Current Status and Perspectives of the Immunotherapy of Leishmaniasis

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Leishmaniasis is a zoonotic protozoan disease caused by *Leishmania*. The disease is widely distributed, affecting approximately 12 million people worldwide, causing a wide spectrum of diseases. The clinical manifestations range from simple cutaneous lesions to progressive disseminated visceral fatal disease. The cutaneous form of the disease caused by *L. major, L. tropica* and *L. aethiopica* in the Old World, and *L. mexicana*, *L. braziliensis* and *L. amazonensis* in the New World, is generally self-resolving with long-life immunity. The disease is characterized by the development of ulcerative skin lesions lasting several months and in most cases is resolved by Th1 T cell activity. However, in patients with a defective cellular immune response, a long-lasting chronic disease (recurrent cutaneous leishmaniasis or diffuse cutaneous leishmaniasis) may develop. American CL caused by *L. braziliensis* is a chronic disfiguring disease. Most of the patients recover from the disease after primary exposure to the parasite, but a small percentage may develop severe secondary life-persisting lesions in the mucocutaneous tissue (mucocutaneous leishmaniasis). The disease is well developed in the presence of Th2 T cell-mediated immunity. The visceral form caused by *L. donovani*, *L. infantum* (also known as *L. chagasi* in Latin America) is characterized by systemic infection of the reticuloendothelial system. Advanced stages of the disease are generally characterized by suppression of anti-*Leishmania* T cell responses mediated by interleukin-2 deficiency.

Leishmaniasis is characterized by a wide range of clinical manifestations that result from the parasite species and the immune status of the host

Until recently, only two vaccines (one live and one killed) were licensed for use in humans, and one for prophylaxis in dogs [1]. Also, the number of available drugs for treating the disease is limited and most of the parasites develop drug resistancy. Over the last two decades, immunotherapy (immune based therapies), either alone or combined with chemotherapy (immunochemotherapy), has been developed as an additional approach in the treatment of leishmaniasis. Immunotherapy has been used to accelerate the specific immune response in immunologically responsive patients and to establish an effective reaction in those who are non-responsive.

CL = cutaneous leishmaniasis

IMMUNOLOGY

In leishmaniasis, cellular rather than humoral immune responses are involved in the recovery from the disease. Spontaneous *Leishmania*-specific T cell response generally develops early after infection. The parasites first multiply as amastigotes into the phagolysosome within the macrophage. Either intact parasites or leishmanial antigens are then picked up by antigen-presenting cells (dentritic and Langerhans cells) that transfer them to CD4+ and CD8+ T cells. These cells activate specific T cells in the production of different cytokines (IL-22, interferon-gamma and granulocyte macrophage colony-stimulating factor) and induce the macrophage to kill the intracellular parasite [2]. CD8+ T cells are involved in the cytolytic anti-leishmanial activity by inducing the production of IFNγ3 and transforming growth factor-beta. Activation in the presence of IL-2 or TGFβ4 led to the development of cytolytic cells producing IFNγ. However, activation in the presence of IL-4, non-cytolytic CD8-CD4- T cells are developed and lead to IL-4, IL-5, and IL-10 production and exacerbation of the disease. IL-12 is an additional essential cytokine involved in inducing cell-mediated immunity, cell proliferation and secretion of IFNγ by T and natural killer cells. It promotes the development of CD4 Th1 cells, which are crucial in the protective immunity against the disease.

Recovery and protection against leishmaniasis result from a strong and specific cellular immune response, followed by

IL-2 = interleukin-2
IFNγ = interferon-gamma
TGFβ = transforming growth factor-beta

the development of long-lasting protection. In CL infection, the Th2 type of immune response induces vulnerability and exacerbation of the disease, while Th1 activity mediates long-life protection against reinfection. Following infection, an early Th2 response is established and a switch from Th2 to Th1 occurs later only in parasite-resistant animals [2]. Resistant mice exhibit pronounced Th1 responses, producing a high level of IFNγ that activates the macrophage to eliminate the intracellular parasite through the production of nitric oxide. Vulnerable mice exhibit a pronounced Th2 response producing IL-4, IL-10 and IL-13, causing down-regulation of nitric oxide production and up-regulation of arginase production. A large number of CD8+ T cells was also detected in the CL lesion during the healing process. It was therefore suggested that CD8+ T cells play an important role in the elimination of parasites during the healing process through the production of specific cytokines [3]. Due to Th2 cell activity, VL5 unlike CL, is associated with systemic depressed cell-mediated immunity which is affected by Th2 T cell activity and its associated cytokines (IL-4, IL-10, IL-13). During the first week of *L. donovani* infection in Balb/c mice, IL-2 induces IL-10, which suppresses IL-12 production and IL-12 receptor expression on T cells, followed by down-regulation of IFNγ production and parasite proliferation within the macrophage [4]. Also, in these mice DCs6 do not migrate well into the spleen and therefore are not involved in destroying the intracellular parasites. In humans, elevated IL-4 and IL-10 production was demonstrated in chronic CL, MCL7 and VL infections. In leishmaniasis, high levels of immunoglobulin G, M and E are also produced, but their effect on disease development has not yet been determined. Advanced human MCL was shown to be related to high IFNγ/IL-2-producing T cell proliferative responses. A case-control study performed in Venezuela indicated that MCL development was associated with genetic regulatory polymorphism affecting tumor necrosis factor-alpha production [5]. IL-10 is also considered to be important in human susceptibility to *L. braziliensis* infection [4].

