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Cutaneous *Leishmania* infection: progress in pathogenesis research and experimental therapy

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Abstract: Studies in murine experimental *Leishmania major* infection have helped to understand the requirements for efficient development of T helper (Th)1/cytotoxic T (Tc)1-mediated protection against the parasite. As such they have revealed that Fcγ receptor (FcγR)I and FcγRIII-mediated uptake of *L. major* amastigotes by dendritic cells (DC) is an important prerequisite for Th1 development. In addition, DC-derived cytokines contribute to adequate T-cell education. DC-based vaccines may thus provide an important tool for both the development of a prophylactic vaccine against leishmaniasis and – together with leishmanicidal drugs – for eliciting immune-deviating functions towards protective immunity in non-healing leishmaniasis. This review highlights recent advances in the understanding of the role of DC for the induction of Th1/Tc1-predominant immunity against *L. major* and how this knowledge may translate into clinical approaches.

Key words: dendritic cells – Leishmaniasis – Th1/Th2 – vaccine

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Introduction

Leishmaniasis is a parasitic disease transmitted by the bite of a sand fly. Together with *Trypanosoma* spp., protozoan *Leishmania* spp. belong to the family of trypanosomatidae. Worldwide, ~12 million people are infected with *Leishmania* and ~2 million new infections are reported annually. Approximately 350 million individuals live at risk of infection every day. In the past decade, the number of cases in endemic areas has increased sharply. Additionally, due to co-infections with HIV, leishmaniasis is spreading to several non-endemic areas of the world (e.g. Southern-Europe). Altogether, people in more than 88 tropical/subtropical countries (16 developed and 72 developing countries) are affected by *Leishmania* infections (1–4).

Approximately 90% of all *Leishmania* infections are localized cutaneous forms (5). But even infection with dermatotropic forms of the *Leishmania* complex (e.g. *L. major*, *L. mexicana*, *L. tropica*) can lead to recurrent, multilocular, chronic or mucocutaneous disease. Very often, healing is associated with the development of disfiguring scars at the exposed skin areas.

Treatment options against *Leishmania* infections are limited to a few drugs with inconsistent efficacy and many side effects: pentavalent antimonials (sodium stibogluconate, meglumine antimoniate), second-line pentamidine, amphotericine B (also formulated as liposome), allupurinol and ketoconazole. In addition, oral miltefosine with fewer side effects has recently been introduced, which appears to be efficient against visceral and cutaneous leishmaniasis (6–8). Local treatment includes paromomycin-containing ointments. In a number of cases, failure of treatment, relapses or more severe side effects are seen. A vaccine against *Leishmania* infection is not available at present.

Immunity against *L. major* infection

Infection with *Leishmania* parasites leads to lifelong immunity against the same subspecies, after the infection is healed. To understand why the development of a vaccine has proven to be difficult, studies exploring the anti-*Leishmania* immune response of humans and mice have been helpful. Infections have been initiated by experimental injection of *L. major*. Most studies have been carried out in mice. To induce experimental leishmaniasis, generally supra-physiologic high doses of *L. major* (2×10^5–2×10^7 stationary-phase promastigotes) have been injected subcutaneously into footpads of various strains of mice. Recently, to more closely mimic natural transmission by sand flies, some variations in the experimental model have been introduced. These include (i) inoculation of only 10–1000 parasites, (ii) the use of metacyclic promastigote preparations of *L. major*, the life form found in the salivary glands of sand flies, and (iii) intradermal, instead of subcutaneous,

inoculation imitating the bite of the sand fly (Fig. 1). This 'low-dose' model reproduced critical observations made previously with standard high-dose inoculation, but also allowed identification of additional factors relevant for resulting immune responses (9–11).

Interestingly, current paradigms of T helper (Th)-subset involvement in infectious diseases are based, in large part, on the results of studies of resistance and susceptibility to *L. major* in inbred mice (Fig. 2). In murine leishmaniasis, BALB/c mice respond to infection with preferential production of Th2-type cytokines, in particular, interleukin (IL)-4 and IL-10, which are associated with disease progression and susceptibility. In contrast, recovery from infection of resistant (e.g. C57BL/6) mice depends on the induction of a polarized Th1-type response, resulting in macrophage (MΦ) activation and killing of the parasites (2).

However, the regulation of immunity against *Leishmania* infection appears to be much more complex. Progress in current pathogenesis research has significantly contributed to a better understanding of protective immunity against *L. major* and most likely will form the basis for the development of an efficacious vaccine.

