TEGUMENTARY LEISHMANIASIS: IMMUNOLOGY AND MOLECULAR BIOLOGY

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The past few years have produced remarkable advances in our understanding of the skin immune response. Some better understandings of T-cell trafficking, the emerging role of Dendritic cells (DCs) and antimicrobial peptides in innate immunity, and the re-emergence of the suppressor cell as the T-regulatory cell, are changing our understanding of the immunopathology in a wide variety of cutaneous diseases and opening new opportunities to manipulate systemic immune responses through the skin. Our group has demonstrated the participation of epidermal DCs in both human and murine cutaneous leishmaniasis, and have shown that most of the tissue-damage observed in tegumentary leishmaniasis is caused by the immune response a not by the parasite. Tissue-damage that is promoted by inadequate epidermal signals directed by DCs. We have also demonstrated increased numbers of IL-10+ Langerhans cells in the lesions of patients with intermediate cutaneous leishmaniasis as compared with localized cutaneous leishmaniasis, and have shown that the adoptive transfer of splenic DCs confers protection to *Leishmania*-infection in a neonatal BALB/c model of the disease. The antimicrobial peptides are mechanisms of the innate immunity that contribute to the host defense. LL-37 is a peptide derived from the human cathelicidin CAP18 predominately expressed on epithelial tissue during inflammation. Besides its antibacterial function, LL-37 has a role as an inflammatory mediator that have not been studied in leishmaniasis. In this study, we evaluated the leishmanicidal activity of LL-37, its effect on the infection with *L. mexicana* promastigotes and murine bone marrow derived DCs, and the lymphoproliferative response against the parasite. The results suggest that LL-37 is a multifunctional regulator of the innate and adaptive immune response against *L. mexicana*, having a leishmanicidal activity, increasing phagocytosis on DCs and macrophages, and acting as activator or suppressor of the adaptive immune response depending on the concentration. In order to understand the immunoregulatory mechanisms that are activated after the resolution of a skin lesion in leishmaniasis, we used a single homotypic stressor just before the inoculation of *Leishmania* parasites in our susceptible and resistant mouse models of *L. mexicana* infection. The stressor consisted of immobilization by placement, for 2 or 8 hours, in restraining cages. The results suggest that stress by immobilization may modulate the genetically predetermined immune response to Leishmania parasite. In addition and perhaps more significant, it indicates that animals manage the same acute and homotypic stress in a different manner, some exacerbating their lesions and others by healing them on the basis of their genetic legacy. Studies by David Sacks' group indicate that nTregs are essential for the development and maintenance of the persistent cutaneous infection, and reactivation of the infections caused by the *Leishmania* parasite.

Key words: Leishmaniasis, molecular biology, immunoregulation, *Leishmania.*

Leishmaniasis is becoming a serious public health problem throughout the World due to the HIV/AIDS pandemic, new transmission routes, and parasite drug-resistance and virulence[1] Immunosuppression by HIV has increased the numbers of silent subclinical infections and it is estimated that 1.5-9.0% of HIV-positive subjects will develop visceral leishmaniasis in southern Europe[2] Infection with HIV/AIDS may increase the risk of developing leishmaniasis by 100-1,000-fold[3]. Other transmission routes for *Leishmania* such as sharing syringes among intravenous drug users may have a significant impact on the epidemiology and pathogenesis of this disease[4]. The incidence of HIV-*Leishmania* co-infections has emphasized the important role played by the immune response against the parasite[5].

Cutaneous leishmaniasis (CL) is caused by different species of *Leishmania* in the Old and the New Worlds[6]. From an evolutionary point of view, the Genus *Leishmania* is very diverse, with dermotropic organisms causing cutaneous disease in the Old World (*L. major*) and New World (*Leishmania mexicana*/ *Leishmania amazonensis*) and viscerotropic organisms (*Leishmania donovani*, *Leishmania chagasi*, and *Leishmania infantum*) causing visceral leishmaniasis (VL)[7,8]. Consequently, this parasite diversity has given rise to different host–parasite interactions with distinct virulence factors and marked differences in the immune mechanisms that mediate susceptibility/resistance to infection and the immunopathology associated with the disease. The study of these variations is of paramount importance to comprehend the host-parasite relationship, necessary for the development of new therapeutic schemes and possible vaccines.

