Review Article

Searching Genes Encoding *Leishmania* Antigens for Diagnosis and Protection


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Leishmaniases are a wide spectrum of parasitic diseases caused by the infection of different species of the genus *Leishmania*. Currently, these diseases are one of the most neglected diseases threatening 350 million people in different countries around the world. Thus, these diseases require better screening, diagnostics and treatment. An effective vaccine, that is not currently available, would be the best way to confront leishmaniases. In the past 20 years the molecular characterization of *Leishmania* genes encoding parasite antigens has been carried out. In this review we summarize the most common strategies employed for the isolation and characterization of genes encoding *Leishmania* antigens. To provide a collective view, we also discuss the results related with diagnosis and protection based on different recombinant DNA-derived *Leishmania* products.

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1. Introduction

Leishmaniases comprise a complex group of diseases caused by several intracellular protozoa of the genus *Leishmania* that infects macrophages from a variety of mammals including human and dogs. These parasites possess a digenetic life cycle and they develop as promastigotes in the gut of the blood-sucking phlebotominae sandflies and as intracellular amastigotes in macrophages of their vertebrate hosts. Depending largely on the species of the parasite and the immunocompetence state of the human host, the disease spectrum ranges in severity from cutaneous (CL), post-kala-azar dermal (PKDL) and diffuse cutaneous (DCL), to mucocutaneous (MCL) and visceral leishmaniases (VL). These infections are endemic in 86 tropical and subtropical countries around the world, accounting for at least 75,000 deaths per year [1]. Canine viscerocutaneous leishmaniases (VCL) is an important emerging zoonosis in countries around the Mediterranean basin, in the Middle East, and in Latin America [2]. This severe form of the disease is caused by *Leishmania infantum* in the Mediterranean area, Middle-East, and Asian countries and by *Leishmania chagasi* in Latin America being wild canids and domestic dogs the major reservoir, playing central role in the transmission to humans [3]. The outcome of infection is determined by interactions between the host immune system and the different parasite species, yet the pathogenesis of leishmaniases remains unclear and the understanding of the mechanisms involved in the immune response to *Leishmania* in humans and dogs is still limited. Generally, protective immunity is associated with a classical cell-mediated immune response that induces macrophage activation by T cells-derived cytokines, while nonhealing disease is associated with the generation of strong humoral responses [4, 5].

Conclusive diagnosis of leishmaniases depends on the detection of amastigotes after staining of bone marrow or splenic aspirates from visceral cases, or biopsy samples taken
from the infected tissues in cutaneous or mucocutaneous patients [6]. The detection of promastigotes by culturing the biopsy samples in a suitable medium can also be employed for diagnosis [6, 7]. The presence of anti-Leishmania specific antibodies during infection has allowed the development of serologic test including immunofluorescent antibody test (IFAT), western blot, immunochromatographic test, and enzyme-linked immunosorbent assay (ELISA) [7]. Sensitivity and specificity of these assays depend on the antigen preparation employed since some of the parasite antigens exhibit cross-reacting epitopes shared by other pathogens. As an strategy to develop specific serodiagnostic test for leishmaniasis, the genes encodings for some parasite antigens were isolated in order to obtain recombinant molecules suitable for serodiagnosis [8].

Different strategies have been employed for the generation of vaccines against Leishmania but, to date, there is no vaccine against this parasite in routine use. The history of Leishmania vaccines has been reviewed by different authors [4, 9–11]. Progress toward vaccine development has included human trials using live vaccines with Leishmania major promastigotes (parasites that cause self healing skin ulcers) performed in the 1980s. Although a high percentage of successful lesion reduction was observed [12, 13] the use of these live vaccines had so many secondary effects that made difficult the standardization of these approaches. Vaccines based on killed promastigotes were also analyzed. The results from several clinical trials using whole parasite antigens showed little or no protection [9, 10]. For the past 20 years, DNA cloning and characterization of genes encoding parasite proteins suitable for the development of defined vaccines have been carried out.

For the development of diagnostic tools as well as second-generation vaccines based on recombinant products (recombinant proteins and/or DNA vaccines) the genes coding for parasite antigens should be cloned and characterized. The first approach for the characterization of Leishmania antigens was the biochemical purification of some parasite membrane fractions or proteins secreted different live stages of the parasite (usually promastigotes). Leishmania secreted factors as well as proteins located in the parasite surface are the most important in the establishment of the infection, either during the first contact of the parasite with the host cell, or interfering with immune cells functions, like cytokine production, antigen presentation, or cell activation. As many of these molecules are complex antigen mixtures, their role in diagnosis and protection is not described in detail in this review. However, it is worth mentioning that several protein fractions were used to assess their immunogenicity. Further examples are FML, LiESAp-MDR, Ric-1, and Ric-2. FML stands for Fucose-Mannose Ligand and it is a membrane-enriched preparation of L. donovani [14] that can be employed for serodiagnosis of human and canine VCL [15, 16]. FML was patented in Brazil as Leishmune and it is being used commercially for canine vaccination [17]. The major immunogenic component of the FML extract is a glycoprotein of 36 kDa (LdGP36) [18].

To date, this protein was not obtained as a recombinant product. The combination of naturally excreted/secreted antigens, purified from culture supernatant of L. infantum promastigotes (LiESAp) with muramyl dipeptide (MDP) as adjuvant, conferred protection to dogs experimentally infected with L. infantum [19]. A double blind trial was performed in naturally infected dogs in France with LiESAp, showing a significant decrease in the incidence of infection two years after the vaccination [20]. Ric-1 and Ric-2 are also protein fractions secreted by L. infantum promastigotes, Ric-1 contains high molecular weight excreted proteins and Ric-2 the low molecular weight ones. These two proteins fractions were able to induce different immune responses, mainly by the modulation of the Th1/Th2 cytokine balance [21]. Immunization with Ric-1 and Ric-2 resulted in a reduction of 50% and 67% in the parasite burden of the spleen from infected mice, respectively [22].

The most common assay for the isolation of genes coding for surface or secreted proteins was the generation of genetic probes designed on the basis of the aminoterminal aminoaacid sequence obtained after their biochemical purification. These genetics probes were employed for the screening of genomic libraries constructed in bacteriophage lambda or cosmid vectors [23–25] (Figure 1). Alternatively, the sera from mice immunized with the purified secreted fractions were employed for the immunoscreening of cDNA or genomic expression libraries (since Leishmania protein coding regions are almost never interrupted by introns [26, 27]) to obtain the genes encoding for the most immunogenic proteins contained in these fractions [28–30] (Figure 1).

After Leishmania infection, humoral responses against different parasite antigens are elicited and significant antibody levels are detected in the sera from patients and dogs suffering the disease (reviewed in [7]). For that reason, the immunoscreening of parasite expression libraries (cDNA or genomic) with the sera from dogs suffering VCL [31–36], or human patients with VL [37, 38] or with MCL [39, 40] was the most common strategy employed in the identification and isolation of Leishmania antigen coding genes. Also, the sera from experimentally infected mice were employed for immunoscreening purposes [41]. Usually, the antigens isolated after these screenings were intracellular proteins. In addition, many of them can be considered as members of conserved families (reviewed in [42, 43]) (Figure 1).

Some other experimental approaches were employed for the characterization of Leishmania antigens. Persistent immunity against Leishmania infection is mediated predominantly by CD4+ T cells of the Th1 phenotype [44–46]. For this reason, the characterization of library clones that stimulate interferon-gamma (IFN-γ) production in T cell clones established from L. major-infected mice was an alternative procedure for the isolation of parasite antigen encoding genes [47, 48]. Also, T cell clones were established from Montenegro (DTH) skin test positive adults residing in regions endemic for VL [49]. Interestingly, many of the genes identified using this methodology were the same genes identified after immunoscreening of expression libraries with the sera from infected human or dogs (Figure 2).

Mice immunization with naked DNA composed of mammalian expression vectors recombinant for heterologous genes induces cellular (mainly CD4+ Th1 and CD8+...
Two parasite genes encoding secreted proteins have been characterized (Table 1). Immunization with Thiol-specific antioxidant (TSA) proteins was identified in 1998 by Webb et al. [28]. This protein was located in the culture filtrate material from in vitro cultivated L. major promastigotes (CFP). The CFP was highly antigenic and immunogenic in the experimental murine leishmaniasis model [47]. To identify immunogenic components of the promastigote CFP, serum samples from CFP-vaccinated BALB/c mice were used to screen an L. major cDNA expression library. The cDNA encoding L. major TSA was isolated and expressed as a recombinant protein in bacteria (rTSA) [28]. Immunization of the recombinant protein using interleukin-12 (IL-12) as adjuvant resulted in the protection against L. major-infected humans. Thus, the sera from some CL (12/27) and VL (12/28) patients contain significant titres of antibodies against rTSA [28]. Also, this antigen elicited in vitro proliferative responses from peripheral blood mononuclear cells (PBMCs) obtained from these patients [28]. The presence of specific anti-TSA antibodies in the sera from dogs with VCL was recently demonstrated [59] (see Table 2).