Only a small number of effective drugs against the disease are available and most of the parasites develop drug resistancy

CHEMOTHERAPY

CHEMOTHERAPEUTIC TREATMENT

Almost all forms of leishmaniasis should be treated. Drug efficacy might be affected by the parasite species, gender and age, body distribution of the disease, immunological status of the host, and mode of treatment. Most patients respond to treatment, but in certain cases of *L. tropica, L. aethiopica*, *L. braziliensis* and *L. amazonensis*, conventional treatment is only partially effective [6]. The pentavalent antimonial compounds, sodium stibogluconate (Pentostam®) and N-methyl meglumine antimonite (Glucantime®), are considered the drugs of choice against all forms of the disease, generally with mild side effects. Alternative therapies include pentamidine and amphotericin-B, which are relatively toxic agents and are sometimes associated with mild or severe side effects. Orally administered agents, i.e., ketoconazole, itraconazole, fluconazole and allopurinol, either alone or in combination, have shown variable levels of efficacy [6]. Miltefosine, a newly developed drug taken orally, has yielded encouraging results against VL and Old and New World CL [7,8]. Physical treatment, including irradiation, cauterization, freezing by liquid nitrogen, infra red and photodynamic climatotherapy, has also been used to treat CL [6]. Topical applications with AMB, ethanolic lipid AMB, and various formulated paromomycin (aminosidine) ointments were also effective against CL [9].

RESISTANCE TO CHEMOTHERAPEUTIC TREATMENT

Parasite drug resistance was demonstrated with most of the available drugs against the disease. In Bihar, India, 40–60% of patients suffering from VL did not respond to pentavalent antimony treatment [10]. Similar results, with 25% unresponsiveness, were recorded with pentamidine [11]. Lack of response to AMB is uncommon, even though resistance to this drug was observed in *L. infantum*/human immunodeficiency virus-infected cases. Resistance of *L. donovani* promastigotes to miltefosine was shown *in vitro*, and resistance of both *L. major* and *L. tropica* promastigotes to paromomycin was induced *in vitro* by repeated exposure of the parasites while gradually increasing the drug concentrations.

COMBINED CHEMOTHERAPY

Combined chemotherapy may act synergistically against the disease, particularly in relapses and in patients who do not respond to available treatment [10]. Combined chemotherapy of sodium stibogluconate and paromomycin was highly effective, curing approximately 95% of the patients suffering from *L. donovani* infection [12]. Also, liposomal AMB combined with miltefosine resulted positively against kala azar resistant to pentavalent antimony in India [13]. A combination of sodium stibogluconate combined with either allopurinol or levamisol was considered more effective than sodium stibogluconate alone. Also, a combination of paromomycin and methyl benzethonium chloride was preferable to either of the drugs administered alone. Combined therapy was further recommended in HIV/AIDS patients suffering from VL.

VL = visceral leishmaniasis
DC = dentritic cells
MCL = mucocutaneous leishmaniasis

AMB = amphotericin-B
HIV = human immunodeficiency virus

IMMUNOTHERAPY AND IMMUNOCHEMOTHERAPY

Immunotherapy and immunochemotherapy have been used to accelerate the specific immune response in immunologically responding and non-responding patients. The concept was to selectively induce Th1 responses that are considered essential for resistance to leishmaniasis. To achieve this goal, various approaches were developed and applied in the treatment of human leishmaniasis [Table 1].

