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REVIEW

ANIMAL MODELS FOR THE STUDY OF LEISHMANIASIS IMMUNOLOGY

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SUMMARY

Leishmaniasis remains a major public health problem worldwide and is classified as Category I by the TDR/WHO, mainly due to the absence of control. Many experimental models like rodents, dogs and monkeys have been developed, each with specific features, in order to characterize the immune response to *Leishmania* species, but none reproduces the pathology observed in human disease. Conflicting data may arise in part because different parasite strains or species are being examined, different tissue targets (mice footpad, ear, or base of tail) are being infected, and different numbers ("low" $1x10^2$ and "high" $1x10^6$) of metacyclic promastigotes have been inoculated. Recently, new approaches have been proposed to provide more meaningful data regarding the host response and pathogenesis that parallels human disease. The use of sand fly saliva and low numbers of parasites in experimental infections has led to mimic natural transmission and find new molecules and immune mechanisms which should be considered when designing vaccines and control strategies. Moreover, the use of wild rodents as experimental models has been proposed as a good alternative for studying the host-pathogen relationships and for testing candidate vaccines. To date, using natural reservoirs to study *Leishmania* infection has been challenging because immunologic reagents for use in wild rodents are lacking. This review discusses the principal immunological findings against *Leishmania* infection in different animal models highlighting the importance of using experimental conditions similar to natural transmission and reservoir species as experimental models to study the immunopathology of the disease.

KEYWORDS: Animal models; *Leishmania*; Immune response.

INTRODUCTION

Leishmaniasis encompasses a group of diseases which are caused by infection with protozoan parasites of the *Leishmania* (Kinetoplastida: Trypanosomatidae) genus. They are still a major worldwide public health problem considering they are endemic in 98 countries or territories, with more than 350 million people at risk[6]. Moreover, it is estimated visceral leishmaniasis (VL) causes over 50,000 deaths annually, a rate only surpassed, among parasitic diseases, by malaria, and 2,357,000 disability-adjusted life years lost, placing leishmaniasis ninth in a global analysis of infectious diseases[6,28]. Despite this very strong data, leishmaniasis is largely ignored in discussions of tropical disease priorities and is one of the most neglected tropical diseases[51,52]. It has been pointed out that this consignment to critical oblivion possibly "results from its complex epidemiology and ecology, the lack of simple, easily-applied tools for case management and the paucity of current incidence data, and often results in a failure on the part of policy-makers to recognize its importance[5,14]. Political and socioeconomic changes may have an even more important role than global warming on the changing epidemiology of the leishmaniases, as has been argued for tick-borne diseases in Europe[91]. The European Centre for Disease Prevention and Control lists the leishmaniases among the ten vector-borne diseases that have the greatest potential to affect European inhabitants[101].

The disease is transmitted to humans by sand flies and displays different clinical manifestations, ranging from asymptomatic or subclinical infection to disfiguring forms of cutaneous and mucosal leishmaniasis or potentially fatal visceral disease[35,36,78,93]. This polymorphic outcome has been considered to depend largely on the virulence of the infecting parasite strain, immunoregulatory effects of sand fly saliva, as well as the host's genetic background and immune response[31,60,62]. In summary, the leishmaniases remain as unpreventable and uncontrollable diseases; moreover, their epidemiological profile is shifting towards an increased prevalence, and therefore novel instruments and approaches to reach their control are urgently necessary.

Leishmaniasis is most likely to be controlled by a successful vaccination program. The relatively uncomplicated leishmanial life cycle and the fact that recovery from a primary infection renders the host resistant to subsequent infections indicate that a vaccine is feasible[55]. Many immunological aspects of the disease have been studied in experimental animal models, such as mice, hamsters, domestic dogs and non-human primates. Although most experimental models of

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leishmaniasis have the major advantage of allowing control over the genetics of both the parasite and the host, none of them, in any way, reproduces the outcome of human infection by *Leishmania* spp[49].

Among the main factors contributing to differences between humans and animal models are the size and nature of the inocula, the infection route and the strain of host or parasite[37,60,70]. Currently, small numbers of *in vitro*-derived metacyclic promastigotes together with strongly bioactive saliva, intradermal infection and host reservoirs as experimental animal models are used to mimic the clinical and immunological features found in human disease[49]. These approaches could contribute to developing improved experimental models for studying leishmaniasis and identifying possible targets to evaluate vaccine candidates.

The present review describes the most common animal experimental models which have been employed to study the immune response to *Leishmania* spp. and includes wild rodents. The main purpose is to discuss the concept of experimental animal models to study leishmaniasis immunology.

MOUSE MODEL

The laboratory mouse owes much of its popularity as a model organism in biomedical research to the existence of a large collection of inbred strains that represent an immortal population of genetic clones derived by repeated brother sister mating. Because mice from each strain are genetically identical it is possible to collect and combine biological data over time and space leading to a depth of phenotype characterization rarely achieved in other mammalian systems. Furthermore, the existence of a definite set of genetic differences among inbred strains allows scientists to explore the effect of genetic diversity on almost any phenotype of interest[107]. Another advantage of the murine model is the simplicity of keeping, breeding and reproducing them[47].