The protein SIR-2 belongs to a highly conserved protein family found in both prokaryotic and eukaryotic species named Hst proteins or sirTuins [60]. Historically, the biological importance of SIR-2-related proteins was attributed to chromatin condensation and transcriptional silencing, having the histones as their physiological substrates [61]. However, other localizations were documented among the diversity of SIR-2 homologues in different organisms. Leishmania SIR-2 cDNA was identified in 1996 by immunoscreening of an L. major cDNA library with the sera from a mouse immunized with L. major antigens that interact with glutathione [58] but subsequently it was found in other Leishmania species with a high degree of homology [62]. The interest of the protein was related to its possible role in the regulation of the parasite cell cycle and as a putative

<table>
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<th>Table 1: Genes encoding Leishmania secreted/excreted antigens.</th>
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<td>Thiol-specific antioxidant (TSA)</td>
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<td>Sirtuin-2 (SIR-2)</td>
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responses) and humoral responses, that are protective against several pathogens (reviewed in [50, 51]). Immunization with genomic expression libraries (ELI) of Leishmania induced protective responses against parasite infection in mice [52]. Thus, ELI emerged like an alternative approach for the characterization of genes encoding parasite protective antigens. For that purpose, sequential fractions of Leishmania libraries constructed in eukaryotic expression plasmids were isolated on the basis of the induction of protective immunity and the genes composing these fractions were identified [53, 54] (Figure 2).

Finally, the search for genes overexpressed in the infective form (metacyclic promastigotes) or in the amastigote stage allowed the characterization of different genes coding for parasite antigenic proteins [55–57].

In this work the strategies employed for the isolation of genes encoding parasite antigens were reviewed. We summarize the results related with diagnosis and protection based on different recombinant DNA-derived Leishmania products.

2. Diagnostic and Vaccines Based on Secreted and Excreted Antigens

Two parasite genes encoding secreted proteins have been characterized (Table 1).

The Leishmania homolog of the eukaryotic thiol-specific-antioxidant (TSA) proteins was identified in 1998 by Webb et al. [28]. This protein was located in the culture filtrate of L. major promastigotes (CFP). The CFP was highly antigenic and immunogenic in the experimental murine leishmaniasis model [47]. To identify immunogenic components of the promastigote CFP, serum samples from CFP-vaccinated BALB/c mice were used to screen an L. major cDNA expression library. The cDNA encoding L. major TSA was isolated and expressed as a recombinant protein in bacteria (rTSA) [28]. Immunization of the recombinant protein using interleukin-12 (IL-12) as adjuvant resulted in the protection against L. major-infected humans. Thus, the sera from some CL (12/27) and VL (12/28) patients contain significant titres of antibodies against rTSA [28]. Also, this antigen elicited in vitro proliferative responses from peripheral blood mononuclear cells (PBMCs) obtained from these patients [28]. The presence of specific anti-TSA antibodies in the sera from dogs with VCL was recently demonstrated [59] (see Table 2).

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![Figure 1: The most common approaches employed for the isolation of genes encoding Leishmania antigens.](Image)
candidate for a cell-division control marker, a biological function that possessed its homologous protein in yeast. However, SIR-2 was shown two years later to be located in the cytoplasm of L. major and also that the protein is among the parasite excreted-secreted antigens (ESAs) [63]. These findings addressed the SIR-2 biological role in Leishmania to an unknown role, rather than the gene expression regulation. The L. major SIR-2 protein was obtained as a recombinant protein expressed in bacteria [63]. rSIR-2 was able to induce the activation of B cells from normal BALB/c mice and its injection induced B-cell differentiation and production of specific antibodies through a T-cell the independent mechanism [64]. Due to all these observations, it was postulated that the presence of SIR-2 specific antibodies induced by immunization with the rSIR-2 can have a protective role since BALB/c-vaccinated mice presented a reduction in the parasite load after L. infantum infection [42].

The use of the L. major rSIR-2 as antigen demonstrated that this protein is highly antigenic during natural canine infections both in symptomatic and asymptomatic infected dogs, showing a preferential IgG2 production [65]. The rSIR-2 was also recognized by the sera from children with VL caused by L. infantum infection [66] (see Table 2).

3. Diagnostic and Vaccines Based on Surface Antigens

Leishmania parasites show different surface molecule composition during their life cycle. Both procyclic and metacyclic promastigotes are covered by glycoproteins and other glycosylated molecules, which are anchored to the surface membrane by glycosylphosphatidylinositol (GPI) forming the glycocalix which is almost completely absent from amastigotes [67, 68]. The main surface molecule of promastigotes is lipophosphoglycan (LPG), although some other surface molecules were described. Four different Leishmania genes encoding surface proteins have been characterized (Table 3). In this section we summarize the role of these membrane proteins in the development of diagnostic tools as well as vaccines (see Table 4).

The Leishmania proteinase GP63 is one of the most abundant surface-exposed proteins on parasite promastigotes [67, 69]. GP63 was identified and purified in 1985 [70]. The most commonly used name, GP63, is derived from glycoprotein of 63 kDa, even though isoforms with different molecular weights were described in many Leishmania species [71]. The first gene encoding GP63 was isolated from L. major [23]. A synthetic oligonucleotide probe based on the aminoterminal protein sequence of purified GP63 was employed in the screening of an L. major genomic library [23]. Since then, the genes encoding this protein were characterized in many other Leishmania species (see [71] for a review). The conservation of the coding sequence of GP63 amongst diverse species of Leishmania provides further support for the importance of GP63 during the life cycle of the parasite [23, 70, 72–74].

Several authors analyzed the antigenicity of the GP63 protein. The use of native L. donovani GP63 as antigen in ELISA seems to distinguish an ongoing from a past VL infection [75]. L. chagasi and L. donovani GP63 were obtained as recombinant proteins expressed in bacteria and were employed in ELISA assays [76]. The sera from the most acute VL patients showed a high reactivity against both recombinant proteins while sera from other forms of leishmaniasis (CL and MCL) and from Chagas’ disease patients showed very low reactivity [76]. L. infantum rGP63 was recognized by the sera from dogs naturally infected with this parasite [77]. These results indicate that rGP63 might be
development of vaccine and that coencapsulation of CpG ODN in liposomes improves the immunogenicity of the GP63 *Leishmania* antigen.

Recombinant *Salmonella typhimurium* [87] or bacille Calmette-Guérin (BCG) [88, 89] expressing *Leishmania* GP63 were also tested for the development of vaccines in murine experimental models. Spleen cells from CBA mice orally immunized with *S. typhimurium* expressing rGP63 developed antibody and proliferative T cell response to *L. major*. The activated T cells are mainly CD4+ and secrete IL-2 and IFN-γ but no IL-4 [87]. These mice did not develop lesions after infection with *L. major* [87]. Mice immunization with recombinant BCG producing GP63 elicited significant protection against a challenge with *L. major* [88] and a strong protective response against challenge with *L. mexicana* promastigotes or amastigotes [89]. Finally, immunization of a eukaryotic expression plasmid containing a cDNA coding for *L. major* GP63 in BALB/c mice induced Th1 responses specific for the parasite GP63 and the mice showed a partial protection against *L. major* infection [90].

Defined vaccines based in GP63 peptides were also tested. Thus, wo peptides representing predicted T-cell epitopes of GP63 were tested on a murine model of CL. Either subcutaneous or intraperitoneal immunization in saline with a peptide representing GP63 amino acids 467–482, significantly protected CBA mice against the development of severe cutaneous lesions only when the peptide was intrinsically modified by covalently adding of a lauryl-cysteine moiety (LC-p467) to its amino terminus during synthesis. In marked contrast, administration of p467 alone, cysteinyl-p467, or GP63 protein in saline resulted in some disease exacerbation. The activated T cells are mainly CD4+ and secrete IL-2 and IFN-γ [88, 90].