PASTEURIZED *LEISHMANIA* PROMASTIGOTES

Immunization of humans using heat-killed promastigotes yielded controversial results. A protective efficacy of 61.6% against *L. major* infection was recorded in uninfected Sudanese volunteers, but to a lesser degree against anthroponotic CL in Iran and VL (43.3% efficacy) in Sudan. Heat-killed parasite antigen + Bacillus Calmette-Guerin produced a protective efficacy of 73% against *L. amazonensis* infection [14] but had no effect against *L. amazonensis* in Colombia. However, better results were achieved with the use of alum-precipitated autoclaved *Leishmania major* plus BCG [15], and killed *L. amazonensis* together with meglumine antimonate showed similar results against CL in Brazil [16]. Pasteurized *Leishmania* promastogotes plus BCG were very effective in treating American MCL and CL [17,18]. Almost all the 5341 Venezuelan patients suffering from American CL and 7 patients suffering from either MCL or DCL who received heat-killed *L. (V.) braziliensis* promastigotes combined with viable BCG recovered from the disease [18]. Autoclaved parasites plus BCG were also found effective in the treatment of human CL in Brazil [19].

LEISHMANIAL ANTIGENS

In our previous study, using guinea pigs with both ears infected with *L. enriettii*, a topical treatment with paromomycin/methylbenzethoniumchloride ointment on one ear also caused a delayed therapeutic effect on the untreated ear. This phenomenon was further demonstrated in humans suffering

BCG = Bacillus Calmette-Guerin
DCL = diffuse cutaneous leishmaniasis

Table 1. Immunotherapy of human leishmaniasis

Immunotherapeutic agent	Chemotherapeutic agent	Country (no. of patients)	Disease/ Parasite	Treatment efficacy	Symptoms	Cellular response	Ref.
Mixed antigens*	–	Brazil (6)	MCL	High	Marked improvement	–	[28]
Pasteurized *L. braziliensis* + BCG	–	Venezuela (5341)	CL	High	95.7% healing	–	[17]
Pasteurized *L. braziliensis* + BCG	–	Venezuela (7)	MCL, DCL	High	~100% healing	High	[18]
Killed *L. amazonensis* + BCG	Glucantime®	Brazil (47 + 405 control)	ACL	87% vs. 88%	Marginal effect	Marginal effect	[19]
Killed *L. amazonensis*	Glucantime®	Brazil (47 +49 control)	CL	100% vs. 8.2% Glucantime®	Marked improvement	Moderate, low IFNγ	[16]
IFNγ	Glucantime®	Brazil (17)	VL	82.3% cure	Marked improvement	–	[26]
IFNγ	Sb	India (16 +15 Sb)	VL	Moderate 87% vs. 60%	Marked in both groups	–	[25]
IFNγ	Sb	Kenya (10 + 14 Sb)	VL	Moderate	Moderate improvement	–	[27]
Alum/ALM + BCG	Sb	Sudan (15 + 15 Sb)	PKDL *L. donovani*	87% vs. 40% Sb	Marked improvement	High (leishmanin)	[15]
Leukinferon (i.m.)	Monomycin (topical)	Uzbekistan (50 + 65 control)	CL	High	Marked improvement	–	[32]
GM-CSF (topical)	Glucantime®	Brazil (5)	CL *L. braziliensis*	100% cure	Marked improvement	High 83%	[29]
Imiquimod (topical)	Glucantime®	Peru (7)	CL	100% vs. 57% Glucantime®	Marked improvement	–	[33]
Imiquimod (topical)	Glucantime®	Iran (59 + 60 Sb)	CL *L. tropica*	44.1% vs. 48.3% Sb	Marked in both groups	–	[34]

*Mixed antigens: *L. major* stress-inducible protein 1 (LmSI1) + *L. major* initiation factor (LeIF) + *Leishmania* heat shock protein 83 + granulocyte macrophage colony-stimulating factor (GM-CSF).

Sb = sodium stibogluconate, BCG = Bacillus Galmette-Guerin, Alum/ALM = alum-precipitated autoclaved *L. major*, PKDL= post-kala-azar dermal leishmanoid.

from CL caused by *L. major* [20]. Treating one lesion affected the healing of other untreated lesions in the same patient. It was suggested that local treatment causing damage to the parasites is followed by the release of leishmanial antigenic constituents, which evoke an immune response that leads to the regression of lesions in distant sites of the body. Indeed, both purified and recombinant specific antigens and DNA encoding antigens have been applied in the development of a protective vaccine against leishmaniasis [1]. Protection against CL was achieved with *L. major* promastigote surface antigen 2, parasite exogenous antigens and DNA encoding antigens. A fucose mannose ligand, either alone or formulated with QuilA saponin, induced a 92–95% protection against canine visceral leishmaniasis caused by *L. donovani* and *L. chagasi* [21]. Also, a commercial leishmanial vaccine, Leishmune® (FML isolated from *L. donovani* promastigotes) enriched with saponin, protected 75– 92% of the vaccinated dogs against VL infection [22]. The vaccine induced a positive delayed-type hypersensitivity reaction and reduced clinical symptoms.