In the New World, CL presents active three forms in humans, which embrace a clinical, histological and immune spectrum with different grades of severity. The resistant form includes immunoresponder individuals with localized cutaneous leishmaniasis (LCL), who develop a Th1 immune response associated with an effective delayed hypersensitivity reaction (Figures 1,2). The susceptible form includes nonresponder individuals with diffuse cutaneous leishmaniasis (DCL), characterized by a Th2 immune response associated with the production of nonprotective antibodies[9]. Mucocutaneous leishmaniasis (MCL) and chronic or intermediate cutaneous leishmaniasis (ICL) are in the middle

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of the spectrum; both are characterized by an exacerbated cell-mediated immunity with a mixed pattern of cytokines[9,10]. Previous studies by our group determine the Th1/Th2 paradigm in human American cutaneous leishmaniasis (ACL)[9] and characterize the cytokine pattern in patients with the atypical intermediate CL[10].

In mice, depending on the mouse strain, the *Leishmania* strain and the number of inoculated parasites, it has been possible to reproduce the distinct clinical forms observed in humans. Thus, syngeneic strains C57BL / 6, CBA, C3H / He and AKR are resistant to *Leishmania major* and *Leishmania mexicana* infection and develop skin lesions that spontaneously resolve, similar to LCL. In contrast, BALB/c, DBA/2 and A/Jax are susceptible and develop chronic and progressive lesions similar to DCL[6]. The Th1 / Th2 concept has been shown to be legitimate in the mouse model of CL[11, 12]. We have established the only reproducible mouse model of *L. mexicana* infection available that gives straightforward Th1 and Th2 responses in C57/BL6 mice and BALB/c mice, respectively, similar to that observed with *L. major* infection[13, 15].

The past few years have produced remarkable advances in our understanding of the skin immune response. Some better understandings of T-cell trafficking[16], the emerging role of antimicrobial peptides in innate immunity,[17, 18] and the re-emergence of the suppressor cell as the T-regulatory cell[19,20] are changing our understanding of the immunopathology in a wide variety of cutaneous diseases and opening new opportunities to manipulate systemic immune responses through the skin.

Dendritic cells (DCs) are in the center of these advances, and the cutaneous DCs are the Langerhans cells, which are a key component of the skin immune system[21, 22]. Classically, epithelial-derived DCs, such as epidermal Langerhans cells, have been defined by their ability to: i) Take up and process antigens in peripheral tissues where they reside, ii) Migrate and transport those antigens from periphery to secondary lymphoid organs and iii) Present antigens to naïve T cells converting them into memory T cells[23]. Thus, epithelial-derived DCs are suited to perform two distinct functions at two discrete locations. In the peripheral tissue, these cells act as professional phagocytes or sentinels for 'dangerous' antigens, including *Leishmania* parasites. Once they migrate into the regional secondary lymphoid organ, they act as antigen-presenting cell, activating T cells that are specific for these antigens. During their migration, DCs shift from an antigen-capturing mode to a T cell sensitizing mode. In addition to switching on the immune response, subtypes of DCs appear to influence the character of T cell differentiation, i.e. the Th1/Th2 balance[23, 24].

Our group has demonstrated the participation of epidermal Langerhans cells in both human and murine cutaneous leishmaniasis[25, 26]. and have shown that most of the tissue-damage observed in tegumentary leishmaniasis is caused by the immune response and not by the parasite. Tissue-damage that is promoted by inadequate epidermal signals directed by DCs[10, 27, 29]. In addition, we have demonstrated increased numbers of IL-10+ Langerhans cells in the lesions of patients with intermediate CL as compared with LCL[10], and have shown that the adoptive transfer of splenic DCs confers protection to *Leishmania*-infection in a neonatal BALB/c model of the disease[30] (Figure 3).