The efficacy of vaccines based on GP63 was firstly tested by the administration of a native purified GP63 reconstituted in liposomes. These liposomes induced appreciable levels of protection depending of the route of immunization in different murine models of CL [81, 82] or VL [83, 84]. Similar results were found by Jaafari et al. studying the ability of the *L. major* rGP63 expressed in bacteria [85, 86]. The recombinant protein lacking the sugar molecules present in the native protein-induced protection against *L. major* infection in susceptible BALB/c mice when it was administered entrapped in liposomes in the absence [85] or in the presence of CpG oligodeoxynucleotides (CpG ODN) [86]. In these works it was shown that immunization of rGP63 alone conferred a partial protection while entrapment of rGP63 in liposomes significantly increased the rate of protection, particularly when coadministered with CpG ODN, inducing high Th1 responses. Taken together, the results indicate that liposomes may be used as a suitable adjuvant for the development of vaccine and that coencapsulation of CpG ODN in liposomes improves the immunogenicity of the GP63 *Leishmania* antigen.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Diagnosis</th>
<th>Vaccination</th>
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<tbody>
<tr>
<td>Glycoprotein of 63 kDa (GP63)</td>
<td><em>L. major</em></td>
<td>rTSA + IL-12 [28]</td>
</tr>
<tr>
<td>Glycoprotein of 46 kDa (GP46/M2/PSA)</td>
<td><em>L. major</em></td>
<td>rSIR-2 [42]</td>
</tr>
<tr>
<td>Kinetoplastid membrane protein</td>
<td><em>L. donovani</em></td>
<td></td>
</tr>
<tr>
<td>Hydrophilic surface protein B1</td>
<td><em>L. major</em></td>
<td></td>
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Table 2: Summary of secreted excreted antigens regarding diagnosis and vaccination.

Table 3: Genes encoding *Leishmania* surface antigens.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Presence of antibodies documented in</th>
<th>Animal model</th>
<th>Immunization mode</th>
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<tbody>
<tr>
<td>TSA</td>
<td>Human VL and CL [28]</td>
<td>Mice CL</td>
<td></td>
</tr>
<tr>
<td>SIR-2</td>
<td>Human VL [66]</td>
<td>Mice VL</td>
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It was described the existence of another family of glycoprotein in the promastigote membrane of different Leishmania species (except L. braziliensis [93]) namely PSA-2 (for promastigote surface antigen 2 [30]), GP46 (because of its molecular weight, or M2 (because of its reactivity against a monoclonal antibody, namely, M2 [94]). L. major GP46 coding gene was isolated after immunoscreening of a genomic expression library with the polyclonal antisera of a mice immunized with surface and integral membrane proteins of the parasite [30]. Purified L. amazonensis M2 protein was partially sequenced for the design of a synthetic oligonucleotide that was employed in the screening of an L. amazonensis library [24].

The antigenicity and immunogenicity of Leishmania GP46 were analyzed. PBMCs from patients recovered of CL proliferate in response to native GP46 (but not to the recombinant protein produced in bacteria) and produce high levels of IFN-γ. In addition, the recombinant protein expressed in bacteria was recognized by the sera from VL patient and VCL dogs, indicating that some B cell epitopes are maintained in the absence of the sugar molecules moiety [95, 96].

The development of vaccines based on the rGP46 protein was evaluated in different experimental models. In the L. major model, protection depends on the source of GP46 antigen and the immune responses induced by the adjuvant. Whereas protection was observed with the native protein (purified from L. major membrane) or expressed as a recombinant protein in L. mexicana, vaccination with the rGP46 expressed in bacteria did not confer protection [97]. The same adjuvant, Corynebacterium parvum, was employed in these assays [97]. These data were taken as an indication that the natural form of the antigen, including the posttranslational modifications was essential for its vaccinating potential. Similar results were obtained when the rGP46 expressed in bacteria was immunized in mice using two different adjuvants (C. parvum or Quilaja saponin) [98, 99]. The lack of protection against L. major infection was correlated with the induction of mixed Th1-Th2 responses. However, the immunization with an rGP46 DNA vaccine induced an exclusive Th1 response and mice were protected against an L. major challenge [98]. The redirection of the immune response towards a Th1 profile after GP46 DNA vaccination also had a significant therapeutic effect. In L. major-infected mice, vaccination with DNA encoding the GP46/M2 after parasite challenge caused reduction in the lesion size and promoted healing in both genetically resistant C3H/He and susceptible BALB/c mice [100]. In other experimental model, DNA vaccination with a recombinant expression plasmid encoding L. amazonensis GP46 induced Leishmania-specific humoral and lymphoproliferative immune responses that partially protected against a challenge with L. mexicana [101, 102]. Finally, immunization of susceptible BALB/c mice with an attenuated recombinant vaccinia virus expressing L. amazonensis GP46 induced robust protection against L. amazonensis challenge [103].

The L. donovani Kinetoplastid Membrane Protein 11 (KMP-11) is a dominant surface membrane glycoprotein associated with the LPG and is a potent T cell-stimulating factor, suggesting that it may be an important molecule for induction of cell-mediated immune responses [104, 105]. KMP-11 gene cloning was made by screening of a L. donovani genomic cosmid DNA library with an oligonucleotide probe designed on the basis of the partial aminoacid sequence obtained from the purified protein [25]. Afterward, L. infantum [106] and L. panamensis [107] KMP-11 coding genes were also cloned.

The use of native L. donovani KMP-11 as well as recombinant L. infantum KMP-11 as antigen in ELISA assays revealed that this protein was recognized as a B-cell antigen during human VL and canine VCL. Reactivity to native KMP-11 was found in a 58% of the serum samples from Sudanese VL patients [108]. L. panamensis rKMP-11 expressed in bacteria was recognized by sera from American VL (76%), MCL (60%), and CL (37%) patients [109]. A high reactivity was found against L. infantum rKMP-11 expressed in bacteria in the sera from VL patients infected with L. chagasi and lower reactivity was found in sera from individuals with subclinical L. chagasi infection [110]. This protein was also recognized by canine VCL sera [106]. All these results indicate that this parasite protein is a major antigenic molecule in the different clinical forms of the leishmaniasis disease. Native L. donovani KMP-11 is also a potent T cell stimulating antigen for CD4+ murine T cells [111] and induces the up-regulation of IFN-γ mRNAs in PBMCs from dogs experimentally infected with L. infantum [112].

There are two studies showing that immunization with rKMP-11 conferred immunoprotection against Leishmania infection in different animal models. Hamsters vaccinated with a KMP-11 DNA vaccine were protected against the development of VL caused by both pentavalent antimonial sensitive and resistant virulent L. donovani strains [113]. The strong protection observed in this highly susceptible model correlated to the generation of Th1/Th2 mixed responses as well as CD8+ T-cell activation. Furthermore, the administration of a recombinant Toxoplasma gondii expressing L. major KMP-11 induced a transient protection against CL in BALB/c mice [114].

Characterization of another Leishmania surface protein (HASPB1) was carried out in a search for genes preferentially expressed in infective stages of the Leishmania life cycle. HASPs represent a heterogeneous family of hydrophilic acylated surface proteins. The initial interest in this protein family was related to the identification of molecules associated with the change in parasite virulence. To address this question, a cDNA stage-specific library was constructed from metacyclic parasites of L. major and after RNA hybridization with stationary phase parasite culture RNA, a specific cDNA was found to be highly expressed in the infective form when compared to the noninfective parasites [56]. Five genes were encoded within this family and the protein products were described as surface peptide markers for infective forms of L. major [115, 116]. Afterward, two homologous genes were found in L. donovani, termed then HASPA and HASPB1. Their immunogenic properties were proved, because there was a strong recognition of infected mice sera to the recombinant HASPB1 [117]. This fact, together with the ubiquity of this protein family in
different Leishmania species, prompted to consider HASPB1 as a suitable candidate for immunodiagnosis and vaccine development.

A study in humans reported that more than 90% of the VL and PKDL patients under study had antibodies able to recognize rHASPB1, confirming the suitable usefulness of HASPB1 for diagnosis [118]. Moreover, rHASPB1 immunized alone induced the protection against experimental VL in the murine model, reducing both the hepatic and splenic parasite burden in vaccinated animals [119]. Immunization using rHASPB1 was assessed not only in mice, but also in a vaccine trial against canine leishmaniasis. Vaccination with rHASPB1 plus Montanide was able to induce a partial protection in 50% of the immunized dogs against infection with L. infantum [120].

4. Diagnostic and Vaccines Based on Intracellular Proteins

Many intracellular parasite proteins interacting with the immune system after Leishmania infection have been characterized. Usually, they are conserved proteins that predominantly stimulate humoral responses in VL and MCL patients, dogs suffering VCL or Th2-mediated humoral responses in experimentally infected mice. Some of these antigens are the heat shock proteins, the acidic ribosomal proteins, the nucleosome forming histones [43], a kinesine-like Leishmania protein [121], cysteine proteinases [122, 123], or actin and tubulin [124]. Remarkably, antibodies against some of these proteins specifically recognize the parasite antigens without cross-reactivity with the host counterparts. This specificity is based on the location of their antigenic determinants in the more divergent regions of these parasite proteins. For that reason, these antigens possess a potential interest for diagnosis. On the other hand, although proteins that induce high humoral responses during the infectious process were not first considered as good vaccine candidates, recent works demonstrated that their vaccination may induce immunity against leishmaniasis. Table 5 summarizes some of the characterized genes encoding parasite intracellular antigens.

