiveness of cross-protection
reported in the literature is variable and several studies have reported its failure, depending on the experimental design and/or the *Leishmania* spp. involved. Furthermore, the cross-protection induced in mice by a previous *Leishmania* infection does not necessarily correlate with complete healing. In contrast, it is sometimes associated with the persistence of a smaller lesion or even a slower progressing lesion than the control mice. Finally, the route of immunization appears to have an influence on the subsequent protection, and subcutaneous immunization has provided variable degrees of resistance. In particular, one mouse study has reported efficient protection to *L. amazonensis* after a subcutaneous infection with an avirulent strain of *L. major*. However, the study did not characterize the associated immune response nor was parasite quantification determined.

Our data indicate that the Th1 response developed by C3HeB/FeJ mice after infection with wild-type *L. major* promastigotes does promote resistance to a *L. amazonensis* challenge, as determined by the development of a transient lesion and decreased parasite load. To our knowledge, the present report is the first to determine the effectiveness of cross-protection using an infectious challenge composed of as much as $5 \times 10^6$ *L. amazonensis* promastigotes and using the subcutaneous route of immunization. Altogether, our data also confirms that the immune response associated with *L. major* infection is a good model for the study of the immune defects associated with *L. amazonensis* infection.

**Materials and Methods**

**Parasites.** Culture of *L. amazonensis* (MHOM/BR/00/LTB0016) or *L. major* (MHOM/IL/80/Friedlin) parasites and the preparation of parasite Ag were performed as previously described. Parasite quantification was determined when the mice had healed their
L. amazonensis infection, i.e. 10 to 12 weeks post-L. amazonensis challenge. For the parasite quantification from cutaneous lesions, the infected feet were disinfected with 70% ethanol and the skin was dissected away. The remaining subcutaneous lesion was homogenized using a Tenbrock tissue homogenizer, washed twice in PBS and resuspended in 2 ml of Grace's insect cell culture medium (Life Technologies, Grand Island, NY) supplemented with 20% heat-inactivated FBS, 2 mM glutamine, 100U/ml penicillin, and 100 µg/ml streptomycin. A ten-fold serial dilution of each parasite suspension was then performed in triplicate and incubated at 27°C for 8 days before parasite quantification was assessed. The lower detection limit of our quantification assay was $10^2$ parasites per foot, and when parasite numbers were lower than the detection limit a value of 10 was given to the sample.

Mice. C3HeB/FeJ mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and bred in a specific-pathogen-free facility. The Committee on Animal Care at Iowa State University approved all protocols involving animals. Five to eight week old mice (3 to 6 mice per group) were inoculated subcutaneously with $5 \times 10^6$ stationary-phase L. major promastigotes in their left hindfoot. Eleven to fifteen weeks later, when mice have healed their primary L. major infection, the mice were infected subcutaneously with $5 \times 10^6$ stationary-phase L. amazonensis promastigotes in their right hind footpad. Lesion size of the footpads was monitored weekly with a dial micrometer (L.S. Starrett Co., Athol, MA), and the results were expressed as the difference between the thickness of the right and left hind footpads. After infection with L. amazonensis, the thickness of the left feet (those that had healed a previous L. major infection) remained stable.
Recall responses. The cells from the popliteal lymph node draining the *L. amazonensis*-infected feet were obtained at 10 to 12 weeks after *L. amazonensis* infection and recall responses were performed as previously described. The cells were incubated with medium containing 50 μg of *L. amazonensis* antigen/ml with or without 1ng/ml of recombinant murine IL-12 (PeproTech Inc, Rocky Hill, NJ). Supernatants were harvested after 72 h, and IFN-γ and IL-4 levels were determined by ELISA. Recombinant IFN-γ (Pharmingen, San Diego, CA) and IL-4 (PeproTech Inc, Rocky Hill, NJ) were used on each ELISA plate to set up a standard curve. The sensitivity of the IFN-γ ELISA ranged between 39 and 78 pg/ml; the sensitivity of the IL-4 ELISA was 39 pg/ml. All ELISA were performed with commercially available antibodies (Pharmingen, San Diego, CA), peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) and ABTS microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) following the manufacturer instructions.