New evidence regarding the origin, function, and remarkable plasticity of DCs are changing the classical role of DCs in many infectious and cutaneous diseases[31, 32]. Basically, in mice epithelia-derived DCs include Langerhans cells and interstitial DCs, whereas blood-derived DCs comprise the so-called classical DCs (CD4+CD205-, CD8+CD205+ or CD4-CD8-) and plasmacytoid DCs CD45RA+[31] Recently, Henri *et al.* has shown: i) A differential *in vitro* susceptibility to infection with *L. major* among DC subtypes[33] ii) Increased numbers of plasmacytoid DCs in the lymph nodes of *L. major*-infected BALB/c mice; and iii) Although plasmacytoid DCs showed a smaller parasite load than Langerhans cells, they suffered a long-lasting infection and are capable of producing alpha-interferon. These results suggest an important role for plasmacytoid DCs in leishmaniasis.

In humans, studies of DC heterogeneity have been extremely difficult due to the unavailability of lymphoid tissue. Indeed, most of our knowledge on human DCs comes from studies of DCs purified from blood, or from *in vitro* cultured models[23]. So far, human blood contains two major DC subsets: plasmacytoid DCs recognized by a high expression of IL-3R and little CD11c, and a second population that expresses high levels of CD11c and is probably the equivalent of murine blood-derived CD11b+. In view of that, the study on human lymph node DCs has been focused on the analysis of epidermal DC immigrants. These can be obtained from epidermal-dermal explants, which represents a unique and irreplaceable model that allows the interception of DCs on their way to skin draining lymph nodes[34].

Another topic of interest is the area of specialized contact between two immune cells, termed the immunological synapse. Initially this concept only referred to the junction between antigen-presenting cells (APCs) and T cells, specifically to sustain T cell receptor (TCR) interaction with MHC-peptide complexes. It was first described in terms of directed secretion of cytokines between APCs and T cells,[35, 36] but later the formation of a geometrically patterned collection of receptors and ligands was discovered[37, 39]. The prototypical immunological synapse is considered for being organized around a c-SMAC (central supramolecular activation cluster) containing a key molecule such as the TCR, surrounded by a p-SMAC (peripheral SMAC)[40]. The underlying molecular mechanism by which proteins are arranged at the immunological synapse also involves spontaneous processes and cytoskeleton-driven mechanisms. This supramolecular organization of proteins has been observed in synapses involving various types of immune cells, including T cells, natural killer (NK) cells, B cells and dendritic cells. However,

between different cell types, there are many differences in the specific organization of the proteins at the junction[39, 40]. Despite the physiological importance of the immunological synapse formed between DCs and T cells, very little is known, except in a few studies where the existence of DC-T synapses in the absence of exogenous antigens has been demonstrated[41, 42]. More recently, a multifocal structure of the synapse formed between mature DCs and naïve T cells has been demonstrated, contrasting with concentric structure observed between macrophages and T cells[43].

Role of the antimicrobial peptide LL-37 against *Leishmania*.

Our results suggest that the antimicrobial peptide LL-37, a human cathelicidin, have a multifactorial effect on the immune response against *Leishmania mexicana*, with a leishmanicide activity at high concentrations (10-20 μM) and a modulatory role over the up-take of parasites by macrophages and DCs at low concentrations (1-5 μM).

Previous works have demonstrated leishmanicide activity of other natural antibacterial peptides as dermaseptin[44], cecropin A-melittin hybrid peptide[45], and indolicidine[46]. In recent studies, we have demonstrated the leishmanicide activity of human cathelicidin LL-37, characterized by morphological alterations of acidocalcisomes and mitochondrial crests as observed by electron microscopy.

LL-37 acts in the initial phases of parasite up-take by macrophages and DCs, increasing the numbers of internalized parasites at 2 hours of infection in both cell types. These results confirm previous observations that myeloid DCs can be infected by few *L. mexicana* promastigotes[47]. Moreover, *L. amazonensis* promastigotes can reproduce inside DCs[48]. It has been established that DCs internalize *Leishmania* parasites in a differential manner depending on the species and life cycle phase[49].