4.1. Ribosomal Proteins. Among the evolutionary conserved antigens of Leishmania, several lines of evidence suggest that ribosomal proteins are immunologically relevant molecules during infection. Some ribosomal constituents can contribute to the host immune system dysfunction through their capacity to modulate cell activities and cytokine release during infection. For example, the genetic immunization of a DNA vaccine encoding the putative 60S ribosomal protein L31 induced Th2 cytokines as well as IL-10 in mice. As a consequence of the immune response elicited, an increase in parasite burden was observed in these animals [54, 135]. Another ribosomal protein that seems to participate in the immunoregulatory processes that play a role in the balance of the Th1 and Th2 responses after infection is the L. major ribosomal protein S3a. This protein was purified from parasite proteins extracts using an S-hexylglutathione agarose affinity column [125]. The purified protein was employed for the immunization of mice and the antibodies elicited were employed to screen an L. major cDNA library [125]. The cDNA encoding L. major S3a protein was cloned into a prokaryotic expression plasmid and the purified recombinant protein was employed for the analysis of its immunogenicity [136]. rS3a was able to induce activation of B cells from normal BALB/c mice and its injection induced a non-specific polyclonal B-cell activation and inhibited T-cell proliferation [136]. Thus, it was hypothesized that the interaction of this protein released by parasite cytology may induce humoral responses and the down-regulation of cellular responses after parasite infection. The presence of high humoral responses against other ribosomal proteins was also documented. Thus, some members of the parasite acidic ribosomal P protein family are recognized by the sera from dogs and humans suffering VL [43, 126, 137, 138].

Three members of the Leishmania P protein family (namely, P0, P2a, and P2b) are constituents of the large subunit of the ribosome. They were classified according to their homology to the Saccharomyces cerevisiae counterparts [33, 139]. Leishmania P2a and P2b are anchored to the ribosomes as complexes interacting with the P0 protein, forming a protruding stalk in the ribosome major subunit. This heterocomplex plays an essential role in the elongation step of protein synthesis.

The genes encoding the L. infantum P2a and P2b [33] and the P0 [32] proteins were isolated and characterized after immunoscreening of a cDNA expression library with a canine VCL serum. The P2a and P2b encoding cDNAs were cloned into different prokaryotic expression vectors [138, 140] and the corresponding purified recombinant proteins were employed to analyze their antigenic and immunogenic properties. Both antigens induced a Th2 mediated humoral responses in BALB/c mice when administered without adjuvant. In addition, these responses cannot be reverted by strong Th1 inducers (DNA vaccination or coadministration of the recombinant protein with CpG ODNs) [140, 141]. According to the preponderance of the Th2 or mixed Th1/Th2 responses elicited in BALB/c mice by these inoculations, no evidence of protection was found after infection with L. major [140].

Both rP2 proteins have shown to be useful for diagnosis of Leishmania infection because substantial titres of anti-Leishmania P proteins antibodies are detected in VCL canine sera and in MCL and VL human patients [141, 142]. Although, the acidic ribosomal proteins were also described as prominent antigens in systemic autoimmune diseases [143], in Chagas’ disease [139] and as major allergens in fungal allergies [144], the anti-Leishmania P2 humoral response is specific for the parasite antigens. This specificity was based on the location of the main antigenic determinant recognized by VCL canine sera [141]. Thus, the epitope recognized during the leishmaniasis process was found to be positioned outside the C-terminal domain, a region highly conserved among the eukaryotic P proteins [141]. Like this C-terminal region contains the antigenic determinants recognized by the sera from Chagas’ disease or autoimmune patients [145], the Leishmania P2 proteins were engineered.
Table 4: Summary of surface antigens regarding diagnosis and vaccination.

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<thead>
<tr>
<th>Molecule</th>
<th>Diagnosis</th>
<th>Vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Presence of antibodies documented in</td>
<td>Animal model</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>GP63</td>
<td>Human VL [76]</td>
<td>Mice CL</td>
</tr>
<tr>
<td></td>
<td>Canine VCL [77]</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Mice VL</td>
</tr>
<tr>
<td>GP46</td>
<td>Human VL [95]</td>
<td>Mice CL</td>
</tr>
<tr>
<td></td>
<td>Canine VCL [96]</td>
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<td></td>
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</tr>
<tr>
<td>KMP-11</td>
<td>Human VL [108–110]</td>
<td>Hamsters VL</td>
</tr>
<tr>
<td></td>
<td>Canine VCL [106]</td>
<td>Mice CL</td>
</tr>
<tr>
<td>HASPB1</td>
<td>Human VL and PKDL [118]</td>
<td>Mice VL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dogs VL</td>
</tr>
</tbody>
</table>

to have deletions in the C-terminal region in order to avoid cross-reactivity with sera from those patients [142].

The antigenicity and immunogenicity of the parasite P0 protein were also analyzed using recombinant versions of the protein produced in bacteria [137, 146, 147]. BALB/c mice immunization with a parasite P0-based DNA vaccine or with the rP0 protein combined with GpG ODN, showed a partial protection after challenge with L. major [146, 147]. P0-vaccinated mice showed an initial significant reduction in lesion size after challenge, but mice ultimately developed nonhealing lesion. The delay in the onset of cell growth was accompanied by a substantial decrease in the parasite load and was correlated to the generation of initial Th1 responses that were changed to a mixed Th1/Th2 response against the parasite rP0 when disease progressed. On the other hand, the Th1 responses induced by vaccination conferred protection against CL in C57BL/6 mice [147].

A high percentage of dogs affected with VCL contains antibodies reacting with the L. infantum rP0 [137]. The humoral response, as occurs for the other acidic ribosomal proteins is specifically directed against the parasite P0 protein that possesses a C-terminal region that differs from higher eukaryotes and resembles the archaeabacterial acidic ribosomal protein C-terminal domain [148]. This divergent C-terminal sequence is also present in T. cruzi P0 and contains the antigenic determinant recognized by the sera from Chagas’ disease patients. In fact, the L. chagasi orthologue was characterized by immunoscreening of an L. chagasi expression library with a pool of five T. cruzi-infected sera [126].

Administration of some other ribosomal constituents using immunization procedures inducing Th1 responses was related to the generation of protective responses. Thus, Melby et al. [53] showed that the genetic immunization of a mixture composed by different cDNAs encoding L. donovani parasite proteins including six ribosomal proteins (S14, S13, S16, S18, L8 y L28) resulted in a partial protection in BALB/c mice infected with this parasite. Also, genetic immunization with eukaryotic expression vectors containing the L22 and S19 L. major ribosomal protein coding genes induced a protective response in the BALB/c mice cutaneous model [54]. In addition, a cDNA clone encoding the L. braziliensis ribosomal protein S4 was recognized by a T-cell clone derived from a VL human donor with a positive DTH skin test, residing in an endemic area for VL [49], indicating that the recognition of some of the parasite ribosomal proteins by the host immune system is not necessarily related to disease progression (see Table 6).

4.2. Stress Proteins. The heat shock proteins (HSPs) are produced by prokaryotic and eukaryotic cells in response to a variety of physiological stresses. They are among the most highly conserved and abundant proteins found in living prokaryotic and eukaryotic organisms. As occurs in many other infectious or autoimmune diseases [149], different members of the heat shock protein family were described as antigenic after Leishmania infection (see Table 7).

The HSP83 protein is a member of the Leishmania HSP90s family located in the cytoplasm of parasite promastigotes [150]. The first parasite HSP83 coding gene was
Table 5: Genes encoding *Leishmania* intracellular antigens.