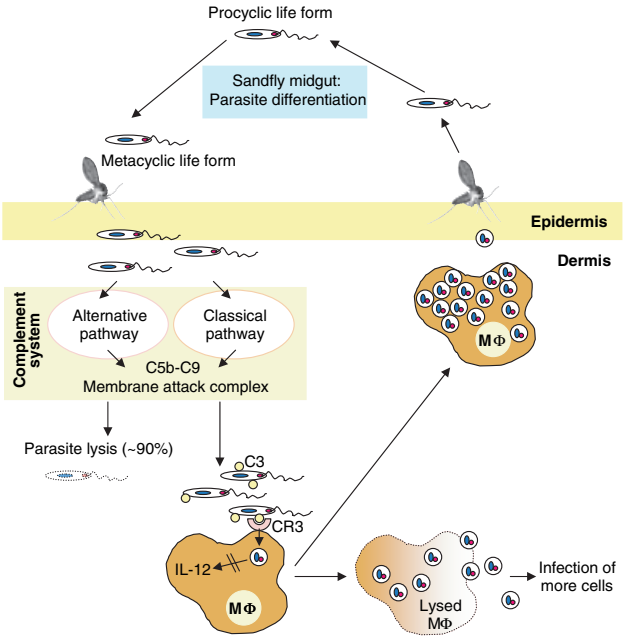

Figure 1. Life cycle of *L. major*. After inoculation of metacyclic promastigotes of *L. major* into the skin by the bite of a sand fly, parasites activate complement and are partially lysed. Surviving parasites utilize C3 to invade macrophage (MΦ) host cells via CR3, and – within these cells – they transform into obligate intracellular amastigotes. MΦ infection does not lead to cell activation (e.g. IL-12 release). Inside of MΦ, parasites replicate and more amastigotes are released into the tissue. The life cycle of *L. major* is completed upon uptake of parasites by another bite of a sand fly.

Inefficient complement lysis of *L. major*

After inoculation of *L. major* into the dermis, the promastigotes interact with serum components. Among our most efficient defense mechanisms of the innate immune system, the complement system helps to clear pathogens from the organism by disrupting the target cell's plasma membrane. Metacyclic promastigotes of *Leishmania* have been shown to activate complement in both the classical and the alternative pathways (12). Opsonization of *Leishmania* metacyclic promastigotes with complement is rapid and lysis via the membrane attack complex (C5b–C9 complex) begins 60 s after serum contact (12). This results in efficient killing of ~90% of all inoculated parasites within 3 min.

Interestingly, *Leishmania* parasites have evolved to resist and circumvent full complement lysis by several mechanisms (13,14): first, when compared with procyclic *L. major* promastigotes, metacyclic promastigotes are more resistant to complement lysis. Intracellular amastigotes are the least sensitive to lysis. This is mediated by a membrane alteration during development that prevents the insertion of the C5b–C9 complex into the parasites' outer membrane (12,15). Second, *Leishmania* parasites are able to express protein kinases that phosphorylate C3, C5 and C9, which leads to inhibition of complement. Finally, two major parasite surface molecules, LPG and gp63, mediate binding of C3bi to the parasite surface. However, elongated forms of the proteins also contribute significantly to complement resistance as they impede complement-mediated lysis.

Complement activation results in binding of C3bi (among other complement factors) to the surface of the parasite, a process called 'opsonization'. *Leishmania* parasites smartly use this opsonization to escape from the hostile environment by promoting phagocytosis via complement receptors (CR). First, C3bi-coated parasites bind to CR1 on eythrocytes (immune adherence) (12). Most likely, this allows for a limited distribution of the parasites in the tissue and further. Upon encounter of MΦ, parasites bind to CR3 on their surface which facilitates uptake of the parasites into their major host cell (Fig. 1). Within these phagocytes, *Leishmania* then transform into the obligate intracellular life form, the amastigote.

Establishment of the infection

Apart from the parasite's usage of the complement system to be opsonized and to efficiently 'hide' in host cells, complement components such as C3a and C5a serve as potent chemoattractants for other cells. Along gradients of C3a and C5a and several chemokines [e.g. macrophage inflammatory protein (MIP)-1α, MIP-2], inflammatory cells such as monocytes/MΦ and neutrophils migrate to the site of parasite invasion in the skin (16,17).