During the past 40 years, murine models of the human disease cutaneous leishmaniasis have been extensively employed to elucidate the cell types, cytokines, signal transduction cascades and antileishmanial effector mechanisms that are necessary for the control of parasites, as well as for the clinical resolution of disease, resistance to a secondary infection, and vaccine development[18,73]. Experimental infection of mice with *L. major* promastigotes has allowed understanding of the immunologic mechanisms governing resistance (C57BL/6 strain) and susceptibility (BALB/c strain) to infection[2].

Susceptibility has been correlated with the development of lesions associated with a Th2 type of immune response, while the healing of lesions in resistant mice has been correlated with the development of a Th1 type of immune response[98]. The resolution of lesions in C57BL/6 mice has been shown to involve several factors contributing to the killing of *L. major* within macrophages. The most efficient mechanism of parasite killing involves the production of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) by CD4+ Th1 cells, which stimulate the synthesis of inducible nitric oxide synthase (iNOS), generating the production of nitric oxide (NO), a potent cytotoxin involved in the clearance or inhibition of *Leishmania* parasites[61,65,77].

In contrast, susceptible BALB/c mice develop severe and uncontrolled lesions that lead to progressive disease and eventual death. This non-healer phenotype has been shown to be associated with a parasite-specific Th2 response characterized by the enhanced expression of deactivating macrophage cytokines such as interleukin 4 (IL-4), interleukin 10 (IL-10) and transforming growth factor-β (TGF-β)[11,94,98]. Studies of mouse models of leishmaniasis have provided important insights into the response of the host to infection. However, the use of different parasite species, tissue targets (mice footpad, ear, or base of tail) and doses (10^5 to 10^7) of metacyclic promastigotes has generated a wide variety of experiments that do not reproduce the natural infection and cannot be extrapolated to human disease.'

In natural infections, the sand fly introduces into the skin a very small number (possibly as few as 100 to 1,000) of metacyclic promastigotes together with strongly bioactive saliva, whereas in laboratory infections thousands to millions of culture-derived promastigotes or tissue-derived amastigotes are injected with a saline solution or culture medium[57].

In order to have a better understanding of natural *Leishmania* infection in the laboratory, investigators are now using small numbers of *in vitro*-derived metacyclic promastigotes and intradermal rather than subcutaneous infection into the ears of mice[49].

It was demonstrated, for example, that in mice inoculated with a mixture of phlebotomine saliva and *L. major* promastigotes, the lesions grew faster and were bigger than those of mice inoculated only with promastigotes[13].

The ability of saliva to enhance *Leishmania* infection has been attributed to modulation of the host immune system, potentially through anti-inflammatory properties such as down regulation of antigen presentation, co-stimulatory molecule expression, and nitric oxide production[13, 81]. However, vaccination by pre-exposure to the bites of uninfected sand flies, with whole saliva or with defined salivary proteins has shown to protect against cutaneous *L. major* infection[97]. The frequent exposure to sand fly bites leads to the production of neutralizing antibodies against salivary proteins and also to the activation of cellular mechanisms that may have an adverse effect on *Leishmania* establishment. In this perspective, characterization of immune responses against sand fly saliva can help estimate both risk of infection and, to some degree, anti-parasite immunity. Although this hypothesis has been proven in animal models, additional large-scale clinical studies are necessary to validate it in humans[15]. Although the regulation of host immune response to *Leishmania* has been well defined in cutaneous *L. major* infection of inbred mice, many studies have demonstrated that the host responses within the same mouse strain could vary according to different *Leishmania* species. Different virulence factors have been identified for distinct *Leishmania* species, and there are profound differences in the immune mechanisms that mediate susceptibility/resistance to infection and in the pathology associated with disease[66]. For example, C57BL/6 or C3H mice, which heal from *L. major* infection, develop chronic disease when infected with either *L. (L.) amazonensis* or *L. (L.) mexicana*.

The characteristic chronic lesions of *L. (L.) amazonensis* infection in C57BL/6 and C57BL/10 mice are independent of IL-4 expression and corresponding Th2 response[1,54]. In addition, *L. (L.) amazonensis* infection in C3H mice results in low levels of production of IL-12 and IFN-γ by antigen-specific CD4+ T cells[54]. However, lesion development and parasite burden have been shown to be exacerbated in the presence

of CD4+ T cells, demonstrating that T cells are activated during *L. (L.) amazonensis* infection and that they contribute significantly to the immunopathology of chronic disease[103].

Recently, it was demonstrated that susceptibility to *L. (L.) amazonensis* in the mouse model of cutaneous leishmaniasis does not depend only on the expression of IL-10. *L. (L.) amazonensis* parasites persistent in IL-10-deficient mice; even in the presence of an enhanced Th1 response during the early stages of infection and in the presence of antigen-specific cells primed for Th1 effectors function during the chronic phase[53].

Although the requirements for effective intracellular killing of *L (L.) amazonensis* by activated macrophages are relatively unknown, it has been demonstrated that the presence of both superoxide and nitric oxide is necessary for efficient killing of amastigotes within LPS/IFN-γ–activated bone marrow-derived macrophages generated from C3H mice[74].

Control of the closely related *L. (L.) mexicana* in C57BL/6 mice has been shown to be IFN-γ and STAT4-dependent and surprisingly independent of IL-12 production while the presence of IL-4, STAT6 and, perhaps as a consequence, the ability to generate Th2 responses, are essential for the rapid lesion growth and nonhealing responses[21,100,106]. Later on, it was demonstrated that endogenous IL-12 is only critical for controlling the late but not the early stage of *L. (L.) mexicana* infection in C57BL/6 mice; however, they fail to resolve lesions, in contrast to *L. major* infection[3].