When we treated parasites with LL-37 before the *in vitro* infection, they were taken-up more efficiently by DCs and macrophages, manifested by increasing numbers of parasites per cell as early as 2h of infection. A possible explanation is that the LL-37 peptide may act as an opsonin on the parasite. Opsonization plays an important role in the internalization of *Leishmania mexicana* by macrophages[50]. Opsonins can bind to antigens helping their ingestion by phagocytic cells, but opsonin receptors are necessary. It has been established that LL-37 can bind to formyl-peptide receptor-like 1 (FPRL1) present on human neutrophyls and monocytes[51].

We also observed that LL-37 modulates in dose-dependent mode the proliferation of T cells after the exposure of murine monocytes to *L. (L.) mexican*a antigens. A low dose of LL-37 (1 μM) increase proliferation in response to *L. mexicana* antigens, whereas higher concentrations (2-5 μM) inhibit T-cell division.

Genetical studies on macrophages have shown that LL-37regulates the expression of chemokines and chemokine-receptors genes[52]. Moreover, LL-37 acts on the macrophages, blocking LPS-dependent activation and cytokine production[53]. In addition, it has been demonstrated that LL-37 inhibits the activation of DCs by TLR ligands, decreasing the concentrations of released inflammatory cytokines and expression of activation molecules, such as MHC-II, CD83, CD86 and CCR7[54].

The chemotactic and immunoregulatory activity of LL-37 on human phagocytes is driven by activation signalling pathways that allow the phosphorilation of MAP kinases, ERK1/2 and p38, through the FPRL-1 receptor coupled to G proteins[55].

Acute stress modulates the immune response in *Leishmania*-infected mice

In order to understand the immunoregulatory mechanisms that are activated after the resolution of a skin lesion in leishmaniasis, we used a single homotypic stressor just before the inoculation of *Leishmania* parasites in our mouse models of *L. mexicana* infection. The stressor consisted of immobilization by placement, for 2 or 8 hours, in restraining cages.

Our results showed that immobilization stress affected the course of *L. mexicana* infection and epidermal DCs (Langerhans cells) in susceptible BALB/c mice. In mice stressed for 8 hr and infected, lesions appeared earlier that in the groups of animals stressed for 2 hr and infected or non-stressed and infected animals[56].

In the same study, acute immobilization stress caused an increase in CGRP innervation and but we also observed that the single inoculation of *L. mexicana* induced a decrease of this peptide. Substance P remained unalterable after infection. The expression of Calcitonin gene related peptide (CGRP) was more evident in the first week after inoculation in mice stressed for 8 hr and infected than in those stressed for 2 hr and infected and or non-stressed and infected animals, coinciding with the observations of Kawaguchi *et al.*[57] using a murine model of delayed-type hypersensitivity. CGRP was not observed in *L. mexicana*-infected mice on weeks 4 and 8 after infection. Ahmed *et al.*[58] observed a significant decrease of CGRP in susceptible BALB/c mice as compared to resistant C57BL/6 mice, from the very first week of infection with *L. major*. This difference in CGRP concentration among mouse strains, may contribute to the susceptibility or resistance to *Leishmania* infection, and explain the nociceptive alterations observed in this illness[59]. CGRP may stimulate the migration of macrophages induced by *L. major*[60], and paradoxically, may inhibit some immunostimulatory functions of Langerhans cells[61]. Overall, a stressor such as immobilization aggravates the susceptibility of BALB/c mice to infection by *Leishmania* spp. In this microenvironment, CGRP may favour the migration of macrophages, which by the action of steroids have a diminished phagocytic capacity, triggering a rapid but not very effective inflammatory response against the parasite. Substance P was observed in the periphery of the granuloma and in the vascular endothelia of *L. mexicana*-infected mice, indicating its possible participation in the inflammatory process.

Figure 1. Interferon-gamma positive cells in the granuloma of a patient with Localised cutaneous leishmaniasis. Avidin-biotin immunoperoxidase.