<table>
<thead>
<tr>
<th><strong>Ribosomal proteins</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal protein S3a</td>
<td>(S3a) L. major [125]</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein L31</td>
<td>(L31) L. major [54]</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein S14</td>
<td>(S14) L. donovani [53]</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein S13</td>
<td>(S13) L. donovani [53]</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein S16</td>
<td>(S16) L. donovani [53]</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein S18</td>
<td>(S18) L. donovani [53]</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein L8</td>
<td>(L8) L. donovani [53]</td>
<td></td>
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<tr>
<td>Ribosomal protein L28</td>
<td>(L28) L. donovani [53]</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein L22</td>
<td>(L22) L. major [54]</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein S19</td>
<td>(S19) L. major [54]</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein S4</td>
<td>(S4) L. major [49]</td>
<td></td>
</tr>
<tr>
<td>Acidic ribosomal proteins</td>
<td>(P2a and P2b) L. infantum [33]</td>
<td>(P0) L. infantum [32]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L. chagasi [126]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Stress Proteins</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shock protein 20 kDa</td>
<td>(HSP20) L. amazonensis [127]</td>
<td></td>
</tr>
<tr>
<td>Heat shock protein 60 kDa</td>
<td>(HSP60) L. major [128]</td>
<td></td>
</tr>
<tr>
<td>Heat shock protein 70 kDa</td>
<td>(HSP70) L. donovani [37]</td>
<td>L. infantum [129]</td>
</tr>
<tr>
<td>Heat shock protein 83 kDa</td>
<td>(HSP83) L. braziliensis [40]</td>
<td>L. braziliensis [40]</td>
</tr>
<tr>
<td>Glucose regulated protein 94 kDa</td>
<td>(GRP94) L. infantum [130]</td>
<td></td>
</tr>
<tr>
<td>Stress inducible 1</td>
<td>(LmSTI1) L. major [41]</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Histones</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone H1</td>
<td>(H1) L. major [55]</td>
<td></td>
</tr>
<tr>
<td>Nucleosome histones</td>
<td>(H2A) L. infantum [34]</td>
<td>(H2B) L. infantum [34]</td>
</tr>
<tr>
<td></td>
<td>(H3) L. infantum [35]</td>
<td>(H4) L. infantum [131]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Cysteine proteinases</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine proteinase A</td>
<td>(CPA) L. major [132]</td>
<td></td>
</tr>
<tr>
<td>Cysteine proteinase B</td>
<td>(CPB) L. major [132]</td>
<td></td>
</tr>
<tr>
<td>Cysteine proteinase C</td>
<td>(CPC) L. infantum [133]</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Other intracellular antigens</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Translation initiation factor 4A</td>
<td>(LeIF) L. braziliensis [39]</td>
<td></td>
</tr>
<tr>
<td>Homolog receptor for activated C kinase</td>
<td>(LACK) L. major [48]</td>
<td></td>
</tr>
<tr>
<td>Amastigote stage specific gene</td>
<td>(A2) L. donovani [57]</td>
<td></td>
</tr>
<tr>
<td>Kinesine</td>
<td>(LcKin) L. chagasi [134]</td>
<td></td>
</tr>
</tbody>
</table>

characterized after immunoscreening of an *L. braziliensis* expression library with a serum obtained from an MCL patient [40]. Two years later, the *L. infantum* HSP83 coding gene was rescued from a cDNA expression library using a VCL canine serum [36]. Both proteins, obtained as recombinant molecules expressed in bacteria, were employed for their serological evaluation. They were recognized as antigenic in a great percentage of patients with active CL and MCL, but anti-HSP83 antibodies were not presented in the sera from individuals with self healing CL [40, 151]. Canine VCL sera specifically recognize the *L. infantum* rHSP83 without cross-reactivity with the host HSP83 [36]. The specificity in the recognition was related with the location of the antigenic determinants in the most divergent regions of the parasite HSP83 [36]. Interestingly, rHSP83 was not recognized by the sera from chronic Chagas’ disease patients [151]. For these reasons, *Leishmania* rHSP83 was considered like a potentially important diagnostic antigen. This protein also contains potent T-cell epitopes which stimulate PBMCs from MCL [40] and VL [152].
Table 6: Summary of ribosomal antigens regarding diagnosis and vaccination.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Diagnosis</th>
<th>Vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>Human VL and MCL [142]</td>
<td>DNA vaccine/rP2 + CpG/prime-boost [140]</td>
</tr>
<tr>
<td></td>
<td>Canine VCL [138]</td>
<td>Mice CL</td>
</tr>
<tr>
<td>P0</td>
<td>Canine VCL [137]</td>
<td>DNA vaccine/rP0 + CpG [147]</td>
</tr>
<tr>
<td>S14/S13/S16/S18/L8/L28</td>
<td>Mice VL</td>
<td>DNA vaccine [53]</td>
</tr>
<tr>
<td>L22</td>
<td>Mice CL</td>
<td>DNA vaccine [54]</td>
</tr>
<tr>
<td>S19</td>
<td>Mice CL</td>
<td>DNA vaccine [54]</td>
</tr>
</tbody>
</table>

Table 7: Summary of stress antigens regarding diagnosis and vaccination.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Diagnosis</th>
<th>Vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP83</td>
<td>Human CL and MCL [40]</td>
<td>DNA vaccine [40]</td>
</tr>
<tr>
<td></td>
<td>Canine VCL [36]</td>
<td>DNA vaccine [36]</td>
</tr>
<tr>
<td>GRP94</td>
<td>Human CL and MCL [154]</td>
<td>DNA vaccine [154]</td>
</tr>
<tr>
<td></td>
<td>Canine VCL [154]</td>
<td>DNA vaccine [154]</td>
</tr>
<tr>
<td>HSP60</td>
<td>Human CL [128]</td>
<td>DNA vaccine [128]</td>
</tr>
<tr>
<td>HSP70</td>
<td>Human VL [37, 155, 156]</td>
<td>DNA vaccine [37, 155, 156]</td>
</tr>
<tr>
<td></td>
<td>Human MCL [40, 155]</td>
<td>DNA vaccine [40, 155]</td>
</tr>
<tr>
<td></td>
<td>Human CL [40, 156]</td>
<td>DNA vaccine [40, 156]</td>
</tr>
<tr>
<td></td>
<td>Canine VCL [31, 129]</td>
<td>DNA vaccine [31, 129]</td>
</tr>
<tr>
<td>HSP20</td>
<td>Canine VCL [127]</td>
<td>DNA vaccine [127]</td>
</tr>
<tr>
<td>STI1</td>
<td>Human CL, VL and PKDL [41]</td>
<td>rSTI1 + IL-12 [161]</td>
</tr>
<tr>
<td></td>
<td>Canine VCL [59]</td>
<td>DNA vaccine [59]</td>
</tr>
</tbody>
</table>

Leishmania glucose-regulated protein 94 (GRP94) is another member of the HSP90s family. This protein, located in the endoplasmic-reticulum is implicated in the LPG synthesis [153]. The antigenicity of the L. infantum GRP94 was evaluated after cloning of the GRP94 coding genes and expression of the rGRP94 in bacteria [130]. This protein constitutes a valuable molecule for diagnostic purposes, since 84% of sera from dogs with VCL reacted with the recombinant protein. The rGRP94 as well as synthetic peptides covering the most variable regions of the protein was also recognized by sera from MCL and VL patients [154]. There is only one report concerning the antigenicity of the Leishmania HSP60. A recombinant version of the L. major HSP60 was recognized by the sera from CL patients [128]. As occurring for the HSP83, the recognition is specific for the parasite protein since the same sera did not show reactivity with mycobacterial rHSP65 or human rHSP60 [128].

The HSP70 coding genes from several Leishmania species were characterized because of their recognition by leishmaniasis sera. The gene encoding L. donovani HSP70 was identified after screening of a cDNA library with serum from a patient with VL [37], L. braziliensis with an MCL serum [40] and L. infantum with the serum from a dog with VCL [129]. Anti-HSP70 humoral responses are highly specific against the Leishmania protein as occurred for the other parasite heat shock proteins. Thus, the anti-HSP70 antibodies present in the sera from infected individual did not recognize host-HSP70 [40, 129]. Leishmania rHSP70 could be a potential candidate for serodiagnosis since it is highly recognized by the sera from patients with VL [37, 155, 156], MCL [40, 155], CL [40, 156], and dogs with VCL [31, 129]. Although the complete protein cannot be used for specific serodiagnosis of VL because it is also recognized by the sera from Chagas’ disease patients, the use of some fragments expressed as recombinant proteins or synthetic peptides covering the most divergent regions of the protein were described as valuable tools for serodiagnosis in geographical areas where mixed infections with T. cruzi and Leishmania occur [155, 156].

L. amazonensis HSP20 was recently described as an antigenic member of the heat shock protein family [127]. It was obtained as a recombinant protein expressed in bacteria and its antigenic properties were analyzed. The rHSP20 was
recognized by the sera from dogs with the VCL disease but not by the sera from human VL patients [127].

It was described that *Leishmania* HSP83 and HSP70 possess interesting adjuvant properties. Thus, the immunization of HSP83 chimeras induced Th1 responses against the fusion proteins [157]. Also, *L. infantum* HSP70 was able to induce a Th1 response against covalently linked protein, when the fusion protein was immunized in mice [158].

Some *Leishmania* heat shock recombinant products were tested as vaccines in the murine model. Thus, *L. major* HSP70 was employed to immunize mice in a prime-boost manner (DNA vaccine as a prime and a boost with the recombinant protein emulsified with Montanide) in two experimental model of murine CL (susceptible BALB/c and resistant C57BL/6 mice). A non-protective, mixed Th1/Th2 response was obtained [159]. As occur for HSP70, DNA vaccination of a HSP20 recombinant eukaryotic expression plasmid did not result in protection in murine experimental models of CL [127]. On the contrary, another member of the parasite stress protein family, the *L. major* stress-inducible 1 protein (LmSTI1) showed a protective role when it was employed as vaccine.