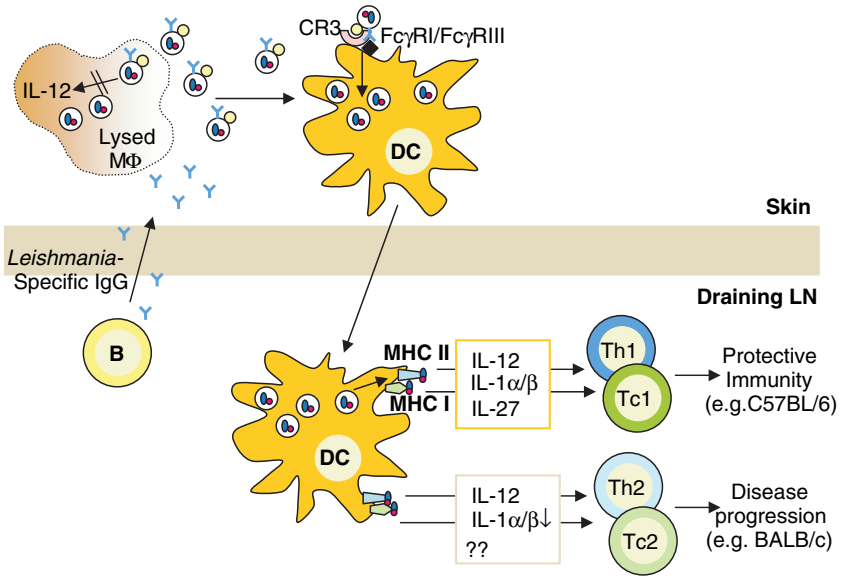

Figure 2. Fcγ receptor-mediated uptake of *L. major* by dendritic cells (DC) induces protective immunity. Free tissue amastigotes bind anti-*Leishmania* IgG released by antigen-specific B cells into the serum of *L. major*-infected mice. Dendritic cells phagocytose *L. major* via FcγRI and FcγRIII. Infection leads to DC activation and migration to draining lymph nodes. FcγR-engagement results in antigen presentation in both the MHC class I context to $CD8^+$ T cells and MHC class II-mediated activation of $CD4^+$ T cells. Cytokine release from DC (most importantly IL-12) induces Th1/Tc1 priming and mediates development of protective immunity. In some stains of mice, genetic differences in the release of Th1-inducing cytokines (e.g. less IL-1) contribute to inefficient Th1 induction, thus, Th2 immunity and disease progression is observed.

Even though MΦ represent potent antigen-presenting cells (APC) of the organism, infection of MΦ via CR3 is a silent process. Parasite infection of MΦ does not lead to cell activation. More importantly, the parasite actively prevents activation of MΦ as demonstrated by the inhibition of cytokine release (e.g. IL-12) from otherwise stimulated infected cells (18–21). Within MΦ, the parasites now replicate until the infected cell is lysed and more surrounding cells are infected (9). Thus, during this stage, the infection is established without knowledge of the immune system.

Another important immune evasion strategy of *Leishmania* that allows for the establishment of the infection is by entering granulocytes (22). Even though MΦ are the final host cells for the parasite, neutrophils are among the first leucocytes infected and upon ingestion of *L. major*, they secrete high levels of MIP-1β, which attracts MΦ. MΦ, in turn, readily phagocytose infected neutrophils, resulting in release of the anti-inflammatory cytokine tumor growth factor-β. Thus, *Leishmania* use neutrophils to silently enter their final host cell.

Induction of protective immunity

Dendritic cells (DC) are APC pivotal in orchestrating T cell immunity and tolerance (23,24) and they are positioned at potential pathogen entry sites such as the skin. Prior data showed that infected DC are the critical APC responsible for T cell priming in *Leishmania* infections (20,25–34). As to which DC subtype contributes to the induction of protective immunity (epidermal Langerhans cells, dermal DC or lymph node resident DC) is still a matter of debate.

Interestingly, DC phagocytose mainly the amastigote life form, but not infectious stage promastigotes (20). We have recently found that DC take up *L. major* via a different receptor than MΦ (11) (Fig. 2). MΦ utilize CR3 for parasite phagocytosis. In contrast, DC acquire the parasite through Fcγ receptor (FcγR)I and FcγRIII-mediated uptake of amastigotes. Thus, in mice without B cells or functional FcγR, decreased numbers of infected lesional $CD11c^+$ DC were found (11). This resulted in worsened disease outcome and significantly larger lesion sizes. Consistent with this, infection of DC is observed later post-infection, when *Leishmania*-specific B cells (producing anti-*Leishmania* antibodies) are present (11). These antibodies are then bound to the parasite surface and allow for recognition of the pathogen by DC.