As an evasion mechanism, *L. (L.) mexicana* promastigotes mediate the phosphorylation of specific transcription factors to enhance iNOS, COX-2 and arginase-1 expression in LPS induced macrophages via TLR-4. The activities associated with all three enzymes are the main factors leading to the downregulation of the IL-12 production in *L. (L.) mexicana* infected macrophages[102].

The significant differences in the immune response between the Old World (*L. major*) and New World (*L. mexicana/L. amazonensis*) *Leishmania* species not only point to interesting features of the host-pathogen interaction and immunobiology of this genus of parasitic protozoa, but also have important implications for immunotherapy and vaccine development. A view of leishmaniasis that only considers mouse model infection with *L. major* misses a wealth of interesting immunobiology associated with other species of *Leishmania*[66]. Therefore, our understanding of the mechanisms involved in mucocutaneous and cutaneous diseases caused by these organisms remains limited.

Mouse models have also been used to study visceral leishmaniasis caused by both *L. donovani* and *L. infantum*. Although the outcome of murine VL infection is genetically determined, most susceptible mouse strains including BALB/c are able to control visceral disease[75]. Following *Leishmania* infection, BALB/c mice develop an organ-specific immune response[113]. During the first weeks of infection, the parasites multiply rapidly in the liver; however four weeks later, the mice develop an effective Th1 immune response, clear the parasites and become resistant to reinfection[76]. The hepatic resistance to *Leishmania* infection in these mice is associated with the development of a granulomatous reaction in the liver[12]. While pathology in the liver is limited, the parasites persist in the spleen and the infection progresses for a longer period of time. Eventually, splenic replication is controlled but parasites are usually maintained for life[58]. Parasite persistence in mice is accompanied by failure of granuloma formation and splenomegaly[39].

Due to the fact that visceral infection in BALB/c mice is chronic but not fatal, it may be more appropriate to use it as a model for studying self-healing or subclinical infection. Although experimental murine models of VL do not allow exact extrapolations with subclinical infection in humans they have been useful to identify genes and predict their functional roles in the protective immune response. Genetically resistant mice have the functional *NRAMP1* gene which is involved in macrophage activation[16]. The *NRAMP1* gene encodes a protein expressed on the membrane of infected macrophages and exerts an enhanced effect on iNOS expression and generation of NO, restricting intracellular *Leishmania* multiplication[17]. In this context, visceral infection in BALB/c mice provides a good model for the evaluation of candidate vaccines.

HAMSTER MODEL

The Syrian golden hamster (*Mesocricetus auratus*) is highly susceptible to infection with visceralizing *Leishmania* species (*L. donovani*, *L. infantum*) and is considered the best experimental model to study visceral leishmaniasis (VL) because it reproduces the clinicopathological features of human disease. However, the wide use of hamsters is still limited due to the scarcity of reagents (e.g., antibodies, cell markers and cytokines) of defined specificity available to study the role of the immune response in disease pathology[48,68,113].

In 1978, CHANG & DWYER provided quantitative evidence indicating an avid ingestion of *L. donovani* amastigotes by hamster macrophages and supported the early findings that lysosome-phagosome fusion ocurrs[26].

In order to understand the immune response to *L. donovani* infection the nucleotide sequences of several hamster cytokine genes (IL-2, IL-4, INF-γ, TNF-α, IL-10, IL-12 and TGF-β) were cloned and used to analyze their expression in a model of visceral infection. In this hamster model there was a pronounced expression of the Th1 cytokine mRNAs (IL-2 and IFN-γ), with transcripts being detected as early as one week post-infection. Surprisingly, although the basal expression of IL-4 was detected in uninfected hamsters, their expression did not increase in response to infection with *L. donovani.* IL-12 transcript expression was detected at low levels starting seven days post-infection and its expression paralleled that of IFN-γ. Additionally, the mRNA for TNF-α was increased within one week of infection but levels did not increase further during the first month of infection. Expression of IL-10, a potent macrophage deactivator, increased in splenic tissue over the first four weeks after infection, suggesting that this cytokine could contribute to progressive disease in hamsters. These studies provided the first description of the molecular immunopathogenesis of disease in hamsters and indicated that progressive disease in this model of VL is not associated with early polarization of the splenic cellular immune response toward a Th2 phenotype and away from a Th1 phenotype, offering important insights into human disease[67].

During progressive disease in the hamster model of VL, uncontrolled parasite replication in the liver, spleen, and bone marrow occurred despite the high activation of the immune response and the strong Th1-like cytokine microenvironment. The failure in the control of VL could be

partially explained by the lymphoproliferative suppression process which occurs during active disease[45]. Visceral infection caused by *L. donovani* led to a gradual impairment of the proliferative response to parasite antigens in hamsters[79]. The latter dysfunction has been attributed to the inability of the infected antigen-presenting cells (APCs) to stimulate specific T cells, the production of TGF-β which triggers the apoptotic death of lymphocytes and the downregulation of protein kinase C activity[9,72,95]. Interestingly, the antigen-dependent immunosuppression observed in *L. chagasi*-infected hamsters with active visceral disease is not related to the cytokine profile[41].