LmSTI1 was identified after screening of an *L. major* amastigote cDNA expression library with the sera from *L. major*-infected BALB/c mice [41]. The STI1 gene is constitutively expressed in both *L. major* promastigotes and amastigotes. However, STI1 transcript levels were upregulated in promastigotes by a shift in culture temperature from 26°C to 37°C [160]. Draining lymph nodes from *L. major* infected BALB/c mice proliferate and produce IFN-γ after in vitro restimulation with rLmSTI1 expressed in bacteria [41]. The prophylactic properties of the *L. major* STI1 were analyzed in experimental murine model of CL. Immunization of the rSTI1 adjuvanted with human IL-12 [161] or as a DNA vaccine [162] induced partial protection against *L. major* infection in BALB/c mice. The degree of protection was enhanced when the rSTI1 was administered as coencapsulated in liposomes with CpG ODN [163].

The antigenicity of the *L. major* rSTI1 was also analyzed. The sera from a great percentage of patients with CL, VL, and PKDL exhibited reactivity toward rSTI1 [41]. Also, the recombinant protein was recognized by canine VCL sera [59].

4.3. Histones. Histone proteins, which are highly conserved through eukaryotic organisms, are nuclear located molecules involved in the structural formation of nucleosomes and chromatin compaction. In spite of their nuclear location, all parasite histones were described as immunodominant antigens during *Leishmania* infection [43]. The identification of an *L. infantum* cDNA encoding the H2A histone after immunoscreening with a canine VCL serum was the first report of a specific immune response against histones elicited during infection with a parasitic pathogen [34]. Afterward, a cDNA coding for the *L. infantum* H3 histone was isolated using the same methodology [35]. Both molecules, together with the other two nucleosome forming histones (H2B and H4), were obtained as recombinant proteins expressed in bacteria [131]. These recombinant molecules were proved to be antigenic in serologic assays employing canine VCL sera. The rH2A was the most frequently recognized (72%) followed by rH3 (68%), rH2B (60%), and rH4 (44%) [131]. As occurs with other *Leishmania* conserved antigens, the anti-histone humoral response elicited during canine infection is specific for the parasite antigens. Canine VCL sera did not recognize the counterpart of mammalian origin because the B cell antigenic determinants were located in the most divergent regions of the parasite histones: the amino-terminal ends of the four core histones and also the C-terminal region of the H2A [131, 164, 165]. In addition, it was found that 58% of patients with American CL have antibodies reacting with the *L. peruviana* H2B [166], that all the sera from patients with VL caused by *L. chagasi* reacted against the *L. infantum* H2A [110] and that high percentages of VL patients from the Mediterranean area had anti-*Leishmania* H2A- and H2B-specific antibodies [95]. All these data indicate that histones can be taken into account in the development of serodiagnosis systems based on recombinant parasite antigens.

The T cell immunogenicity of parasite histones was also demonstrated, since *Leishmania* rH2B protein was able to induce strong in vitro proliferation and IFN-γ production in PBMCs obtained from patients with CL and from a T-cell clone derived from a VL immune donor [49]. Also, a predominant IFN-γ production was observed when PBMCs from CL patients were stimulated with *L. infantum* rH2A and rH3 histones [167].

The prophylactic value of the *Leishmania* histones was evaluated in different experimental models. It was firstly described that the immunization of a mixture of expression eukaryotic plasmids encoding *L. donovani* H2B, H3, and H4 was able to induce partial protection against infection with *L. donovani* in mice [53]. In addition, the co-administration of a mixture of the four *L. infantum* nucleosomal histones as a DNA vaccine [168] protected BALB/c mice against a virulent challenge with *L. major*. The observed protection was related with the generation of histone-specific Th1 response in which both CD8+- and CD4+-dependent production of IFN-γ was detected [168]. Also, the adoptive transfer of BMDCs pulsed with the four parasite recombinant histones plus CpG ODN induced protection in CL [169, 170] and VL [171] murine models. Finally, it was documented that the immunization of a recombinant protein containing the divergent amino-terminal region of the *L. major* H2B was also protective in the murine model of CL when adjuvated with CpG ODN [172].

Histone H1 was related with parasite infectivity because it is overexpressed early during infection. *L. major* H1 histone coding gene was isolated from a parasite cDNA expression library constructed with mRNA from macrophages recently infected with *L. major* [55]. Generation of *L. major* transfectants overexpressing histone H1 causes a delay in the cell-cycle progression [173] that reduces infectivity in vitro [174] and in vivo [173]. Interestingly, a vaccine composed of *L. major* H1 partially purified or obtained as a recombinant protein had protective capacity against experimental murine CL [175]. Furthermore, it was observed that combination of the rH1 protein plus Montanide (an adjuvant employed
for human vaccination) developed protection against CL in outbred vervet monkeys [176] (see Table 8).

4.4. Cysteine Proteinases. Cysteine proteinases (CPs) are key molecules for *Leishmania* virulence. Because of their critical contribution to the ability of the parasites to infect and proliferate in mammals, cysteine proteinases of *Leishmania* were viewed as promising drug targets for many years [177]. These enzymes seem to be predominantly expressed and active in amastigotes and to a lesser extent in metacyclic promastigotes [178, 179]. Three classes of cysteine proteinase genes (types I–III) were identified in *Leishmania*: CPB genes (type I), CPA (type II), and CPC (type III). Although the exact role of cysteine proteinases in *Leishmania* pathogenesis remains unclear, it was demonstrated that *Leishmania* cannot grow within macrophages in the presence of cysteine proteinase inhibitors [180]. Also, the gene product of *cpb* 2.8 of *L. mexicana* is a potent inducer of a Th2 response in BALB/c mice [122]. In addition, it was demonstrated that the CPB of this parasite suppresses the antileishmanial Th1 immune response of C3H and C57BL/6 mice [181]. Moreover, deletion of CPs genes diminishes pathogenicity of *L. mexicana* in hamsters and *cpa/cpb*-deficient *L. mexicana* grew more slowly as promastigotes presenting lower infectivity and growth in human mononuclear phagocyte host cells [182]. All these data provide evidence of the importance of these molecules in the survival of both promastigote and amastigote forms of *Leishmania* parasites.

The CPA and CPB genes from different *Leishmania* species were isolated and the antigenicity and immunogenicity of the corresponding recombinant proteins were analyzed. *L. major* CPA and CPB genes were obtained using specific primers designed from the sequence of *L. mexicana* CPs and genomic DNA from *L. major* promastigotes [132]. PCR products corresponding to *L. major* CPB and CPA genes were amplified, cloned, and subsequently expressed as recombinant proteins in bacteria. The same primers were used to obtain *L. infantum* CPs genes [123]. The *L. chagasi* CPB and the *L. infantum* CPC [133] proteins were also obtained as recombinant proteins expressed in bacteria.

Sera from active or recovered cases of human CL patients showed a high reactivity against *L. major* rCPs [132]. Similar and intensive recognition of *L. infantum* rCPs (with a higher recognition toward rCPB than rCPA) was observed in active cases of human VL [123]. These proteins were also recognized by the sera from dogs affected with VCL [123]. The *L. chagasi* CPB (that presents a high degree of conservation with the *L. infantum* CPB) was described as a tool suitable for the diagnosis of human VL since it was recognized by the sera from VL patients (80% of sensitivity) without cross-reactivity with the sera from other diseases, including Chagas’ disease and tuberculosis (96% of specificity) [183]. Also, *L. infantum* rCPC was recognized by the sera from active and cured VL patients [133].

The immunogenicity of the rCPs was also evaluated. *L. major* rCPA and rCPB induced Th1 responses in patients with localized CL due to *L. guyanensis* (neither IL-4 nor IL-13 and low levels of IL-10 were detected) [184]. In addition, it was shown that the PBMCs obtained from individuals recovered from CL, produced IFN-γ after in vitro stimulation with a chimerical protein composed by both *L. major* CPs [185]. Also, and for canine VCL, asymptomatic dogs exhibited specific lymphocyte proliferation to *L. infantum* rCPs in contrast to the symptomatic cases [123]. Finally, it was described that *L. chagasi* rCPB induces the in vitro proliferation and secretion of IFN-γ in PBMCs obtained from *L. chagasi* infected asymptomatic humans and dogs [186]. Interestingly, symptomatic subjects produced lower levels of IFN-γ, and also IL-4 and IL-10 in response to the stimulation with rCPB [186].