Chemotherapy of canine VL, caused by *L. chagasi, L. donovani* and *L. infantum*, is only partially effective, and immunotherapy is a new approach to control the disease [22]. Approximately 100% protection against canine VL was achieved in dogs vaccinated with LiESAp vaccine (a 54 kDa excreted protein of *L. infantum*) combined with muramyl dipeptide [23]. However, while crude parasite extracts together with Glucantime® were only partially effective, purified antigen (LiF2, *L. infantum*-derived fraction 2) combined with Glucantime cured all the dogs within 6 months. The saponin-enriched Leishmune vaccine given alone was found only moderately effective against *L. chagasi* [22], but the polyprotein recombinant vaccine Leish-110f®, together with the adjuvant MPL-SE® and meglumine antimonate induced elevated cellular immune responses, improved the clinical symptoms and reduced the mortality rate in symptomatic dogs [24].

Accumulated immunological data and new vaccine research are being channeled into developing effective immunotherapy and immunochemotherapy

CYTOKINES AND VARIOUS IMMUNOMODULATORS

A combination of anti-leishmanial chemotherapeutic agents together with various immunomodulators (MDP13, IFNγ, IL-12) accelerated treatment efficacy in ACL, MCL and DCL patients, caused by various *Leishmania* strains. Either a moderate or a synergistic effect of IFNγ plus pentavalent antimonial compounds was demonstrated against intracellular *L. donovani* amastigotes *in vitro* and against VL in mice and humans [25-27]. In murine leishmaniasis, recombinant Th1-stimulating cytokine IL-12 given alone or combined with either sodium stibogluconate or paromomycin cured mice from *L. major* infection. Treatment with recombinant IFNγ and Th1-stimulating cytokine IL-12 also has a positive effect. IL-18, a potent inducer of IFNγ production by T cells, did not influence the therapeutic activity against *L. donovani* infection. Human DCL and MCL respond very poorly to conventional therapy, and chemotherapy generally produces only transitory remission of the disease. Immunotherapy, using antimonials combined with IFNγ was only marginally effective. However, GM-CSF14 plus either a mixture of *L. major* antigens (LmSI1 + LeIf + HSP 83) [28] or meglumine antimonate [29] was reported as being highly effective in treating American CL and MCL. Recently, a combination of Z-100, a polysaccharide obtained from *Mycobacterium tuberculosis* combined with pentavalent antimonial, was found effective against *L. amazonensis*, *in vitro* [30].

In experimental VL, neutralizing IL-10 or blocking its receptors was preferable for the induction of Th1 cytokine activity, leading to IFNγ secretion, granuloma formation, macrophage activation and parasite killing, which were further increased in combination with pentavalent antimonials [31]. However, suppression of other cytokines, including receptor fusion antagonists of IL-13, IL-4 and TGFβ, inhibited parasite replication but only marginally affected parasite killing without the induction of a synergistic effect with pentavalent antimonials [31].

TOPICAL APPLICATION

A combination of topical monomycin (Leishmacol®) and injectable leukinferon (a complex of IL-1, TNFα, INFγ and macrophage migration inhibitory factor cytokines) was successful in treating zoonotic CL in Uzbekistan [32]. Imiquimod® (IMQ, 3M Pharmaceuticals, USA), a low molecular weight imidazoquinoline, is a novel immune response-activating agent, found to induce IFNγ, TNF, IL-1β, IL-1α, IL-6, IL-10, IL-1 receptor antagonist, GM-CSF and granulocyte CSF. IMQ applied topically has been shown to act synergistically with meglumine antimonate in the treatment of CL in experimentally infected mice. This combination was also found highly effective in the treatment of CL caused by *L. peruviana* in Peru [33], but less so against CL caused by *L. tropica* in Iran [34]. No synergistic effect was observed with an ointment comprising Imiquimod combined with Leshcutan® (15% paromomycin + 12% methylbenzethonium chloride) in the treatment of Balb/c mice infected with *L. major*.