Furthermore, the fatal outcome of the disease in the hamster model has been related to the loss of macrophages effector functions. Indeed, throughout the course of infection, inducible NO synthase (iNOS, NOS2) mRNA or enzyme activity in liver or spleen tissue was not detected. Thus, although a Th1-like cytokine response was prominent, the major antileishmanial effector mechanism that is responsible for control of infection in mice was absent throughout the course of progressive VL in the hamster[68].

Later on, it was shown that the lack of NO production was due to a defect in the transcriptional activation of NOS2. Luciferase reporter assays demonstrated that the hamster NOS2 promoter, like the human NOS2 promoter, has reduced basal and IFN-γ/LPS-induced activity compared with the mouse promoter. The mechanism described above is the most probable reason for the inability of hamsters to control *Leishmania* infection[84].

The role played by infected macrophages in the development of the cellular unresponsiveness present in visceral leishmaniasis has been studied. Adherent spleen cells from infected hamsters were unable to present *L. donovani* antigens to antigen specific T cells, however they were able to present KLH. Conversely, T cells from infected animals did not respond to parasite antigens even when these antigens were presented by normal syngeneic macrophages. Interestingly, lymphocytes from inguinal lymph nodes of infected animals sensitized in their footpad with parasite antigens proliferated well when stimulated *in vitro* with *L. donovani* antigens. These results suggest that the defect in the cellular immune response of the *L. donovani* infected hamsters is a consequence of a selective inability of their antigen presenting cells to process and present parasite antigens to T cells[95].

To date, progressive disease in hamsters has been mostly achieved by the injection of a large number of parasites via the i.v., intracardial, or i.p. routes. However, these routes of infection do not mimic natural transmission by sand fly bite where the parasites are delivered into the skin of a mammalian host in the presence of saliva. Recently, it was demonstrated that a salivary protein of the sand fly vector *Lutzomia longipalpis* protects against the fatal outcome of visceral leishmaniasis caused by *L. infantum* in a hamster model. Immunization with 16 DNA plasmids coding for salivary proteins of *Lu. longipalpis* resulted in the identification of LJM19, a novel 11-kDa protein, that protected hamsters against the fatal outcome of VL. LJM19-immunized hamsters maintained a low parasite load that correlated with an overall high IFN-γ/TGF-β ratio and iNOS expression in the spleen and liver up to five months postinfection. Importantly, a delayed-type hypersensitivity response with high expression of IFN-γ was also noted in the skin of LJM19-immunized hamsters 48 hours after exposure to uninfected sand fly bites. Induction of IFN-γ at the site of the bite could partly explain the protection observed in the viscera of LJM19-immunized hamsters through direct parasite killing and/or priming of anti-*Leishmania* immunity. These findings reinforce the concept of using components of arthropod saliva in vaccine strategies against vector-borne diseases[44].

To better understand the hamster immune response to important pathogens such as *Leishmania*, a duplex real-time reverse transcriptase (RT) PCR assay was developed for the relative quantification of the mRNAs of hamster cytokines, chemokines, and related immune response molecules. The application of this assay to a biological model was demonstrated in a cutaneous hamster model by comparing mRNA expression in skin and lymph node tissues between uninfected and *L. panamensis* infected hamsters. As a result, there was a relatively greater basal expression in the LN compared to the skin for most transcripts (IL-4, CCR4, IL-21, TNF-α, TGF-β, IFN-γ, IL-12p40, IL-10, and Foxp3). Conversely, the assay identified that the basal expression of CCL22 and CCL17 mRNAs was significantly greater in the normal skin compared to the LN. At an early stage of infection(one week p.i.) there was concomitant upregulation of the type 1 (IFN-γ and IL-12p40) and type 2 (IL-4, IL-10, IL-13, and IL-21) cytokines at the site of cutaneous infection, suggesting that a balanced type 1 and type 2 cytokine response contributes to the chronicity of the disease caused by *L. panamensis* in hamsters[40].

Undoubtedly, the hamster model may be helpful for understanding the immunological mechanisms involved in the pathogenesis of visceral leishmaniasis. However, it is necessary to continue the efforts of producing specific reagents (i.e. cytokine-specific and cell surface markers of monoclonal antibodies) and develop more sensitive techniques that allow the study of the immunopathogenesis of the disease, which has important implications for the generation of therapeutic and vaccine targets.

DOG MODEL

Wild canines and domestic dogs are the main reservoirs of zoonotic visceral leishmaniasis caused by *L. infantum* in the Mediterranean area, Middle-East, Asian countries and Latin America. The role of dogs as the main reservoir of visceral leishmaniasis has led to an increased interest in studying the immune response and finding *Leishmania* antigens implicated in protective cellular immunity in canine visceral leishmaniasis. Recent research has provided new insights on the epidemiology, pathology and immunology of canine leishmaniasis and its genetic basis. These new findings have led to better understanding of the disease, and have also helped in the development of new diagnostic methods and control measures against the infection, such as insecticide-impregnated collars for dogs, new drugs, and second generation vaccines[4,10].

Canine visceral leishmaniasis is a multisystemic disease with variable clinical signs. Infected dogs may develop symptomatic infection resulting in death, while others remain asymptomatic, or develop one or more mild symptoms and are classified as oligosymptomatic[27]. The typical histopathological finding in the skin, liver and spleen, is a granulomatous inflammatory reaction associated with the presence of *Leishmania* amastigotes within macrophages[10].

