Potential biomarkers of immune protection in human leishmaniasis

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Abstract

Leishmaniasis is a vector-borne neglected tropical disease endemic in over 100 countries around the world. Available control measures are not always successful, therapeutic options are limited, and there is no vaccine available against human leishmaniasis, although several candidate antigens have been evaluated over the last decades. Plenty of studies have aimed to evaluate the immune response development and a diverse range of host immune factors have been described to be associated with protection or disease progression in leishmaniasis; however, to date, no comprehensive biomarker(s) have been identified as surrogate marker of protection or exacerbation, and lack of enough information remains a barrier for vaccine development. Most of the current understanding of the role of different markers of immune response in leishmaniasis has been collected from experimental animal models. Although the data generated from the animal models are crucial, it might not always be extrapolated to humans. Here, we briefly review the events during Leishmania invasion of host cells and the immune responses induced against Leishmania in animal models and humans and their potential role as a biomarker of protection against human leishmaniasis.

Keywords Leishmaniasis · Immunology · Biomarkers · Protection · Vaccine · Macrophage

Introduction

Leishmaniasis is a vector-borne neglected tropical disease endemic in over 100 countries around the world. Clinical manifestations of the disease are mainly cutaneous (CL), mucocutaneous (MCL) and visceral (VL) and post kala-azar dermal (PKDL) leishmaniasis [1, 2]. The pathogenesis of leishmaniasis is influenced by elements from the triad of parasite–host–vector interplay. At least in murine model of leishmaniasis, the type of immune response generated upon infection with Leishmania plays a crucial role in the outcome of the disease either cure and protection or progression and even death. Although plenty of data concerning the factors involved in pathogenesis of Leishmania infection and the effector mechanisms of the host immune response are collected in animal models and patients during the last decades, but yet the immune biomarkers of cure/protection or exacerbation in human leishmaniasis are not well defined.

It is well known that CL caused by natural infection or leishmanization induces strong protection against further CL lesion development, which justifies to develop vaccine against leishmaniasis [3]. Wealth of information which is accumulated over the past years on the biology of intracellular parasites, map of Leishmania genome, and numerous experimental studies on the immunology of leishmaniasis, supported search to develop an effective vaccine (reviewed in [4, 5]). In the last decades, numerous Leishmania vaccine candidates have been introduced as vaccine candidate including whole live, attenuated, genetically modified, killed parasites, and subunits or fusion proteins, but only a few have been tested in clinical trials [4]. The absence of a vaccine against leishmaniasis is primarily attributed to the absence of clear understanding of correlates of protection [6]. Moreover, animal models of leishmaniasis do not always mimic human leishmaniasis [7], and extrapolating results of protection assays obtained with the experimental murine models to humans is doubtful.
Methods

A literature search using the PubMed, Scopus and Google Scholar databases has been conducted for publications with full text or abstract in English language over the last 45 years. Relevant additional articles identified during review by authors were also included.

Search terms included were “Leishmania* AND (immune response OR immunology OR protection)” or “leishmaniiasis AND biomarker”. The initial search strategy identified more than 10,000 results. To limit search hits, other fields such as title [Ti], title abstract [Tia], English [lang] and publication year [dp] were used which decreased the number of results to 7897 articles. Based on the title and the abstract, 523 articles including 46 review papers and 477 original papers were carefully analysed and finally 280 were cited.

Macrophages and initiation of Leishmania infection

Macrophages (MΦ) are known as the primary antigen presenting cells (APCs), other phagocytic cells including monocytes, dendritic cells and neutrophils are also recruited to the site of infection and play important roles (reviewed in [8]). It has been proposed that Leishmania parasites use neutrophil polymorphonuclear leukocytes (PMNs) as temporary host cells to silently enter macrophages without activation of defence mechanisms (Trojan horse hypothesis) [9]. Subsequently, macrophages phagocytose free parasites and apoptotic PMNs infected with Leishmania parasites and serve as the definitive host cells and permit parasite growth. However, macrophages are naturally responsible for killing of invading parasites by activation of effective microbicidal mechanisms (reviewed in [10]). The effective elimination of parasites by macrophages and development of protective immune response against Leishmania require involvement of dendritic cells (DCs) [10].