Interestingly, lesion involution and healing start right after infected DC are found in *Leishmania* lesions (9). This confirms that infected DC directly contribute to the development of protection by two ways (Fig. 2): First, infection of DC with *L. major* results in activation of the cells associated with upregulation of major histocompatibility complex (MHC) class I and II expression as well as costimulatory molecules and migration to the draining lymph node (20,25), leading to efficient T cell priming. Second, cytokines released by DC are important for Th1 differentiation. Th1 induction against *L. major* is critically dependent on IL-12 (35). Although IL-12 was initially detected in MΦ, recent data indicate that DC are the primary source of IL-12 in leishmaniasis (20,36). IL-12 and related cytokines (e.g. IL-27) released by infected DC induce Th1-mediated protection (1,20,25–27,37). Finally, factors other than IL-12, e.g. IL-1α/β, that also contribute significantly to efficient Th1 induction have been identified (1,38–40).

T cell-dependent protection against *L. major*

In human and experimental cutaneous leishmaniasis, development of protective immunity is dependent on the generation of interferon (IFN)-γ-producing T cells. IFN-γ activates infected MΦ to eliminate the parasites via production of nitric oxide (NO) (2,41). Th2 cytokines, in contrast, induce arginase 1 in so-called 'alternatively activated' MΦ. Inhibition of arginase 1 improved disease outcome by efficiently controlling parasite replication (42). In addition, MΦ/neutrophils unresponsive to IL-4/IL-13 triggering show reduced arginase activity and improved leishmanicidal effector functions (43). Therefore, the balance of NO-producing, classically and arginase 1-expressing alternatively activated MΦ regulates disease outcome.

Healing in cutaneous leishmaniasis is thus critically dependent on the generation of *Leishmania*-specific $CD4^+$ Th1 cells. Recent data suggested that IFN-γ release by $CD8^+$ *L. major*-specific T cells, so called cytotoxic T (Tc) cells type 1, also promotes the development of protective immunity (Fig. 2) (10,44,45). Interestingly, the role of CD8 T cells for the establishment of protection against *L. major* became most prominent when physiological low-dose inocula were used for experimental infection (10,45). As low-dose infection more closely mimics natural infections, both CD4 and CD8 T cells are thus required to mediate full protection.

To be effective in mediating parasite clearance, T cells have to migrate to lesional skin in *Leishmania*-infected organisms. Recently, some insight has been gained into the factors that contribute to tissue-selective generation and homing of T cells. $CD4^+$ and $CD8^+$ T cells in contact hypersensitivity or infectious diseases have been shown to preferentially localize to the skin by expressing cutaneous lymphocyte-associated antigen (CLA), E- and P-selectin ligand, CCR4, CCR8, CCR10 and CTACK, but not $\alpha4\beta7$ (46–51). $CD4^+$ T cells homing to intestinal lymphoid organs display other (in part reciprocal) expression of these adhesion molecules (47). Thus, during an infection the local microenvironment within the skin directs chemokine and adhesion receptor expression by T cells, targeting the resulting effector T cells to the inflamed skin.

How do antigen-specific T cells learn about the site of infection, so that they express the 'right' chemokine receptors and migrate to the appropriate organ? Intracutaneous injection of DC induced skin-homing $CD8^+$ T cells with upregulated E-selectin ligand expression and effector function in contact hypersensitivity (52). In contrast, intraperitoneal injection of antigen-bearing DC induced T cells expressing the gut-homing integrin $\alpha4\beta7$. In addition, DC from different tissues (skin, gut) induced the corresponding homing markers on T cells *in vitro* (52). These data suggest that only antigen-presentation via the skin route leads to the efficient generation of skin-homing $CD4^+$ and $CD8^+$ T cells. The local microenvironment within the skin thus directs both expression of chemokines and endothelial adhesion molecules, resulting in *L. major*-reactive effector T cells that direct their own recruitment to the skin by inducing sustained endothelial expression of E-selectin (53,54). Therefore, it appears that skin DC, for example, are specialized to target T cells into inflamed skin in various skin diseases, e.g. contact hypersensitivity and infections with *Leishmania* spp. or HSV-1 (20,31,52,55,56).

Memory responses dependent on parasite persistence?

In lymph nodes of long-term infected mice that have recovered from infection with *L. major*, both MΦ and DC harboured viable parasites. However, only DC were able to induce a vigorous T cell immune response to *L. major in vitro*. Thus, *L. major*-infected DC in lymph nodes [incapable of expressing inducible NO synthase to eliminate the parasite (57)] represent long-term host cells, which suggests that parasite persistence supports the maintenance of T cell memory (58). In addition, IL-12 had to be continuously present to maintain efficient protective immunity (59). The source of this IL-12 at later stages post-infection is still not known, but infected DC are a likely source. Thus, mice deficient in IL-12, IFNγ or NO are extremely susceptible to infection (2,9).