Studies on experimentally infected dogs have demonstrated that three years after infection, asymptomatic or resistant dogs responded to

L. infantum antigen both in lymphocyte proliferation assays *in vitro* and in delayed-type hypersensitivity reaction, whereas no serum antibodies to parasite antigen were shown. In contrast, symptomatic or susceptible animals failed to respond to the parasite antigen in cell-mediated assays both *in vitro* and *in vivo* and showed considerably higher serum antibodies to leishmanial antigens, which are not immunoprotective. In addition, peripheral mononuclear cells from asymptomatic dogs produced significantly higher levels of IL-2 and TNF-α than symptomatic and control uninfected dogs. Similar results were observed with a group of mixed-breed dogs with natural *Leishmania* infections, also grouped as asymptomatic or symptomatic on the basis of clinical signs of canine visceral leishmaniasis[85].

The main effector mechanism involved in the protective immune response of dogs infected with *L. infantum* is the activation of macrophages by IFN-γ and TNF-α to kill intracellular amastigotes via the L-arginine nitric oxide pathway, as has been observed following successful chemotherapy of *L. infantum*-infected dogs[112]. NO production and anti-leishmanial activity has also been detected in a canine macrophage cell line infected with *L.infantum* after incubation with IFN-γ, TNF-α and IL-2[88], as well as in macrophages from dogs immunized with killed *L.infantum* promastigotes[82]. Later on, it was demonstrated that NO production may be involved in the long-term protection of dogs against natural *Leishmania* infection and in the clinical presentation of canine leishmaniasis[83].

The local tissue cytokine response of dogs naturally infected with *L. infantum* has been evaluated. The analysis revealed an enhanced INF-γ mRNA accumulation in infected dogs which was positively correlated with humoral, (IgG1) but not with lymphoproliferative, responses to the *Leishmania* antigen. However, infected dogs with detectable IL-4 mRNA had significantly more severe symptoms[90]. A balanced production of Th1 and Th2 cytokines was detected in the spleen of *L. infantum* infected dogs, with a predominant accumulation of mRNA for IL-10 and IFN-γ that was related to the parasitic load and to clinical progression[59]. Additionally, a mixed cytokine profile with high levels of expression of IFN-γ, TNF-α and IL-13 was determined in the skin of asymptomatic dogs naturally infected with *L. infantum*. Moreover, the levels of transcription factors GATA-3 and FOXP3 were correlated with the asymptomatic disease. These results indicate that in addition to the mixed cytokine profile, the enhanced expression of their associated transcription factors plays an important role in the clinical status of *Leishmania* infected dogs[69]. The role of Th2 type cytokines in canine VL has not yet been defined. Evidence for Th1 and Th2 mixed responses has been reported in antigen-stimulated PBMC from asymptomatic dogs experimentally infected with *L. infantum*, which displayed IL-2, IFN-γ and IL-10 mRNA transcripts. However, IL-2 and IFN-γ predominated in asymptomatic dogs and the development of symptomatic infections could not be related to IL-10 expression[25]. IL-10 mRNA transcripts were detected in Con A-stimulated PBMC derived from dogs with clinical signs of VL[87]. All of these results are in agreement with experiments in which PBMC obtained from symptomatic VL dogs were stimulated by a recombinant *L. infantum* cysteine proteinase and high levels of IL-10 were detected by an ELISA assay. In contrast, low or undetectable concentrations of this cytokine were found in PBMC supernatants from oligosymptomatic and asymptomatic animals, respectively[89].

Although IL-10 secreted by CD25+ CD4+-regulatory T cells has been implicated in murine and human leishmaniasis, the involvement of these cells in canine visceral leishmaniasis has not been explored.

Few studies have demonstrated the involvement of CD8+ lymphocytes in resistance to canine VL. These lymphocytes were detected in asymptomatic dogs experimentally infected with *L.infantum* but not in symptomatic animals, suggesting that direct lysis of *L. infantum*-infected macrophages by cytotoxic T lymphocytes represents an additional effector mechanism in resistance to VL[86]. In dogs naturally infected with *L. infantum*, a reduction in both CD4+ and CD8+ populations was observed, while restoration of these cells occurred after drug treatment[19].

The use of dogs as experimental models to study visceral leishmaniasis has led to elucidate the role of immune cells and their principal products to better understand the possible mechanisms mediating immune response during *Leishmania* infection, which may contribute to the development of vaccines or immunotherapy.

Natural infection of domestic dogs with *L. (V.) braziliensis*, *L. (V.) peruviana*, *L. (V.) panamensis*, *L. (V.) colombiensis* and *L. (L.) mexicana* has been reported in Latin America[30]. To date, there is no solid evidence that dogs act as reservoir hosts for the domestic transmission of CL[92,99]. Most studies are designed to determine the prevalence of CL in dogs, however, little is known about the parasitologic and immunologic course of infection.

NON-HUMAN PRIMATE MODEL

Non-human primates are valuable models for biomedical research because of their similarities to humans in anatomy, immunology and physiology. However, they are expensive laboratory animals that are difficult to obtain and to handle. Availability of a non-human primate model of leishmaniasis would facilitate the study of different aspects of this disease and would accelerate the development of vaccines and testing of new drug candidates.

