How basic research have contributed to the knowledge of tegumentaty leishmaniasis

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INTRODUCTION

Leishmaniasis is caused by different species of *Leishmania* that result in distinct cutaneous, mucosal and visceral forms of the disease. In the last 30 years great advances in the knowledge about the pathogenesis of leishmaniasis have been observed. The majority of the contributions in this field had as focus the role of the host immune response in protection or pathology, but the interest is growing regarding the role of host genetics as well as parasite and vector factors in determine the different clinical forms of the disease. Before 1980 very little was known about basic research in leishmaniasis. The identification that IFN-γ produced by CD4 T cells was the major mechanisms of *Leishmania* killing was one of highly relevant observations for the understanding of the disease[1,2]. It is known that cytotoxicity mediated by T cells can also kill parasite-infected cells[3,4] but functional studies as well as data correlating cytotoxicity mediated by CD8 T cells and clinical expression of the disease are lacking. In the last years there has been an increasing interest about innate immunity in the control of Leishmania infection but, very little has been shown about innate immunity in human leishmaniasis, although studies have been performed in experimental models. In this review we show the major advances regarding the role of host, parasite and vectors in the pathogenesis of tegumentary leishmaniasis, how these studies have contributed to control the disease and how experiments performed in the laboratory have been translated to attenuate the clinical manifestations of tegumentary leishmaniasis.

EARLY EVENTS IN *LEISHMANIA BRAZILIENSIS* INFECTION

Early after infection *Leishmania* interact with different cell types of innate immune system including, neutrophils, macrophages and dendritic cells (DC). Mononuclear phagocytes constitute the main cell type harboring *Leishmania* and consequently, killing of *Leishmania* parasites depend upon activation of these cells. Activation of mononuclear phagocytes consists in up-regulation of HLA-DR and co-stimulatory molecules expression, and production of pro-inflammatory cytokines (IL-12 and TNF-α) and nitric oxide (NO). IFN-gamma produced by T and NK cells is known to be the main cytokine responsible for activation of mononuclear phagocytes for *Leishmania* killing. However, despite the enhanced production IFN-gamma observed in CL and ML patients due to *L. braziliensis* infection, a few parasites persist causing disease[5,6].

The researches attempting to study the interactions between *Leishmania* with monocytes, macrophages and DCs have identified many ways these parasites use to evade immune responses, including interference with antigen presentation, cytokine production, cell migration and NO synthesis[7-9]. Despite the knowledge gained regarding the interaction *Leishmania*/mononuclear phagocytes, important questions remain unanswered. One example is the *Leishmania* killing mechanism used by human phagocytes. While in mice it has been documented an important role for NO synthesis in *Leishmania* killing process, in human it is still debatable[10-12]. An analysis using different *Leishmania* isolates of CL patients from an endemic area, identified some isolates that were resistant to NO, which positively correlated with lesion size[13]. This suggests that, in the case NO production is not the main mechanism used by human macrophage to kill *Leishmania*, it may contribute to control disease severity. Another way *Leishmania* uses to avoid immune response is by decreasing the ability of mononuclear phagocytes to become activated and present antigen. Studies have shown that *L. braziliensis* infection of macrophages lead to protesome-mediated degradation of STAT-1, production of TGF-beta and impairment in adhesion to extracellular matrix[14-16]. Interestingly, inhibition of phagocytes function by *Leishmania* is quite contradictory with the exaggerated immune response observed in CL and ML patients. It is important to notice that most of the studies addressing *Leishmania* infection and phagocyte function were not done using a single cell basis analysis. Our recent data using mouse DCs and carboxyfluorescein succinimidyl ester (CFSE)-labeled parasites reveled that DCs that were not infected (bystander) became activated and were better antigen presenting cells than the *Leishmania*-infected ones, which appeared immature[17]. Similarly, in an *in-vivo* study where mouse model were infected with a transgenic parasite it was found that bystander DCs were activated[18]. Considering that these studies was performed in experimental model, and in lesion of CL and ML patients most of the phagocytes are found are not infected, it is important to determine the role of bystander cells in these patients.