FML = fucose mannose ligand
MDP = muramyl dipeptide

GM-CSF = granulocyte macrophage colony-stimulating factor

LIPOSOMES

The use of liposomes as a vehicle for drug delivery has been extended to the delivery of antigen to antigen-presenting cells. Liposomes are taken by endocytosis and presented by the major histocompatibility class I and class II. A recombinant gp63 (rLmSTI1) of *both L. major* and *L. donovani* encapsulated in cationic liposome induced significant protection in Balb/c mice [35]. A similar effect was achieved using soluble leishmanial antigen entrapped in oligomannose-coated liposomes against *L. major* [36]. *L. donovani* promastigote antigen encapsulated in non-phosphatidylcholine liposomes protected hamsters against the disease through specific CD8+ and CD4+ T cell immune response. Also, subcutaneous immunization of Balb/mice with *L. major* stress-inducible protein 1 antigen (rLmSTI1) encapsulated in liposomes resulted in a significant defense against the disease, associated with elevated specific IgG activity with a predominant IgG2a titer [37]. Liposomal amphotericin B is considered superior to free AMB in the treatment of visceral leishmaniasis [38]. L-AMB combined with rHuGM-CSF or meglumine antimonate was found effective against VL in AIDS patients and against CL caused by *L. braziliensis* in Brazil.

DC-BASED IMMUNOTHERAPY

Another novel approach is the introduction of dentritic cells for the induction of antigen-specific T cell immunity. DCs are the most potent antigen-presenting cells and play a critical role in the activation of T, B and NK cells. These cells are able to capture antigens, transfer them to local lymph nodes where they present the antigenic epitope to naïve T cells, and activate specific Th1 response. In CL, DCs rather than macrophages induce the initiation of the immune response [2]. DC-based vaccine was determinant in mounting a protective Th1 response and in reducing the concentration of amastigotes in the liver and the spleen of mice infected with *L. major*. DCs pulsed with peptide 154-169aa of gp63 or soluble promastigote lysate reduced lesion formation and parasite load in Balb/c infected with *L. major*. Also, a protection against CL in Balb/c mice was further achieved using DCs pulsed with *L. infantum* nucleosomal histones [39]. DC-based immunotherapy combined with antimony-based chemotherapy was very effective against murine VL [40]. While three weekly injections of *L. donovani*-soluble antigen-pulsed DCs into mice infected with *L. donovani* only reduced the number of spleen and liver amastigotes, treatment with sodium stibogluconate combined with antigen-pulsed DCs resulted in a complete deletion of the parasites.

Ig = immunoglobulin
L-AMB = liposomal amphotericin B
NK = natural killer

SUMMARY

There is still a need for innovative and alternative therapies against leishmaniasis. Despite recent advances in immunology, effective immunotherapy against the disease has not yet been proven. Live, attenuated and dead parasites, purified and recombinant specific antigens, DNA vaccines as well as DC-based immunization that have been employed in the development of protective vaccine have not yet been adopted as immunotherapeutic agents. Recently, a commercially prophylactic vaccine (Leish-110f®) was developed by BioPharm International, by constructing a recombinant fusion protein consisting of TSA (thiol-specific antioxidant), LmSTI1 (*L. major* stress-inducible protein 1) and LeIF (*Leishmania* elongation initiation factor). This vaccine, when administered together with the adjuvant monophosphoryl lipid A (MPL®), either alone or plus squalene (MPL-SE®) or AdjuPrime, protected mice against *L. major* and *L. infantum* infections. Also, Leishvacin® (Leishvacin, Biobrs, Montes Carlos, State of Minas Gerais, Brazil), a commercial non-living promastigote polyvalent *Leishmania* vaccine administered either alone or combined with BCG, was found to be highly immunogenic against American CL in humans. Leishvacin alone was also found to be effective as a prophylactic vaccine, sensitizing lymphocytes from normal uninfected humans, which was further accelerated by recombinant GM-CSF. Standardization and additional carefully controlled studies in animals and humans, using these new vaccines and other immunomodulators in conjunction with various chemotherapeutic agents, are still required to determine the optimal conditions for the development of a potent anti-leishmanial immunotherapy and immunochemotherapy.

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"All I ask is this: Do something. Try something. Speaking out, showing up, writing a letter, a check, a strongly worded e-mail. Pick a cause – there are few unworthy ones. And nudge yourself past the brink of tacit support to action. Once a month, once a year, or just once"

Joss Whedon (b. 1964), American writer and film director

"Do not fear to be eccentric in opinion, for every opinion now accepted was once eccentric"

Bertrand Russell (1872-1970), English philosopher, logician, mathematician, historian and social critic. He is considered one of the founders of analytic philosophy along with his protégé Wittgenstein and is widely held to be one of the 20th century's premier logicians. He was a prominent anti-war activist, championing free trade between nations and anti-imperialism. He won the Nobel Peace Price in 1950