The Asian rhesus macaques (*Macaca mulatta*) are quite susceptible to *Leishmania* infection: they develop a human-like disease, exhibit antibodies to *Leishmania* and parasite-specific T-cell mediated immune responses both *in vivo* and *in vitro*, and can be protected effectively by vaccination[46]. Distinct histopathological patterns were observed in *Macaca mulatta* lesions at biopsy, but healing lesions contained more organized epithelioid granulomas and activated macrophages, followed by fibrotic substitution in response to *L. (L.) amazonensis* infection[7]. Interestingly in *L. (V.) braziliensis* infection, the presence of antigen-specific IFN-γ or TNF-α-producing CD4+ and CD8+ cells are likely important for the immunological effectiveness of granulomas. However, their resolution can be attributed to the concomitant recruitment of IL-10-producing CD4+CD25+ regulatory T cells that suppress the effector T-cell mediated inflammatory response[32,105]. The progression and resolution of skin lesions caused by both *Leishmania* species appears to be very similar to that observed in humans, confirming the potential for this monkey as a viable surrogate to study the immune response in human cutaneous leishmaniasis[7].

Macaques have also been used to explore immune response against *L. major* infection. Infected animals develop a simple cutaneous lesion which progresses spontaneously to ulceration and complete resolution

within about three months which is associated with a non-specific chronic inflammation and/or tuberculoid-type granulomatous reaction. Additionally, macaques develop varying levels of resistance against homologous re-infection as it happens in humans. Thus, the importance of this model in experimental CL lies in the reproduction of clinical and histopathological features that are common in *L. major*-infected humans and in the resistance to secondary infection, indicating the development of an acquired immunity[8].

New World primates, such as owl monkeys *(Aotus trivirgatus),* squirrel monkeys *(Saimiri sciureus),* and marmosets *(Callithrix jaccus jaccus)* have been considered potential hosts for studying visceral leishmaniasis. Owl monkeys develop a visceral disease characterized by weight loss, anemia and hepatosplenomegaly. Its high susceptibility to *L. donovani* infection suggest it may be useful for the study of VL[20]. In contrast, squirrel monkeys develop a visceral disease when infected with *L. donovani* but are able to recover from disease and became resistant to reinfection[34].

Although little is known about immune response to *Leishmania* infection in monkeys, they are frequently used as models for preclinical testing of *Leishmania* candidate vaccines. The safety, immunogenicity, and efficacy of a vaccine combining heat-killed *L. (L.) amazonensis* with human rIL-12 (rhIL-12) and alum (aluminium hydroxide gel) as adjuvants was evaluated in rhesus macaques. The single s.c. vaccination was found to be safe and immunogenic, although a small transient s.c. nodule developed at the vaccination site. Groups receiving rhIL-12 had an augmented *in vitro* Ag-specific IFN-γ response after vaccination, as well as increased production of IgG. Furthermore, intradermal forehead challenge infection with 10^7 metacyclic *L. (L.) amazonensis* promastigotes at four weeks demonstrated protective immunity in all monkeys receiving rhIL-12 with alum and Ag. Thus, a single dose vaccine with heat-killed *Leishmania* using rhIL-12 and alum as adjuvants was safe and fully protective in a primate model of cutaneous leishmaniasis[56].

Successful vaccination has been achieved against visceral leishmaniasis by intradermal inoculation of alum-precipitated autoclaved *L. major* with BCG (bacile Calmette-Guérrin) and autoclaved *L. donovani* with BCG in Indian langurs. Vaccinated animals show a delayed protection and significant lymphoproliferative response with high levels of IFN-γ and IL-2[38,71].

Attempts were made to reproduce the spectrum of human visceral leishmaniasis due to *L. donovani* in Vervet monkeys (*Chlorocebus pygerythrus*). Both symptomatic and asymptomatic/cryptic infections were observed. However asymptomatic infected animals had competent humoral and cellular responses to homologous parasites[43].

The development of a non-human primate model of leishmaniasis, which largely mimics the human situation, is described for studies of different aspects of the disease that would not be possible in humans for ethical reasons. However, for financial and ethical reasons, the use of primates in biomedical research is limited. Studies involving these animals have, therefore, been tailored to solve questions that cannot be answered in other animals. Monkeys are normally the final experimental animals to be used in studies of the safety and efficacy of vaccines and drugs developed in other laboratory animals[48].

WILD RODENTS

Classical laboratory inbred strains of mice have been extremely helpful for research in immunology and oncology. Unfortunately, because they all derive from a relatively small pool of ancestors, their genetic polymorphism is rather limited[47].

A new approach to study host-parasite relationships has been the use of wild rodents, particularly primary reservoirs, as experimental animal models. They are, as the human being, genetically polymorphic and represent an emerging system for the genetic analyses of the physiological and behavioral bases of habitat adaptation[47]. Laboratory studies using natural hosts as experimental models provide a suitable indication of the importance of these hosts as reservoirs, since it allows a better understanding of the dynamics of infection, especially concerning the ability to retain the infection and amplify parasite populations in a given environment, due to features that favor parasite transmission (e.g., presence of parasites in the skin). Moreover, the study of these rodents could allow the understanding of the mechanisms involved in immune activation during nonpathogenic and pathogenic infections, to clarify how the reservoir immune response regulates *Leishmania* infection and how the parasites evade a sterilizing immune response.