While *in-vitro* studies have shown that, in presence of IFN-gamma, macrophages are able to kill *L. braziliensis*, clearly *in-vivo*, other unknown factors may contribute to the persistence of the parasites and disease expression, once cells from CL and ML patients produce high amounts of IFN-gamma and cannot completely clear parasitemia[19,20].

NK cells contribute to immunity as the first line of defense in numerous infectious by early cytokine secretion and cytotoxicity[21]. Studies to determine the role of NK cells in *Leishmania* infection have been developed mainly in experimental models infected with *L. major*, *L. infantum* and *L. braziliensis*[22]. These observations described an important role for NK cells in the early phase of *Leishmania* infection as they produce IFN-γ and assist in directing the immune response towards type 1, which is essential for successful control of the parasites. Liese et al showed that in *L. braziliensis*-infected IL-12p35$^{-/-}$ or IL-12p40$^{-/-}$ mice, the early NK cell response to *L. major* or *L. infantum* was absent, which clearly demonstrates that IL-12 is essential for triggering NK cells activity *in-vivo*[23].

In human CL lesions, we found a heterogeneous but usually strong expression of TIA-1, a marker of cytotoxic granules of T and NK cells, suggesting that cytotoxic activity occurs *in-situ* and that both NK cells and activated CD8+ T cells are involved in this process[24]. Interestingly, studies using co-cultures of human mononuclear phagocytes infected with *Leishmania* and NK cells, reveled that while *L. major* promastigotes do not activate NK cells to produce IFN-γ, other *Leishmania* spp. were potent inducers of IFN-γ secretion by NK cells[25].

THE ROLE OF MACROPHAGES AND T CELLS IN THE PATHOGENESIS OF CUTANEOUS AND MUCOSAL LEISHMANIASIS

The observation made by Mosman and Coffman[26] about the heterogenicity of CD4 T cell population brought a great advance in the knowledge of host parasite relationship. After the observation that CD4 Th1 cells were responsible for the cell mediated immunity through the secretion of IL-2, IFN-γ and TNF-α and Th2 cells secreted predominantly IL-4, IL-5, IL-10 and IL-3, studies in experimental models of *Leishmania* showed that while activation of Th1 cells were associated with control of parasite infection, a predominant activation of CD4 Th cells was associated with susceptibility and parasite dissemination[27]. In humans, it was also observed that, in the absence of lymphocyte proliferation and production of IFN-γ, children infected with *L. chagasi* progress to visceral leishmaniasis[28]. Absence of Th1 activation in patients infected *L. amazonensis* is also associated with parasite growth and dissemination of the disease as observed in diffuse cutaneous leishmaniasis[29, 30]. However, the Th1 x Th2 paradigm does not explain the pathogenesis of clinical forms in *L. braziliensis* infection. In contrast with the observation of a down modulation of a Th1 immune response observed in visceral leishmaniasis and diffuse cutaneous leishmaniasis, patients infected with *L. braziliensis* produce high amounts of pro-inflammatory cytokines (**Figure 1**). This predominant activation of Th1 cells is observed in all the clinical forms of *L. braziliensis* infection: cutaneous leishmaniasis, mucosal leishmaniasis, disseminated leishmaniasis and sub-clinical *L. braziliensis* infection. Interestingly, subjects who live in endemic area and control *L. braziliensis* infection (sub-clinical *L. braziliensis* infection) produce lower IFN-γ and TNF-α than patients with cutaneous, disseminated and mucosal leishmaniasis. In diseases caused by *L. braziliensis* in addition to the immune response do not eradicate parasites from the host, the inflammatory response participates of the pathology. Evidence of the role of the immune response in ulcer development in tegumentary leishmaniasis include: 1) intense inflammatory response in tissue in the absence or paucity of parasites[6]; 2) exacerbation of the immune response is associated with severity of the disease[5, 19]; 3) correlation between the frequency of cells expressing TNF-α and IFN-γ and lesion size[31]; 4) correlation between the frequency of cells expressing T cell activation markers and lesion size[31]; 5) evidences that a granulomatous vasculitis and expression of mediators of cytotoxicity precedes the appearance of the ulcerated lesions[24]. The mechanisms of the lesion development in cutaneous and mucosal leishmaniasis is not completely understood but the following sequence of events are more likely to explain disease development in *L. braziliensis* infection: 1) despite of the exaggerated Th1 immune response individuals are not free of parasite; 2) increasing in number of activated T cells sensitized to leishmania antigen; 3) lack of modulation of the immune response due to decreased efficacy of IL-10, TGF-β and regulatory T cells to control T cell activation[5]; 4) occurrence of tissue damage secondary to the exaggerated inflammatory response[32]. Although several molecules are involved in trigger tissue destruction in cutaneous and mucosal leishmaniasis, emphasis has been given to the role of TNF-α and cytotoxicity in tissue damage associated with the immune response. 1) TNF-α and TNF-α receptor are highly expressed in tissue of CL and ML patients[33]; 2) TNF-α induce NO production and stimulate apoptosis[34-36]; 3) Molecules associated with cytotoxicity, such as granzyme and TIA are highly expressed in tissue[24]; 4) Drugs that down modulate TNF-α production accelerate healing of cutaneous and mucosal ulcers and cure patients refractory to antimony therapy[37].