Results

Mice infected with *L. major* are resistant to a subsequent *L. amazonensis* infection. C3HeB/FeJ mice were infected subcutaneously in their left hind footpad with 5 x 10⁶ *L. major* stationary phase promastigotes. The resulting lesion consisted of a discrete cutaneous nodule that occasionally ulcerated and consistently healed over the following 11 to 15 weeks (data not shown). At these timepoints, the mice were re-infected subcutaneously with 5 x 10⁶ *L. amazonensis* stationary phase promastigotes in their right hind footpad and the associated lesion size was measured weekly. As a control, naïve mice were also infected with 5 x 10⁶ *L. amazonensis* stationary phase promastigotes in their right hindfoot. As shown in Figure 1,
control mice infected with *L. amazonensis* developed chronic lesions, whereas in mice previously infected with *L. major*, the *L. amazonensis* lesion was transient and healed in 10 to 12 weeks. The chronic lesion of *L. amazonensis*-infected mice contained an average of $10^7$ parasites (Fig. 2). In contrast, the healing response of mice infected with *L. major* and subsequently with *L. amazonensis* was associated with a significant decrease in parasite load (Fig. 2).

**Resistance to *L. amazonensis* infection is associated with an enhanced Th1 response within the draining lymph node.** In each experiment the mice were sacrificed at ten to twelve weeks after *L. amazonensis* infection, a time at which the associated cutaneous lesion had resolved. The cells from the draining lymph node were cultured in a 3 day recall response with or without *L. amazonensis* antigens and the levels of IL-4 and IFN-γ were determined in the culture supernatants. While lymph node cells from both groups of mice produced low to undetectable amounts of IL-4, mice sequentially infected with *L. major* and *L. amazonensis* produced significantly higher levels of IFN-γ than the *L. amazonensis*-infected control mice (Fig. 3A). The levels of IFN-γ detected were similar whether *L. amazonensis* or *L. major* antigens were present during the 3 day recall response (4.5 ± 1.0 ng/ml and 5.9 ± 1.5 ng/ml with *L. amazonensis* and *L. major* antigens, respectively). To further determine the phenotype of the healing immune response, we also determined the IL-12 responsiveness of the cells from the draining lymph node by measuring the levels of IFN-γ produced *in vitro* in response to *L. amazonensis* antigen and recombinant murine IL-12. IL-12 responsiveness is a marker of Th1 responses and as shown in Figure 3B, draining lymph node cells from mice sequentially infected with *L. major* and *L. amazonensis* produced
significantly more IFN-γ than the *L. amazonensis*-infected control in response to IL-12.

Altogether, these data indicate that the healing phenotype observed in co-infected mice is associated with an increased Th1 response at the level of the draining lymph node.

**Discussion**

The Th1 immune response elicited after *L. major* infection has been used as a model to describe T cell defects associated with *L. amazonensis* infection. However, whether the immune response of *L. major*-infected mice represents an appropriate model to study *L. amazonensis* infection, i.e. if the Th1 response of mice infected with virulent *L. major* would promote resistance to a subsequent *L. amazonensis* infection, is not known. Our data indicate that mice previously infected with virulent *L. major* were able to control a *L. amazonensis* infection, characterized by lesion resolution and a decreased parasite load (Fig. 1 & 2). The healing phenotype was associated with a Th1 response as determined by low levels of IL-4 and high levels of IFN-γ in the *in vitro* recall response from the draining lymph node (Fig. 3A). This is consistent with previous studies in which the vaccination of mice with parasite antigen or DNA, with or without an adjuvant, was shown to promote resistance to *L. amazonensis* infection. When determined, the healing response following vaccination correlated with increased production of IFN-γ in the draining lymph node, the spleen or at the lesion site. Furthermore, the adoptive transfer of *L. amazonensis*-specific Th1 cells into C3H mice was recently shown to promote resistance to subsequent *L. amazonensis* infection. Altogether, this demonstrates that resistance to *L. amazonensis* infection can be associated with a Th1 response. IL-12 responsiveness is another marker of Th1 responses that was reported to correlate with the potential of mice to heal *Leishmania*
infection. Therefore, the increased IL-12 responsiveness of DLN cells from mice with *L. major* and subsequently with *L. amazonensis* further supports the Th1 phenotype of their healing response (Fig. 3B).