The role of several species of rodents as wild reservoirs of *Leishmania* species is well known[24,33,96,110]. However, there are only a few studies that followed up experimentally infected wild hosts by *Leishmania* species, mostly due to the difficulties of managing wild mammals in captivity. To date the host-parasite interactions involved in persistent infections in different *Leishmania* reservoirs are unknown.

Sigmodon hispidus has been identified as *Leishmania* reservoir, however no studies of experimental infection have been carried out with this pathogen[33]. Currently *Sigmodon hispidus* is used as a model for the study of various infectious diseases, mainly caused by viruses and bacteria, due to its high susceptibility to a wide variety of pathogens[80]. A large number of cytokine and chemokine genes have been cloned and sequenced and monoclonal antibodies have been generated in order to facilitate its use as an experimental animal model. Recently, low levels of NO production and iNOS expression similar to human macrophages were found in *Sigmodon hispidus* infected with bacteria[23]. These similarities could explain the high susceptibility of this rodent to human pathogens.

Thrichomys laurentius is a South American caviomorph rodent formerly included in a monospecific genus, in which the importance of the retention of infection and transmission of *Leishmania* species has been established. These rodents were found infected with *Leishmania* species of different complexes – *L. (L.) mexicana* and *L. donovani* – in an endemic area of both visceral and tegumentary leishmaniasis in Brazil. *Thrichomys laurentius* was adapted to captivity and experimental patterns of *L. infantum* and *L. braziliensis* infections were identified in this rodent. Both *Leishmania* species demonstrated the ability to invade and persist in the viscera and skin of *T. laurentius*, yet no rodent displayed skin lesions, histological changes in skin, spleen or liver, nor clinical evidence of infection[96].

In the Yucatan peninsula of Mexico, *Peromyscus yucatanicus* has been identified as primary reservoir of *L. (L.) mexicana*[22,109]. It has been adapted to the laboratory and a colony was established for experimental studies.

P. yucatanicus inoculated with 10^6 promastigotes of *L. (L.) mexicana* on the base of the tail reproduced both the clinical and histopathological picture of CL in humans, supporting its utility as a novel experimental model to study CL caused by *L. (L.) mexicana*[104]. Moreover 100% of *P. yucatanicus* inoculated with 10^2 ("low inoculum") developed subclinical infection (absence of clinical signs and evidence of parasite´s DNA at the site of inocula) and when immunosupressed with cyclophosphamide a reactivation with the appearance of lesions was observed[29]. Nitric oxide production was documented in co-cultured macrophages and lymphocytes from *P. yucatanicus* with clinical and subclinical infection caused by *L. (L.) mexicana*[64]. Although NO production was observed in these wild rodents, they were unable to clear the infection, which differs with the response observed in murine models where the generation of NO is the main effector mechanism involved in the control of *L. major* infection. Similarly, the role of this cytotoxic molecule in the antileishmanial activity of human macrophages remains controversial[42].

Recently cDNAs of Th1, Th2 and Th17 cytokines have been amplified from *P. yucatanicus* spleen cells by PCR using *P. maniculatus* primers cloned into TOPO TA cloning vector and sequenced[63,111]. These results strongly support employing *P. maniculatus* specific primers to study the kinetics of cytokines involved in the immune response against clinical and subclinical *L. (L.) mexicana* infection in *P. yucatanicus*. This approach will allow the quantifying and analyzing of the expression of important cytokines, transcription factors and cellular markers involved in the immune response to *L. (L.) mexicana* infection in a specific manner. It will also permit to determine the immune response leading to the clinical and subclinical infection in Yucatan deer mice and compare with the immune response observed in humans, in order to confirm its importance as an experimental model to study LCL caused by *L. (L.) mexicana.*

CONCLUSIONS

First of all, there is a worldwide agreement regarding the concept that experimental animal models are expected to mimic the pathological features and immunological responses observed in humans when exposed to a variety of *Leishmania* spp. with different pathogenic characteristics[50]. This approach deserves to be re-analyzed based on updated studies.

What does it mean to "mimic the patholgical features"? It is clear to date that the outcomes of the infection depend on a variety of factors in each particular laboratory animal including: a) the *Leishmania* spp. inoculated; b) the virulence of the parasite isolate used; c) the parasite stage, via, size, and route of the inoculum. In addition, the nature of each laboratory animal, i.e. the genetic makeup that relates to the immunological background, which plays an important role in host-parasite realtionships. Moreover, when we say "leishmaniasis" as so "the leishmaniases", we are referring to a group of diseases that are caused by different species of protozoan parasites of the genus *Lesihmania*. We have been trying (as it has been done in other pathologies such as "cancer") to include different diseases as a single pathological entity. Therefore, shouldn't we develop a different experimental animal model for each leishmaniasis?

It is well known that infection begins when an infected female sand fly takes a blood meal from a human host in a leishmaniasis endemic area. Following inoculation into the skin by the sand fly bite, the flagellated promastigotes penetrate into the macrophage, transform into amastigotes and multiply. The infected macrophage eventually bursts and the released parasites are able to infect new phagocytic cells. When the infected host is bitten by another female sand fly, parasites are ingested and the life cycle continues. The course of the disease is variable ranging from spontaneous healing to chronicity, but most infected individuals remain asymptomatic or subclinical. Therefore, there is a wide infection spectrum as a result of the parasite inoculation. As a consequence it is necessary to study the significance of subclinical infection in humans and other hosts. Therefore, when building a "good" animal model to study leishmaniasis immunology, should we consider all the possible outcomes of the *Leishmania* spp. infection, particularly subclinical infection?