In contrast to the wide knowledge of T cell response in human leishmaniasis, very little is known about macrophage behavior in human *Leishmania* infection. Macrophages are preferentially infected by *Leishmania* and serve both as a site of parasite multiplication and

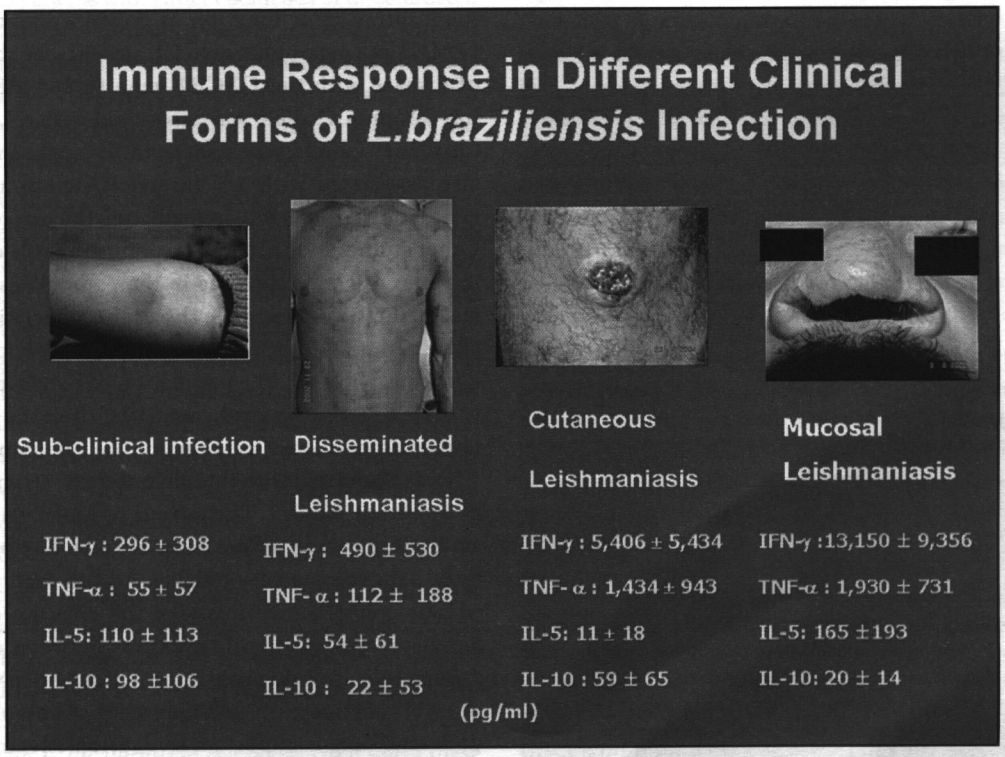

FIGURE 1 - Immune response in different clinical forms of *L. Braziliensis* infection.