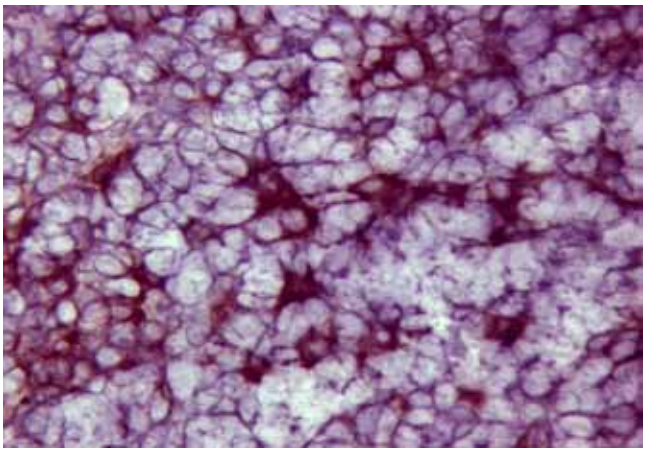


Figure 2. Activated epidermal dendritic cells expressing CD83 in the lesion of a patient with Localised cutaneous leishmaniasis. Avidin-biotin immunoperoxidase.

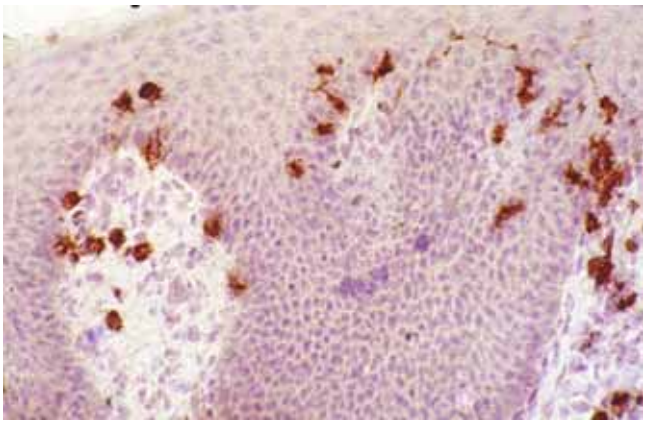
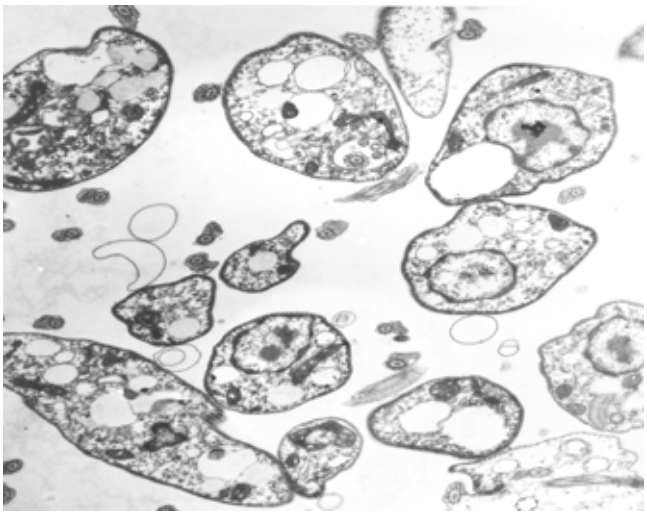


Figure 3. *Leishmania mexicana* promastigotes treated with 100mg of LL-37 for 1 hour. Transmission electron microscopy.



Similar experiments in resistant C57BL/6 mice showed an early appearance and fast decrease of the lesion in the group of mice subjected to 8hr stress, contrasting with that observed in susceptible BALB/c mice infected with *L. mexicana*[62]. Stressed C57BL/6 mice showed an increase in Langerhans cell density and CGRP expression, which was marked in the 8hr stressed group. In these animals, acute and homotypic stress induces an adaptive response capable of better controlling the infection[63].