With reference to the suggested requirement to "mimic immunological responses observed in humans" when exposed to a particular *Leishmania* spp., the problem becomes more complex if we consider that it varies depending on multiple factors according to present knowledge of the host-parasite interaction. In a recent work of the Working Group on research Priorities for Development of Leishmania Vaccines, a good review was made on vaccine trials in the last three decades, the profile of strategies, and animal models used in leishmaniasis trials[108]. The main questions raised encompassed issues concerning all of the leishmaniases. They have addressed the employment of live attenuated or genetically modified parasites, the role of vectors, and elucidation of protective immunity. Regarding the last issue, they considered it crucial to test vaccine candidates in different models using different species, and to test the effects of including salivary proteins of vectors. The major challenge is the absence of an experimental animal model that mimics the whole picture of human leishmaniasis, i.e. different subclinical and clinical outcomes and protective immune response. This situation leads to the necessary development of research studies focused mainly on building new animal models capable of evaluating the same criteria in both models and humans.

The use of wild rodents, primary reservoirs, as experimental models for studying *Leishmania* infection could be very useful to elucidate their role as reservoirs so as to improve our knowledge about the parasite-vector and parasite-host relationship, in order to understand what happens in human *Leishmania* infections.

FINAL CONSIDERATIONS AND PERSPECTIVES

The use of experimental animal models remains a good alternative for designing immunological studies that, for ethical reasons, cannot be performed in humans. Certainly, the increased interest in studying the immune response against *Leishmania* infection in different animal models has contributed to our understanding of parasite-host relationship. However no model can develop all the possible outcomes of the *Leishmania* infection or entirely reproduce the disease in humans. Thus, there are still so many questions to answer in order to find control strategies or a successful vaccination program.

Although mouse model has widely contributed to the understanding of immune response against *L. major* infection many studies have demonstrated profound differences in the immune mechanisms related to infections with New World *Leishmania* species. Furthermore, visceral infection in mice does not mimic the pathological features and immunological responses observed in human cases. The hamster

result is a better model to study the progressive disease of visceralizing *Leishmania* spp. The lack of reagents for immunological analysis and the strong immunosuppression of the lymphoproliferative response in hamsters make difficult its use for the evaluation of vaccine candidates. The increased interest of researchers to use dogs as experimental models lies in the possibility of studying the immune response in natural infection. Since dogs are important reservoirs of visceralizing *Leishmania*, vaccination of these animals would constitute a major step towards the control of human infections. Finally, the use of monkeys has been explored for testing vaccine candidates, however little is known as to whether the immune response to *Leishmania* infection is similar to that observed in humans.

In order to obtain more meaningful data regarding immune response that parallels human disease it is very important to continue with the efforts in developing strategies to mimic natural transmission such as the use of low infectious doses, bioactive saliva and natural reservoir hosts to have a better approximation of the dynamics of natural infection. These approaches could contribute to developing improved experimental models for studying leishmaniasis and identifying possible targets to evaluate vaccine candidates.

RESÚMEN

Modelos animales para el estudio de la inmunología de la leishmaniosis

Las leishmaniosis siguen siendo un importante problema de salud pública a nivel mundial y se clasifican como categoría I por el programa TDR/WHO, debido principalmente a la ausencia de control. Muchos modelos experimentales tales como roedores, perros y monos han sido desarrollados, cada uno con características específicas, para caracterizar la respuesta inmune a las diferentes especies de *Leishmania*, sin embargo ninguno reproduce la patología observada en la enfermedad humana. La diversidad en los resultados obtenidos podría deberse en parte a que diferentes cepas de parásitos o especies están siendo examinadas, diferentes tejidos (cojinete plantar, oreja o base de la cola) han sido infectados y diferente número ("bajo" $1x10^2$ y "alto" $1x10^6$) de promastigotes metacíclicos han sido inoculados. Recientemente, nuevos enfoques han sido propuestos con el fin de obtener datos más significativos en cuanto a la respuesta inmune del huésped y a la patogénesis, de tal forma que reproduzcan lo que ocurre en la enfermedad humana. El uso de la saliva del insecto y de un número de parásitos menor en las infecciones experimentales ha permitido reproducir la transmisión natural, identificar nuevas moléculas, así como mecanismos inmunes que deberían ser considerados en el diseño de vacunas y estrategias de control. Adicionalmente, se ha propuesto como una buena alternativa el uso de roedores silvestres como modelos experimentales tanto para el estudio de las relaciones huésped-patógeno como para probar nuevas vacunas. A la fecha, el uso de reservorios naturales para estudiar la infección por *Leishmania* ha sido un reto, debido a la carencia de reactivos inmunológicos para uso en roedores silvestres. Esta revisión describe los principales hallazgos inmunológicos ante la infección por *Leishmania*, en los diferentes modelos animales, destacando la importancia del uso de condiciones experimentales similares a la transmisión natural y de reservorios como modelos experimentales para el estudio de la inmunopatología de la enfermedad.

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