are also involved in *Leishmania* killing. In addition to present antigens to T cells, macrophages have an important role in T cell activation and secretion of cytokines involved in the inflammatory response. As TNF-α play a pivotal role in the inflammatory response observed in cutaneous leishmaniasis, and macrophages are the main cells that produce this cytokine in human leishmaniasis[5, 38] it's important to investigate the role of these cells in the inflammatory response in cutaneous leishmaniasis.

In human blood two monocyte populations can be distinguished. The classical monocytes, which are strongly positive for the CD14 cell surface molecule (CD14++CD16-) and another populations of monocytes, which co-express CD16 (CD14+ CD16++). The classical monocytes represent 90-95% of the total monocytes in a healthy person while the CD14+ CD16++ consists of 5-10% of the total monocytes in the blood[39, 40]. These monocytes subsets differ in many aspects, including adhesion molecules and chemokines receptors[41]. Additionally, the CD14+ CD16++ monocytes subsets express high levels of the pro-inflammatory cytokine TNF-α but no detectable levels of anti-inflammatory IL-10 in response to LPS[42, 43]. Based on these findings these cells have been termed pro-inflammatory monocytes. A considerable increase in the number of CD14+CD16++ monocytes had been described for a variety of systemic, infectious agents in humans, including bacterial sepsis and HIV[44, 45]. Recently, a significant increase in the levels of CD16 expression on monocytes was observed in human leishmaniasis caused by *L. braziliensis*[46].

Table 1 shows data from studies performed with peripheral blood-derived macrophages from cutaneous and mucosal leishmaniasis patients infected with *L. braziliensis*.

Production of CCL2 was higher in ML patients than in healthy subjects (HS) group. The levels of CXCL9 were significantly higher in CL patients and in ML patients when compared with controls and we also observed a difference in the CXCL9 production between ML and CL patients. The production of the inflammatory cytokine TNF-alpha was higher in ML and in CL than HS. The frequency of $CD14^{+}CD16^{+}$ monocytes was higher in patients with CL and ML than HS. These data pointed out that macrophages from cutaneous and mucosal leishmaniasis patients produce higher amount of pro-inflammatory chemokines and cytokines indicating that independent of T cells, these cells may participate in the inflammatory response and pathology associated to *L. braziliensis* infection.

TABLE 1 - Characterization of monocytes/macrophages subsets in human tegumentary leishmanisis.

	Healthy Subjects	Cutaneous leishmaniasis	Mucosal leishmaniasis	P value
A. CCL2 (pg/ml)	2,828± 4591[a]	6,775 ± 8152	11,129± 8562[a]	0.0005[a]
A. CXCL9 (pg/ml)	99 ± 108[a],[b]	5,396± 6610[b],[c]	14,222± 8002[a],[c]	<0.0001[a] <0.0001[b] 0.0026[c]
A. TNF-α (pg/ml)	18 ± 26[a],[b]	61 ± 56[b]	101 ± 99[a]	0.0018[a] 0.0177[b]
B. Frequency of CD14+CD16++ cells (%)	17 ± 9[a],[b]	26 ± 10[b]	27 ± 13[a]	0.0337[a] 0.0143[b]

The production of CCL2, CXCL9 and TNF-α by infected human macrophage cultured *in vitro* in the presence of *L. braziliensis* was performed in six days monocyte derived- macrophages from CL (n= 16), ML (n=8) and HS (n=14).The cells were infected with *L. braziliensis* and chemokines/cytokine were determined by ELISA. For statistical analysis, Mann-Whitney nonparametric test was used.