Leishmania parasites are able to engage different cell surface receptors including complement receptors [11, 12], fibronectin receptor [13], Toll-like receptors 2, 3 [14] and 4 [15] and mannose receptor [16] to enter into the host cells. The leishmanial membrane protease gp63 cleaves C3b attached to its surface, converts it to C3bi inactive form which binds to CR3 receptor, and mediate entry of opsonized promastigotes into macrophages. This strategy protects the parasites from lysis by complement activity [17].

Leishmania parasites are engulfed by macrophages and are eliminated by production of interferon gamma (IFN-γ), reactive oxygen species (ROS) and nitric oxide (NO) derivatives inside phagolysosome; however, intracellular amastigotes modulate various antimicrobial defense pathways and interfere with a number of critical macrophage functions to sustain and multiply inside the cell (reviewed in [18]). Macrophages successfully phagocytose Leishmania parasites, but the production of IL-12 is inhibited by the intracellular parasites [19]. It was shown that internalization of L. major through CR3 receptor, which is a mechanism of silent entry into macrophages, leads to blockade signaling cascade and synthesis of interleukin 12 (IL-12) [20, 21]. IL-12 is necessary for the killing of Leishmania parasites by macrophages, as it allows for upregulation of inducible nitric oxide synthase (iNOS or NOS2) and NO synthesis and subsequent parasite elimination [22, 23] by promoting the development of CD4+ T cells and production of IFN-γ (basic findings on the role of Th1/Th2 cytokines in reference [24]). Leishmania infection also leads to induction of other regulatory cytokines such as IL-10 and transforming growth factor β (TGF-β) which interfere with macrophage effector functions in favor of parasite survival and disease progression [25].

Macrophage: arginine metabolisms and NO production

Among the most important players are arginine-derived metabolites which significantly influence the parasite survival in macrophage (reviewed in [26]). Polyamines are essential metabolites in trypanosomatid protozoa and play a role in the synthesis of thiol trypanothione. Polyamines are synthesized by a metabolic process involving arginase I enzyme (arg1) which catalyzes the hydrolysis of L-arginine to L-ornithine. Animal studies showed that induction of arg1 enzyme promotes Leishmania growth and dissemination in vivo, and induction of non-healing leishmaniasis [27]. In contrast, inhibition of arg1 activity is associated with limited pathology, the lower parasites burden and delays in disease outcome in BALB/c mice [28]. Spleen macrophages isolated from L. donovani-infected hamster showed low iNOS but high arg1 enzyme along with increased polyamine synthesis [29].

In human leishmaniasis, higher levels of arg1, TGF-β, ornithine decarboxylase (ODC), and prostaglandin E2 in plasma and higher expression of arg1 and ODC in lesion biopsies have been shown in L. amazonensis-infected patients with diffuse CL (DCL) compared with patients with localized CL (LCL), indicating a role for arg1/polyamines in DCL development [30]. It was shown that the level of arg1 activity is higher in blood PMNs of patients with chronic CL than that of acute CL, suggesting a possible role of arg1 in chronicity of CL lesions caused by L. major/L. tropica [31]. A high arg1 activity was shown in peripheral blood mononuclear cells (PBMCs) and plasma of VL and VL-HIV
co-infected patients and as such an increase level of arg1 is suggested as a marker of VL severity [32].

Polarising signals activate macrophages leading to their development into functionally distinct subsets which influence intracellular *Leishmania* survival and determine disease outcomes. Th1 type of cytokines particularly IFN-γ, induce classically activated (M1) macrophages which produce a significant amount of NO and initiate parasite killing. Classically activated macrophages may contain lower concentrations of arginine, as a result of NO production. In contrast, by activation of Th2 type of cytokines such as IL-4 and IL-13, alternatively activated macrophages (M2) are developed which characterized by increased expression of arg1 and polyamine biosynthesis, favouring amastigote growth in macrophages and disease progression [33, 34]. Genetic disruption of amastigote arginase resulted in reduction of parasite replication [35] and significantly attenuated infection in murine model [36], an indication of amastigotes reliance on de novo synthesis of polyamines.