Several studies determined the efficacy of vaccines based on CPs against CL in mice. DNA vaccines encoding *L. major* CPs were tested, administrated intramuscularly in BALB/c mice, either separately or as a cocktail [187]. It was only when the *cpa* and *cpb* genes were coadministered in mice that long-lasting protection against parasite challenge was achieved. Analysis of the immune response showed that protected animals developed a specific Th1 immune response, which was associated with an increase of IFN-γ production. A similar protection was also observed when animals were primed with *cpa/cpb* DNA followed by recombinant CPA/CPB boost [187]. The recombinant *L. major* rCPB or rCPA inoculated together with polysaccharide as adjuvant was tested in the same infection model [188]. Vaccination with rCPB, but not

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### Table 8: Summary of histone antigens regarding diagnosis and vaccination.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Diagnosis</th>
<th>Animal model</th>
<th>Immunization mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2s/H3/H4</td>
<td>Canine VCL [131, 164]</td>
<td>Mice CL</td>
<td>DNA vaccine [168]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mice CL</td>
<td>BMDCs + CpG [169]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mice VL</td>
<td>BMDCs + CpG [171]</td>
</tr>
<tr>
<td>H2B</td>
<td>Human VL [95]</td>
<td>Mice CL</td>
<td>rH2B-Nt + CpG [172]</td>
</tr>
<tr>
<td>H2A</td>
<td>Human VL [95]</td>
<td>Mice VL</td>
<td>DNA vaccine [53]</td>
</tr>
<tr>
<td>H2A/H3/H4</td>
<td></td>
<td>Mice CL</td>
<td>rH1 [175]</td>
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<td>H1</td>
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<td>Monkey CL</td>
<td>rH1 + Montanide [176]</td>
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rCPA, allowed BALB/c mice to mount a partial protective response, with a delay in the clinical outcome. This partial protective effect was abrogated if a CD8 depleting antibody was given intravenously to rCPB-immunized mice, at the time of parasite challenge. In fact, only one immunological response, with a delay in the clinical outcome. This partial protection achieved by the injection of rCPB plus poloxamer. Therefore, to reduce the production costs, the cpa and cpb genes were fused in tandem together to give rise to a single hybrid protein [185]. The protective potential of the CPA/B hybrid protein plus poloxamer against L. major infection was then assessed in BALB/c mice, showing a delay in the expansion of lesions size compared to control groups. A predominant Th1 immune response characterized by in vitro IFN-γ production was found) [193]. In addition, rLeIF stimulates the production of Th1 cytokines and correlates the higher levels of total IgG and IgG2 (but not IgG1) to rCPA and rCPB. PBMCs from vaccinated dogs showed higher level of proliferation than controls when stimulated with total parasite antigens or rCPs [190]. Proliferation correlated to the presence of higher IFN-γ mRNA and less IL-10 mRNA levels [190]. Notwithstanding, other study reported that administration of L. infantum rCPA and rCPB adjuvated with recombinant canine IL-12 alone or in combination with Quil A was not effective to vaccinate dogs against an intravenous challenge with L. infantum [191] (see Table 9).

4.5. Other Intracellular Antigens. The homologous of the higher eukaryotic initiation factor 4A (eIF4A) in Leishmania (LeIF) was described as an immunostimulatory antigen. It was identified by screening of an L. braziliensis genomic expression library with a serum from a patient suffering MCL [39]. Subsequently, it was shown that the recombinant protein stimulates the production of Th1 cytokines and the proliferation of the T cells from this patient [39]. This study revealed several evidences indicating that LeIF protein promotes preferentially Th1-type responses after infection. Thus, rLeIF induced higher proliferative response in patients with MCL and self-healing CL disease than those with cutaneous lesions. Whereas the parasite lysate stimulated patient PBMCs to produce a mixed Th1/Th2-type cytokine profile, rLeIF stimulated the production of IFN-γ, IL-2, and TNF-α but not IL-4 or IL-10, besides down-regulating IL-10 mRNA expression. rLeIF also stimulated the production of IL-12 in culture and the IFN-γ production was IL-12 dependent [39]. Further, it was observed that rLeIF protein stimulates human mononuclear antigen-presenting cells to produce IL-12, concomitantly with an upregulation of costimulatory and intercellular adhesion molecules [192]. The immunogenicity of the parasite LeIF was also analyzed in experimental CL infected mice. The in vitro stimulation of draining lymph node cells from L. major-infected BALB/c mice with rLeIF preferentially secreted IFN-γ (no detectable IL-4 production was found) [193]. In addition, rLeIF downregulated Leishmania Ag-specific IL-4 production by lymph node cells from infected BALB/c mice. Subsequently, BALB/c mice immunized with rLeIF were partially protected
IFN-γ stimulated fresh spleen cells from naive SCID mice to secrete L. major protective CD4+ T cell clone (Th1) from immunized BALB/c mice. Characterized antigen of L. major receptor for activated C kinase is probably the best characterized antigen for leishmaniasis (see Table 10).

Adjuvant as well as a therapeutic and prophylactic vaccine for Leishmania, LeIF might be used as a Th1-type cytokine inducer IFN-γ. The putative receptor of LeIF is unknown, yet Toll-like receptor 4 (TLR4) was excluded, since LeIF was able to stimulate splenocytes from mice defective for this receptor (C3H/HeJ) [194]. Thus, LeIF was able to stimulate splenocytes in both stages of the parasite life cycle [48]. Interestingly, it was shown that this parasite protein was implicated in the induction of the early IL-4 response against L. major [197]. In summary, all these data support the outstanding protection induced by several vaccination protocols based on LACK antigen against L. major infection in BALB/c mice [198–202] a CL model that depends critically on a Th1/Th2 balance. However, vaccines based on LACK were not so efficient to induce protection against other Leishmania species that are not so strictly dependent on this Th1/Th2 balance, namely, L. mexicana [102, 203], L. amazonensis [203–205], L. donovani [206], or L. chagasi [207]. Notwithstanding, it was demonstrated that dogs experimentally infected with L. infantum were protected against VL following an heterologous prime-boost vaccination regime with a DNA vaccine encoding LACK and recombinant vaccinia virus (rVACV) expressing LACK [208], or its corresponding nonreplicative modified vaccinia (MVA-LACK) [209]. Similarly, the same prime-boost regime protects BALB/c mice against an intradermal infection with this parasite [210] (see Table 10).

The L. donovani A2 multigene family encodes for a group of proteins that are composed predominantly of multiple copies of a 10 aminoacid repeated sequence ranging in molecular weight from 45 to 110 kDa, depending on the number of repeats within the protein [211]. The A2 family was characterized looking for molecules specific of the amastigote stage. These genes were isolated after the screening of an amastigote cDNA library with life cycle stage-specific DNA probes [57]. L. chagasi A2 gene was also identified after a double screening of an amastigote cDNA library using in a first step a pool of sera from Brazilian VL patients and a second step T cells obtained from immune mice [212]. Since the Leishmania A2 antigen showed immunogenic properties, it was obtained as a recombinant protein expressed in bacteria and tested for a possible role in diagnosis and vaccination. A study using the

<table>
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<th>Molecule</th>
<th>Diagnosis</th>
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<th>Vaccination</th>
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<td>Presence of antibodies</td>
<td>Mice CL</td>
<td>rLACK [193]</td>
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<td>documented in</td>
<td>Mice CL</td>
<td>rLACK + IL-12 [48, 203]</td>
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<td>DNA vaccine [102, 196, 198, 205]</td>
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<td>Listeria expressing rLACK [199]</td>
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<td>Vaccinia expressing LACK [200–202]</td>
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<td>LACK</td>
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<td>Mice VL</td>
<td>Prime-boost [210]</td>
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<td>DNA vaccine [206, 207]</td>
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<td></td>
<td>Human VL [213, 214]</td>
<td>Dogs VL</td>
<td>Prime-boost [208, 209]</td>
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<td>Canine VL [214]</td>
<td>Mice VL</td>
<td>rA2 + DNA vaccine [215]</td>
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<td>A2</td>
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<td>rA2 + P. acnes [216]</td>
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<td>K39</td>
<td>Human VL [95, 121, 134, 218–222]</td>
<td>Dogs VL</td>
<td>rA2 + saponine [217]</td>
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<td>Kinesine motor domain</td>
<td>Human VL [227]</td>
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Table 10: Summary of other intracellular antigens regarding diagnosis and vaccination.

A result of transgenic expression of this molecule in the thymus, were resistant to infection with L. major and develop a Th1 response after infection [197]. In summary, all these data support the outstanding protection induced by several vaccination protocols based on LACK antigen against L. major infection in BALB/c mice [198–202] a CL model that depends critically on a Th1/Th2 balance. However, vaccines based on LACK were not so efficient to induce protection against other Leishmania species that are not so strictly dependent on this Th1/Th2 balance, namely, L. mexicana [102, 203], L. amazonensis [203–205], L. donovani [206], or L. chagasi [207]. Notwithstanding, it was demonstrated that dogs experimentally infected with L. infantum were protected against VL following an heterologous prime-boost vaccination regime with a DNA vaccine encoding LACK and recombinant vaccinia virus (rVACV) expressing LACK [208], or its corresponding nonreplicative modified vaccinia (MVA-LACK) [209]. Similarly, the same prime-boost regime protects BALB/c mice against an intradermal infection with this parasite [210] (see Table 10).