The ex vivo expression of CD16+ molecule was performed from PBMC of CL (n= 16), ML (n=8) and HS (n=8) using direct staining with fluorescein isothiocyanate (FITC) conjugated anti-CD14 monoclonal antibody and phycoerythrin (PE)-conjugated anti-CD16. Flow cytometric analysis of cell surface expression of CD14 and CD16 was performed by gating on monocytes according to light scatter profile. For statistical analysis, Mann-Whitney nonparametric test was used.

HOST GENETICS IN *L. braziliensis* INFECTION

The role of genes on host resistance or susceptibility to infectious diseases has been demonstrated in several studies performed in different populations. Although recognizing the role of these genes in humans is complex, due to the influence of environmental, ethnic and occupational factors, there is increasing evidence of genetic differences between populations concerning the infection by intracellular parasites. Although the environment and parasite strains can contribute to the development of leishmaniasis, specific polymorphisms at the host genome, are relevant to the clinical outcome of this disease, whose spectrum is highly variable and characterized by a complex immune response. The endemic area of Corte de Pedra, Bahia, Brazil has the highest incidence rates of cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML) in the state. We have previously shown by an epidemiological study involving affected and highly exposed families, that familial aggregation of disease occurs in this area[47]; and, that a polymorphic variant of the IL-6 gene, a cytokine that modulates the Th1 immune response, is strongly associated with ML in this population. Our data also point to functional differences in the production of IL-6 in cells stimulated with leishmania antigen in individuals with different genotypes, suggesting that polymorphism as a regulatory element[48]. Recently, investigating the role of genes that might regulate the infiltration or function of polymorphonuclear neutrophils (PMN) and macrophages in CL and ML, we show an association of disease with the CXCR1 gene (rs2854386). Interestingly, the common C allele was associated with CL, whereas the rare form, the G allele, was associated with ML. In addition, CL was also associated with a 3' insertion/deletion at the SLC11A1 gene, known for its role in regulating the macrophage activation. Other studies in the literature points the role of polymorphisms in genes of great relevance in the pathogenesis of tegumentary leishmaniasis. An example shows the association between the G allele of the TNFA gene, related to high production of the TNF-α cytokine, with ML in a population of Venezuela[49]. Recently, the C allele of the polymorphism -819 C/T of the IL-10 gene, major immunomodulatory cytokine of the inflammatory response in CL was associated with this phenotype in an endemic population of Southern Bahia[50]. The advent of molecular techniques such as Genome-wide association studies has been generating a huge data volume very quickly. Add to that, the establishment of new consortiums involving the creation of public domain databases, promoting interactions between different research groups will also bring new information and make it clear the role of host genetics in the development of complex diseases. Such understanding is important to develop effective treatments and ultimately, to develop prevention strategies.

PARASITES AND VECTOR FACTORS IN THE CLINICAL EXPRESSION OF *L. braziliensis* INFECTION

For many years it is known that disease expression in leishmaniasis is associated with parasite factors. While visceral leishmaniasis in Brazil is caused by *L. chagasi*, cutaneous leishmaniasis is predominantly associated to *L. braziliensis*, *L. guyanensis* and *L. amazonensis* species. However, as the same species as in the case of *L. braziliensis* can cause different clinical form of leishmaniais such as cutaneous, disseminated cutaneous and mucosal leishmaniasis, it is possible that intra-species genetic variability between the parasites strains/isolates may be responsible, in part, for the wide disease spectrum. For instance Saravia et al described an increased frequency of mucosal involvement among human cases by particular *L. braziliensis* zymodemes or strains[51]. We recently described a multiclonal population structure among *L. braziliensis* isolates from the endemic area of Corte de Pedra defined by randomly amplified polymorphic DNA (RAPD). Specifically we observed different eletrophoretic profiles in leishmania strains isolated from patients with cutaneous, mucosal and disseminated leishmaniasis[52]. This data indicate that *L. braziliensis* in Corte de Pedra is polymorphic and that there is an association between the different genetic patterns determined by RAPD and clinical forms of the disease[52].