Previous studies have shown that the exogenous administration of the Th1 cytokines IL-12 or IFN-γ was not able to promote resistance to *L. amazonensis*. In addition, the neutralization or the absence of IL-4 or IL-10, respectively could not restore resistance to *L. amazonensis*. Altogether, this suggests that these cytokines are not, by themselves, altering the course of *L. amazonensis* infection. Rather, resistance may require an array of immune factors that the *L. major*-induced immune response, as a whole, can provide. This would also suggest that some elements of a parasite-specific immune response may be needed prior the *L. amazonensis* challenge in order to provide protection. Our data confirm that the immune factors necessary to promote resistance to *L. amazonensis* are present after a *L. major* infection. Finally, our data also support a previous study suggesting that subcutaneous vaccination may be effective in promoting resistance to *L. amazonensis* infection, even in an experimental system using a high dose challenge.

In conclusion, our data show that the Th1 response of C3HeB/FeJ mice infected with *L. major* is able to promote protection to a subsequent *L. amazonensis* challenge. Although leishmanization has been currently abandoned, this co-infection model confirms that the immune response developed after *L. major* infection is a good model to study the defects associated with *L. amazonensis* infection. This model will allow us to better understand and define the immune factors necessary or sufficient to promote resistance to *L. amazonensis*. 
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References


Figure 1. Previous *L. major* infection provides protection to a subsequent *L. amazonensis* infectious challenge. C3HeB/FeJ mice were infected with $5 \times 10^6$ *L. major* stationary phase promastigotes in the left hind footpad. When the mice had healed their *L. major* infection, they were re-infected with $5 \times 10^6$ stationary phase *L. amazonensis* promastigotes in their right hind footpad, as described in the Material and Methods. The lesion size of the *L. amazonensis*-infected feet was measured over time after the re-infection. Mice sequentially infected with *L. major* and then *L. amazonensis* (*Lm - La*) developed transient lesions in comparison to *L. amazonensis*-infected control mice (*La* control). The values shown are the mean ± standard deviation from 1 experiment (5 mice per group) representative of 5 independent experiments.
Figure 2. The healing response of mice sequentially infected with *L. major* and then *L. amazonensis* (*Lm*-*La*) correlated with a significant decrease in parasite load. The number of parasites within the *L. amazonensis*-infected feet of *Lm*-*La* mice or *L. amazonensis*-infected controls (*La control*) was determined 10 to 12 weeks after *L. amazonensis* infection. * Indicates a significant statistical difference (p<0.05, t-test). The values shown are pooled from 5 independent experiments and expressed as the mean ± SEM.
Figure 3. The cytokine profile of the draining lymph node (DLN) of mice sequentially infected with *L. major* and *L. amazonensis* (*Lm - La*) is consistent with a Th1 response. Mice were infected as described in Material and Methods. Ten to twelve weeks post *L. amazonensis* infection, DLN cells were plated *in vitro* with *L. amazonensis* antigens with or without IL-12 for 3 days and the total amounts of IFN-γ (closed histograms) and IL-4 (open histograms) were measured in the culture supernatants. A: Both *L. amazonensis*-infected
control mice (La control) and Lm – La mice produced low to undetectable levels of IL-4. However, Lm – La mice produced significantly more IFN-γ than control mice. * Indicates a significant statistical difference (p<0.05, t-test). B: When IL-12 is added to the *in vitro* cell cultures, DLN cells from Lm – La mice produced more IFN-γ than La control mice. The values shown are pooled from 5 independent experiments and expressed as the mean ± SEM.
CHAPTER 5: General Conclusions

In experimental studies of *L. major*, the protective role of Th1 CD4⁺ T cells is widely recognized. In mice infected with *L. amazonensis*, the impaired Th1 response has been shown to correlate with CD4⁺ T cells defects such as an impaired expression of IL-12Rβ2. The intravenous adoptive transfer of Th1 CD4⁺ T cells was also recently shown to promote protection against a subsequent *L. amazonensis* promastigote, but not amastigote, challenge.