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L. donovani rA2 protein, performed with kala-azar patients from an endemic region, showed that it was possible to detect by ELISA anti-A2 antibodies in 82% of the VL patients in Sudan and 60% in India, meanwhile using antibody immunoprecipitation the detection rate increased up to 92% [213]. A similar study was done in VCL dogs and Brazilian patients of VL. Anti-A2 antibodies were found in the 87% of dogs and 77% of the symptomatic patients, suggesting that the rA2 protein would be of particular interest for serodiagnosis [214].

Immunization of both L. donovani A2 DNA vaccines [215] or rA2 protein combined with Propionibacterium acnes [216] was proved to induce a significant protection against VL caused by L. donovani in experimentally infected mice. Protection correlated to the generation of a Th1/Th2 mixed response and with the A2-specific splenocyte proliferation and production of IFN-γ, and also with the generation of anti-A2 antibodies that induce a complement-mediated reduction of the viability of amastigotes that results in a reduction of macrophage infection [216]. Immunization of the L. donovani rA2 protein formulated with saponine, induced protection against L. chagasi experimental infection in dogs [217] (see Table 10).

An L. chagasi antigenic protein of great value for the generation of canine and human VL diagnosis test was named rk39. This recombinant protein contains an extensive repetitive domain located in the C-terminal region of the L. chagasi kinesine (LcKin) and was isolated after screening of an L. chagasi genomic expression library with a serum of a VL patient infected with L. donovani [134]. LcKin belongs to a conserved microtubule-based motor protein superfamilly and possesses a conserved sequence in the motor domain but little sequence similarity outside of the domain, including the repetitive aminoacid sequence encoded by the K93 clone [134]. The rK39 protein was employed for human diagnosis of VL caused by the related L. chagasi and L. infantum species as well as VL caused by L. donovani [95, 121, 134, 218–222] and for diagnosis of canine VCL [223–226]. Tests based on this antigen have high sensitivity (around 100%) and large specificity, since anti-K39 antibodies are virtually absent in the sera from patients with CL, MCL, or Chagas' disease [134]. Since sera from early infected of self-healing subjects were nonreactive with rK39 and the antibody titres to this antigen directly correlated to active disease, it can be employed as marker for disease progression in VL [121]. In addition rK39 ELISA has a high predictive value for detecting VL in immunocompromised persons, like those with AIDS [219]. A kit (InBios, USA) using this antigen is now commercially available in the form of antigen-impregnated nitrocellulose paper strips adapted for use under field conditions.

Remarkably, and as also occurs for other intracellular antigens, the aminoacids repeats of the parasite kinesine contaiend in the rK39 protein possessed epitopes that cause proliferation and IFN-γ production in T cells isolated from immune mice [212].

The antigenicity and immunogenicity of the conserved motor domain region located in the N-terminal region of the L. chagasi kinesine, were also analyzed. For that purpose a recombinant protein containing the N-terminal region of L. donovani kinesine was expressed in bacteria [227]. This recombinant protein induced the proliferation of PBMCs and was recognized by the sera from VL cured patients [227] (see Table 10).

5. Vaccines or Diagnosis Based on Mixtures of Nonrelated Antigens or Poly-Protein Quimeric Molecules

Humans or dogs naturally exposed to Leishmania mounted both humoral and cellular responses to some of the above antigens. The fact that none of the antigen elicited responses in all exposed individuals underscores the fact that a vaccine formulation and optimized diagnostic test based on recombinant proteins may require a mixture of parasite antigens. Then, efficient diagnosis as well as the development of vaccines based on recombinant proteins will require the combination of nonrelated antigenic molecules.

In this sense, Dumonteil et al. tested DNA vaccines encoding L. mexicana GP63, CPB, and LACK, as well as L. amazonensis GP46 in the BALB/c model of L. mexicana infection [101]. Although each one of the four DNA vaccines induced Leishmania-specific humoral and lymphoproliferative immune responses, only mice immunized with GP46, GP63, and CPB were partially protected against challenge and, moreover, the immunization of mice with a mixture of these three plasmids further increased protection. A DNA-vaccine based on a mixture of TSA, STI1, and LACK genes was evaluated in the cutaneous model that more accurately reproduces the clinical-pathological findings associated with human disease, consisting in the intradermal inoculation of a low number of L. major parasites into the ear of resistant C57BL/6 mice [228]. Vaccination with a mixture of these genes protected C57BL/6 and BALB/c mice even when challenged 12 weeks after the immunization (no pathology and a 1000-fold reduction in dermal parasite loads) [229]. Concomitantly, administration of the rSTI1 and rTSA proteins adjuvated with alum and human IL-12 induced a remarkable protection in Rhesus monkeys, being a promising candidate subunit vaccine against human leishmaniasis [161]. The same combination of STI1 and TSA antigens was tested as a DNA vaccine in BALB/c mice challenged with L. major in the footpad. TSA-DNA vaccine conferred a substantial protection greater than the partial protection induced by STI1-DNA. Interestingly, this different degree of protection correlated to the activation of CD8+ T cell responses, since cytotoxic-T-lymphocyte activity was generated after immunization with TSA DNA but not STI1 DNA [162]. The immunogenicity of TSA, LeIF, and STI1 recombinant antigens combined with MPL-SE or AdjuPrime was also analyzed in dogs as potential vaccine candidates for VCL [230]. When dogs immunized with the recombinant antigens plus MPL-SE were experimentally exposed to low numbers of culture forms of L. chagasi promastigotes a clear boost in the immune response was observed. Moreover, immunoglobulins were predominantly of the IgG2 isotype, whereas animals primed with the recombinant antigens formulated in AdjuPrime as well as animals vaccinated with...
In summary, all these data suggest that this vaccine represents a good candidate for use in human clinical trials and has completed phase 1 and 2 (safety and immunogenicity testing in normal, healthy human subjects) [9].

Other parasite multiepitope proteins were also constructed. Boarino et al. assayed a recombinant chimeric antigen containing the 39 aminoacid unit of *L. chagasi* K39 fused with other two *L. chagasi* antigens (K9 and K26) for the diagnosis of human VL and canine VCL [234]. Also, a chimeric protein containing the antigenic determinant of the *L. infantum* histone H2A and the acidic ribosomal protein P2a, P2b and P0 (namely, protein-Q) was constructed for the specific diagnosis of canine VCL with a sensitivity ranging from 79% to 93% and specificity ranging from 96% to 100% dependent of the negative control sera employed [235]. The ability of this multitargeting protein to induce protection was tested in a murine model of VL [236]. Administration of this protein with CpG ODN induces a mixed Th1/Th2 response that results in a significant protection after *L. infantum* challenge in BALB/c mice [236]. Administration of the protein-Q mixed with live BCG conferred protection to dogs experimentally infected with *L. infantum* [237].

6. Concluding Remarks

In the detection of leishmaniasis cases, serodiagnostic methods are of great importance prior to attempts to parasite detection. Different parasite antigenic proteins were isolated and expressed as recombinant proteins but the potential diagnostic usefulness of these recombinant proteins for human and canine leishmaniasis deserves further research. This includes the analysis of all the available recombinant proteins against the sera from patients or dogs living in endemic areas and the evaluation of their reactivity with the sera from other cross-reacting diseases. The identification of new parasite specific antigens or specific antigenic determinants in the already characterized proteins will allow the development of more sensitive and specific tests.

Although a common feature of most of the *Leishmania* proteins described above is their frequency of recognition by sera from leishmaniasis patients, many of them were also considered of interest for the development of vaccines. Surface, secreted and excreted antigens were tested for their...
prophylactic potential because they are the first parasite factors that interact with the host immune system and are usually implicated in the establishment of the infection. Moreover, many intracellular proteins referred as “patho-antigens” because an inadequate humoral response against them is thought to result in pathology [42, 238, 239] are good candidates for vaccination if their Th2 mediated responses are redirected by the use of Th1 adjuvants (Figure 3).

Generation of vaccines against such a complex parasite as *Leishmania* would be optimized by incorporating different target antigens in the vaccine formulation, taking advantage of these antigens that induce the required immunity (mainly CD4+ and CD8+ IFN-γ-mediated responses) and redirecting towards a Th1 bias the pathoantigenic-driven immune responses that result in pathology (IL-4 Th2-driven and IL-10 deactivating responses).

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