It is known that host immune response in cutaneous and mucosal leishmaniasis is more exacerbated than in disseminated leishmaniasis. To determine whether host immune response in these different forms of disease could be influenced by the isolate of *Leishmania*, soluble leishmania antigen (SLA) of *L. braziliensis* was performed with isolates obtained from patients with cutaneous leishmaniasis and with isolates obtained from patients with disseminated leishmaniasis. These studies showed that *L. braziliensis* antigen from isolates of disseminated leishmaniasis induced more TNF-α and IFN-γ than antigens from isolates of cutaneous leishmaniasis patients, when stimulated cells from patients with either cutaneous or disseminated cutaneous leishmaniasis[53]. Putting together these data showed that not only isolates from disseminated cutaneous leishmaniasis differ genetically from isolates of cutaneous leishmaniasis, but also antigens from these isolate induced cells to produce more or less pro-inflammatory cytokines.

While it is clear that genetic differences among *L. braziliensis* isolates may influence the clinical forms of the disease, studies regarding the role of the vector in the clinical spectrum of *L. braziliensis* are lacking. Most of the studies regarding the role of sand fly saliva in pathogenesis of leishmaniasis come from data with *Lutzomya longipalpis* and *Phlebotomus paptasis*. Initially it was observed that co-inoculation of *L. longipalpis* or *P. paptasis* salivary gland sonicate (SGS) and *L. major* led to a significant exacerbation of lesion size and parasite load in BALB/C mice[54, 55]. Similar effects were also observe with *L. braziliensis* (56) and *L. amazonensis*[57]. On the other hand, BALB/C mice are protected against *L. major* when immunized with SGS[54].

The main vectors isolated in Corte de Pedra, an area of *L. braziliensis* transmission are *L. intermedia* and *L. whitmani*. The immune response to *L. intermedia* SGS have been studied in mice and humans. In BALB/C mice it was observed that mice immunized with *L. intermedia* SGS produce both IFN-γ and IL-4. Moreover, when challenged with *L. braziliensis* in the presence of saliva these animals were not protected. In fact parasite load was increased in mice previously immunized with SGS[58]. To evaluate the human immune response to *L. intermedia* saliva antibodies were determined in patients with cutaneous leishmaniasis and in subjects with subclinical *L. braziliensis* infection. It was shown that patients with active lesions displayed higher levels of anti *L. intermedia* saliva antibodies than individuals who had subclinical *L. braziliensis* infection. This study showed that immunization with whole SGS from *L. intermedia* do not protect BALB/C mice against challenge with *L. braziliensis* and that high titers of antibodies against SGS were associated with development of disease rather than with protection in human *L. braziliensis* infection[58].

BASIC RESEARCH IN THE CONTROL AND THERAPY OF TEGUMENTARY LEISHMANIASIS

The observation that killed or attenuated leishmania associated with cytokines was able to protect mice against *L. major* infection led to a great interest in vaccine development for cutaneous leishmaniasis. As control of leishmania is dependent of T cells the identification of parasite antigen able to induce a strong type 1 immune response became a major goal in this field. Several antigens have been identified as potential vaccine candidates but attempts to control *L. braziliensis* infection have not been successful. One major question is regarding the strategy to induce a potent type 1 immune response with vaccine candidates. Patients with cutaneous and mucosal leishmaniasis have an exacerbated type 1 immune response, but rather than control infection this immunological response is associated to pathology[5]. The best examples of putative resistant individuals are subjects that live in the endemic area and despite exposure to leishmania infection, control parasite growth and do not develop pathology[59]. These individuals with sub-clinical *L. braziliensis* infection produce less IFN-γ and TNF-α than leishmaniasis patients as shown in **Figure 1**. In such case control of parasite growth can be made by the innate immune response. However, very few studies have been performed to evaluate human innate immune response to leishmania. In Colombia it was shown that macrophages from individuals with sub-clinical *L. braziliensis* infection have a greater ability to kill leishmania than cells from patients with relapsing leishmaniasis[60]. In Africa, NK cells from individuals exposed to *L. aethropica* but who do not develop disease are the major source of IFN-γ upon stimulation *in vitro* with leishmania antigen[61]. Putting together these data indicate that studies need to be done regarding the role of the innate immune response in protection of humans against *L. braziliensis* infection.