We have shown here evidence, under 2 different experimental designs, i.e. immunotherapy and vaccine, that the presence of Th1 CD4⁺ T cells is not sufficient to mediate protection to *L. amazonensis*. In our immunotherapy studies, the high numbers of amastigotes present during the chronic *L. amazonensis* infection may have limited the effectiveness of the Th1 CD4⁺ T cells induced by the DC-based treatment. Indeed, *L. amazonensis* amastigotes have been shown to impair DC responses, including the production of the Th1 cytokine IL-12. As a result, although the CD4⁺ T cells of mice that received the immunotherapy did upregulate their expression of the Th1 marker IL-12Rβ2, it is possible that the potentially protective immune response was now limited by the lack of available IL-12. Our data would support this hypothesis and would confirm the thought that during a *L. amazonensis* infection, not only the T cell response is impaired but also the APC response. If both T cells and DC are defective, it would be interesting to understand which defect occurred first. In fact, it is tempting to hypothesize that during the priming of T cell responses, the DC exert some imprinting function on the naïve T cells. As a result, this would suggest that if some functional pathways are defective in the DC, the T cells interacting with those particular defective DC will subsequently be defective in those same pathways. In fact, our immunotherapy studies could support such hypothesis. Indeed, the DC-based
immunotherapy contained appropriate APC that could effectively prime T cells toward a Th1 phenotype. However, the injected DC did not likely persist very long in the draining lymph node and T cells subsequently had to interact with the defective, pre-existing host DC again. This could explain why the efficiency of the T cell response remained limited. Future studies will be needed to better understand what the APC defects are and how they could be overcome. Finally, despite the failure to promote protection, the fact that our DC-based immunotherapy was able to modulate and polarize the immune response of mice with established chronic cutaneous lesions provides hope for future therapeutic strategies aimed at modulating the immune response, either in this infectious model or in chronic diseases caused by other intra-cellular pathogens.

Our vaccine studies were designed to test if the failure of the immunotherapy was mainly mediated by the high parasite loads present during chronic infection. Our hypothesis was that the Th1 response elicited by Ag-pulsed BM-DC and IL-12 as a vaccine instead of an immunotherapy would promote protection against a subsequent *L. amazonensis* challenge. However, to our surprise, the Th1 CD4$^+$ T cells induced by the vaccine did not confer resistance to a *L. amazonensis* infection. This is in contrast to a study in which the intravenous adoptive transfer of *L. amazonensis*-specific Th1 CD4$^+$ T cell lines promoted protection against a subsequent promastigote challenge. Several hypotheses may account for these discrepancies. First, it is possible that the DC-based vaccine from our study did not prime a central memory response as effectively as the adoptive transfer of CD4$^+$ T cells. This seems unlikely as the subcutaneous injection of Ag-pulsed BM-DC and IL-12 did induce a *L. amazonensis* Ag-specific Th1 response in the spleen. Second, the numbers of Th1 CD4$^+$ T cells induced by our vaccine was probably lower than the high numbers that were adoptively
transferred i.v. Finally, qualitative differences between the Th1 CD4+ T cells from the vaccine and the adoptive transfer may explain the different disease outcome observed. In particular, in our experimental system, the expression of IL-12Rβ2 may not be as indicative of an efficient Th1 response as in the mouse models of *L. major* infection. In addition, the qualitative differences in the Th1 CD4+ T cells may account for differences in other cell subsets. In particular, it has been recently shown that adequate CD8+ T cell responses required the presence of efficient CD4+ T cells. Therefore, it is possible that in our system, the Th1 CD4+ T cells resulting from vaccination did not stimulate adequate CD8+ T cell responses. In conclusion, a more in depth characterization of the CD4+ T cells induced by the vaccine is needed. In addition, it would be useful to compare them to Th1 CD4+ T cells that can mediate protection to a *L. amazonensis* infection.

As a result, we thought that it would be useful to design an experimental system (different from the artificial intravenous transfer of high numbers of Ag-specific cells) in which mice could resist a *L. amazonensis* challenge. In particular, as the immune response elicited by *L. major* is used as a model to characterize the immune defects associated with a *L. amazonensis* infection, it was of interest to determine if mice infected with *L. major* could resist a *L. amazonensis* challenge. As shown in our third study, mice that have healed a *L. major* infection can heal a subsequent *L. amazonensis* infection. This confirms that it is appropriate to use the Th1 response associated with *L. major* infection to study the defective response of *L. amazonensis*-infected mice. In addition, this model provides a useful tool to determine which immune factors are necessary to promote resistance to *L. amazonensis*. In particular, we are currently undertaking experiments aimed at determining which cell
subset(s) from the *L. major*-infected mice can promote protection to a subsequent *L. amazonensis* challenge.

In conclusion, our data challenge dogma and indicate that the presence of Th1 CD4$^+$ T cells is not always indicative of protection to a *L. amazonensis* infection. It further supports the thought that the immune response necessary to heal a *L. amazonensis* infection may not have the same requirements as the one that mediates protection to a *L. major* challenge. In addition, our model of sequential infection with *L. amazonensis* and *L. major* will help us determine the immune factors that can mediate protection to *L. amazonensis* infection.
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