Altogether the results suggest that stress by immobilization may modulate the genetically predetermined immune response to *Leishmania* parasite. In addition and perhaps more significant, it indicates that animals manage the same acute and homotypic stress in a different manner, some exacerbating their lesions and others by healing them on the basis of their genetic legacy.

Regulatory T cells in cutaneous Leishmaniasis

The study of regulatory T cells (Tregs) in infectious diseases has acquired much interest in the last years due to their capacity to limit the magnitude of the effector immune response (Th1/Th2) that is triggered through recognition of pathogen antigens during the course of infection, causing failures in the adaptive control of infections[64, 66]. These lymphocytes have been grouped into natural (nTregs) or induced regulator T cells based on their development, antigenic specificity, mechanisms of action, dependency of TCR and signals of costimulation for their activation[67, 68].

The long-term persistence of pathogen antigens in the host after a clinical cure is what provides resistance to reinfection (concomitant immunity) and is a flagrant characteristic of chronic diseases such as tuberculosis, toxoplasmosis, herpes virus infection and leishmaniasis[69, 72]. Different studies have focussed on the mechanisms involved in latency and chronicity of infections. In leishmaniasis, the role of nTregs in persistence and immunity to *L. major* was evaluated by Belkaid *et al.*[71], using resistant C57BL6 mice injected intradermally with low doses of *L. major* promastigotes. Natural regulatory T cells rapidly accumulate in the dermis, where they suppress, by both interleukin-10-dependent and interleukin-10-independent mechanisms, the ability of CD4+CD25- effector T cells to eliminate the parasite from the site. During *L. amazonensis* infection, a high percentage of endogenous CD4+CD25+CD86+ regulatory T cells, as well as high levels of FoxP3, TGF-beta1, and IL-10RI transcripts, were detected in the skin and draining lymph nodes, indicating local accumulation of Tregs[72].

Natural regulatory T cells also controls the early production of IL-4 in response to *L. major* that leads to the maturation of Th2 cells and the development of disease in susceptible BALB/c mice[73].

Traffic and retention of nTregs to the sites where regulation is required involve integrins and chemokines[74, 76]. Suffia *et al.*[77] evaluated the expression of the CD103 (alpha E beta 7 integrin) in dermis and draining lymph nodes during the course

of *L.major* infection in susceptible BALB/c and resistant C57BL/6 mice, showing that CD103 is differentially expressed by nTregs and CD4+CD25- T cells in both resistant and susceptible mice. In steady-state conditions dermal nTregs expresses CD103, whereas only 48% of CD4+CD25- T cells expressed the integrin. In addition, in resistant mice during the acute phase of infection, when accumulation of dermal nTregs ceases[77], the expression of CD103 decreases, whereas in the chronic phase this integrin increases. Moreover, the percentage of CD4+CD25- T cells that express CD103 decreases during the course of the infection.

Another study showed that chemokines may guide nTreg cell homing in sites of infection and show that CD4+CD25+ nTreg cells, compared with normal CD4+ T cells, preferently express the CCR5 chemokine receptor, which enables them to migrate in response to CCR5 ligands *in vitro*. CCR5 directs the homing of CD4+CD25+ nTreg cells to *L. major*-infected dermal sites where they promote the establishment of infection and long-term survival of the parasite in the immune host[78].

Mendez *et al.*[79] showed that the reactivation of an infection by a mechanical, enviromental or antigenic trigger is associatted with an influx of Tregs, usually relsted to immunosuppression. Their results showed that depletion of CD25+ T cells at the time of secondary challenge prevented disease reactivation at the site of persistent infection while strengthening the expression of immunity at the site of secondary challenge. In addition, they showed that the adoptive transfer of T reg cells obtained from infected mice into chronically infected mice was sufficient to trigger disease reactivation and prevent the expression of an effector memory response. These results may explain the Koebner phenomenon[80] that refers to skin lesions appearing on lines of mechanical trauma, observed in some cutaneous diseases such as psoriasis, lichen planus and leishmaniasis.

Altogether these results indicate that nTregs are essential for the development and maintenance of the persistent cutaneous infection, and reactivation of the infections caused by the *Leishmania* parasite.

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