While more knowledge about the human protective immune response against *L. braziliensis* is necessary for vaccine development, therapy is the main way to attenuate clinical manifestations of the disease. In this field an increasing I the number of isolates resistant to antimony have led to an increasing in therapeutic failure. In Northeastern Brazil failure to two courses of 20mg/kg/weight for 20 days of pentavalent antimony, is up to 40% in cutaneous leishmaniasis, 80% in disseminated leishmaniasis and in more than 90% of patients with atypical cutaneous leishmaniasis[62, 63]. Based in the understanding that an exaggerated inflammatory response is responsible for the pathology in *L. braziliensis* infection, we have proposed the use of regulatory cytokines as well as drugs that down modulate TNF-α production to treat cutaneous and mucosal leishmaniasis.

The granulocyte macrophage colony stimulating factor (GM-CSF) can improve antigen presentation and accelerate the healing of ulcers due to different etiologies. A randomized double-blind placebo controlled trial was performed comparing antimony associated to GM-CSF applied topically in the ulcer with antimony plus placebo. At day 60 all patients in the GM-CSF group had their ulcers healed. In contrast 30% of the patients who received antimony plus placebo failure to heal and needed a second course of antimony to cure their lesions[64]. In an open trial we showed that GM-CSF associated with antimony cure cutaneous leishmaniasis patients that were refractory to at least three courses of antimony therapy[65].

Based on the knowledge that TNF-α is highly produced in cutaneous and mucosal leishmaniasis, and that TNF-α participates of the development of the lesion we have proposed to use pentoxifylline, a potent TNF-α inhibitor, associated to antimony to treat leishmaniasis patients infected with *L. braziliensis*. Pentoxifylline is a methylxanthine originally licensed in the United States for peripheral vascular disease. Pentoxifylline also suppresses TNF-α mRNA transcription. In leishmaniasis pentoxifylline in the dose of 400mg tid for 30 days was initially used associated to antimony in patients with mucosal leishmaniasis refractory to antimony. In 10 patients that did not respond to at least two courses of antimony, the combination therapy cured 9 of 10 patients[66]. More recently in double blind placebo controlled trials the association of pentoxifylline plus antimony was more effective than antimony plus placebo in cure cutaneous and mucosal leishmaniasis[67].

CONCLUSIONS

Basic research in leishmaniasis has given important contributions in the understanding of the pathogenesis and in control of *Leishmania* infection. A highly relevant aspect of *L. braziliensis* infection is that control of parasite does not occur despite an exaggerated pro-inflammatory immune response. In fact production of TNF-γ and IFN-γ are associated with pathology. In addition to T cells, activated monocytes participate of the pathogenesis of cutaneous and mucosal leishmaniasis secreting pro-inflammatory chemokines and cytokines when infected with *L. braziliensis*. Moreover, it is clear that genetic differences among isolates of *L. braziliensis* participates of the pathogenesis and are associated with the expression of different clinical forms of *L. braziliensis* infection.

Despite the identification of a large number vaccine candidates antigens and the attempt in the development of a vaccine against *L. braziliensis* infection prevention of *L. braziliensis* has not been reached. The documentation that individuals with sub-clinical *L. braziliensis* infection control *Leishmania* and produce small amounts of pro-inflammatory cytokine indicate that studies should be developed to identify the mechanisms of *L. braziliensis* killing by human cells.

The basic research that lead to the understanding of the pathogenesis of *L. braziliensis* infection and have contributed to the use of alternative therapies based in cytokines or anti-cytokines compounds. Specifically it has been shown that association of pentavalent antimony plus GM-CSF or pentavalent antimony plus pentoxifylline are more effective than antimony, decrease the healing time of cutaneous and mucosal ulcers and cure patients that are refractory to antimony alone.

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