Interestingly, in IL-$10^{-/-}$ mice, complete elimination of the parasites and sterile healing of lymph nodes is observed. This was associated with a loss of protective memory responses (60). Thus, long-term protection from re-infection appeared to be associated with persistence of residual parasites in secondary lymphoid organs of immune animals. However, a recent study suggested that the $CD4^+$ T cells responsible for protective immunity against *L. major* include two populations: parasite-dependent T effector cells and parasite-independent central memory T cells. The existence of a long-lived population of central memory T cells did not require the continued presence of live parasites (61). Upon re-contact with *L. major*, these cells expand and promote efficient protection. Thus, protein-based vaccines instead of vaccination with life or attenuated *Leishmania* became a feasible strategy for the development of an efficacious vaccine.

Tregs as mediators of immunity

Interleukin-10 in chronic cutaneous leishmaniasis is produced by regulatory T cells (Treg) which serve to limit the magnitude of effector responses and thus are responsible for the failure to adequately control infection (62). Already

very early after parasite inoculation, Treg rapidly accumulate at the site of infection. Thus, removal of $CD4^+CD25^+$ Treg from infected mice enhances the capacity of mice to mount effective immunity early on and control parasite numbers. Transfer of Treg to chronically infected mice stimulates local parasite growth and reactivation of disease (63,64). Interestingly, intradermal vaccination with life *L. major* into ears induced delayed effector cell recruitment due to a rapid influx of IL-10-producing $CD4^+CD25^+$ T cells that prevented parasite clearance (63). Subcutaneous vaccination was not associated with a similar recruitment of Treg to the vaccination site thus leading to better vaccination results.

Experimental treatment utilizing DC and prevention strategies

Considering that maximally 100 parasites are inoculated during natural transmission by the sand fly and that 90% of the inoculated parasites are immediately killed by the complement system, the chances for parasite survival and establishment of an infection are only slight (12). The success rate of infection is not known, most inoculations of *Leishmania* parasites may be aborted early on. Substances that would enhance complement function and/or inhibit parasite resistance to complement lysis would be interesting tools for infection prevention.

Resistance to *Leishmania* infections is mediated by T cells. As *Leishmania*-specific T cells provide life-long immunity against reinfection with the same species, deliberate infection of infants on the buttocks was encouraged (preventing skin lesions in the face later on). This process was called 'leishmanization' (6,7). However, the development of a recombinant vaccine that results in similar protection without the risks that are associated with a viable pathogen would be desirable. Usage of antigens expressed by several *Leishmania* species or a combination of antigens may also provide protection against mixed infections. These mixed infections with different *Leishmania* strains have shown to be frequent and might represent a special problem with regard to treatment response rates (65,66).

As described above, several groups have demonstrated that infected DC are the most potent inducers of protective immunity. Using viable parasites for DC-based vaccination in humans is problematic, thus, antigen-pulsed DC have been utilized to induce long-lasting protection (26,27,67,68). DC pulsed with parasite lysate (26,27,64,68) or recombinant parasitic proteins (67) have been tested. The use of adjuvants such as CpG motifs may be useful to further promote the antigen-presenting and IL-12-releasing capacity of DC used for vaccination (68).

Due to the laborious and expensive task of isolating DC from humans, DC-based vaccinations can only serve as proof-of-principle experiments. The translation into protein-based vaccines involving an *in vivo* activation of skin DC would be desirable. Here, to achieve induction of skin-homing effector T cells, activation of 'skin DC' appears critical. Several approaches have been undertaken to apply antigen intradermally and/or subcutaneously together with adjuvant (CpG, IL-12, etc.) (64,69–72). These have shown promising results. Interestingly, DC activation via adjuvant has proven to be responsible for the vaccination efficacy (73), and CpG-mediated DC activation reduced the number of *Leishmania*-specific Tregs in an IL-6-dependent fashion (64). Future studies will have to exploit these vaccination strategies in detail to improve their success and to test the capacity to induce long-lasting immunity.

Finally, when the patient presents to the doctor with obvious *Leishmania* skin lesions, treatment options are limited as described above. Apart from eliminating the parasite by anti-parasitic drugs, immune deviation protocols that shift prevailing Th2 immunity to Th1-mediated protection may facilitate healing. Even though in human infections with *L. major*, the T cell response may not follow a clear Th1/Th2 profile, once a Th2 immune response has developed, experimental studies showed that it is difficult to achieve a shift towards an IFN-γ-dominated immunity at all. Some approaches have been successful if the parasite burden of an infected individual was reduced together with the application of Th1-inducing IL-12 or CpG oligonucleotides (74–76). These encouraging data may represent a strategy how the 'classical' anti-parasite drugs combined with modern immune-modulating agents, e.g. CpG motifs or imiquimod (77), may provide effective therapies.

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