Mononuclear cells produce two major anti-*Leishmania* components; ROS which is generated by respiratory burst during phagocytosis, and NO, which is produced by iNOS in response to IFN-γ (reviewed in [37, 38]). T cells are the main source of IFN-γ production, an initial macrophage activation through IFN-γ is necessary for parasite killing through oxidative-burst mechanisms [39]. In addition to IFN-γ, there are a number of other inflammatory cytokines, such as IL-1, tumor necrosis factor (TNF), interferon alpha (IFN-α), and interferon beta (IFN-β) which are also involved in macrophage activation and induction of iNOS expression and NO production (reviewed in [40]).

Production of ROS showed to be an important part of host cell immune response to induce anti-parasitic effector mechanisms, albeit the role of ROS in *Leishmania* infection control in murine model varies and depends on the parasite species and mouse strains. Unlike what observed in *L. major* infection, NADPH oxidase, which is required to generate ROS, showed no impact on the course of *L. braziliensis* infection in mouse model [41]. In human, however, production of ROS is shown to be an important part of control mechanisms of *L. braziliensis* infection [42].

Similarly, NO is an essential factor in control of *Leishmania* infection in mouse model, genetic deletion or functional inactivation of iNOS in *L. major* infected mice on a resistant background at early stage of infection, abolished IFN-γ release by NK cells and increased TGFβ expression, resulted in a progressive parasite dissemination throughout the infected mouse. Furthermore, induction of iNOS was dependent on IFNα/β production [43, 44]. The function of iNOS and NO in human leishmaniasis is less known, while production of ROS is shown to be involved in killing of *L. braziliensis* by human macrophages, NO alone was found not to be sufficient to control the infection of monocytes from CL patients in vitro [42]. It was reported that NO production is not traceable in supernatants of human macrophages infected with *L. chagasi*, but in vitro blockade of NO affected parasite growth in human macrophages [45].

**Dendritic cells and interactions with Leishmania**

Macrophages and DCs are both professional APC, but in regard to *Leishmania* infection, they use different strategies for parasite uptake, internalization and antigen presentation. DCs preferentially uptake *Leishmania* amastigotes opsonized with IgG through surface FcγRI or FcγRIII receptors [47]. Although the phagocytosis capacity of DCs is not completely comparable with that of macrophages [48], but antigen presentation and IL-12 production by DC is critical in CD4+Th1 and CD8+T cells development to mediate protective immune response against *Leishmania* infection [22, 49]. In contrast to *L. major* and *L. donovani* parasites which promote production of IL-12 by murine DCs, infection with *L. mexicana* and *L. amazonensis* amastigotes failed to activate DCs or to induce IL-12 production [50, 51].

Several subtypes of DCs have been identified in both humans and mice that have distinct functions and molecular features (reviewed in [52]). Plasmacytoid DCs (pDCs) are considered resident DCs which specially produce type I interferon, and classical DCs (cDCs), mediate antigen processing and presentation to T cells [53]. cDCs express the integrin CD11c and MHC class II, cDCs can be further divided into two major subsets cDC1s and cDC2s. A transcription factor Zbtb46 (BTBD4) was identified which specifically expresses by all cDCs in both human and mouse but not by pDCs, monocytes, and macrophages [54].

Langerhans cells (LCs) are seen in human skin epidermis and mucosal tissues similar to murine langerhans cells which are identified by the presence of a transmembrane lectin with mannose binding specificity called langerin. LCs are differ markedly from other migratory DCs in their ontogeny and
may have a protective or suppressive function in skin pathology (reviewed in [55]).

During inflammation, monocytes recruit to the site of inflammation and differentiate into “inflammatory DCs” which present DC markers (CD11c and zbtb46) and DC functions [56].

During recent years, numerous mouse models for the specific depletion of DC subsets have been generated which are used for elucidating specific functions of DCs (reviewed in [57]). In one approach, mouse models are developed that express diphtheria toxin (DT) receptor (DTR) under the control of a cell type-specific promoter, and subsequent administration of DT mediates selective depletion of the DTR-expressing cells. DT disrupts protein translation by involving elongation factor 2 which eventually leads to cell death.

Several DTR mouse strains have been generated for depletion of specific DC subsets, such as Zbtb46-DTR strain for specific depletion of cDCs, Cd207-DTR strain for depletion of LCs as well as cDC1s in skin-draining LNs, Ly75-DTR strain for depletion of CD205+ cDCs (the majority are CD8α+ cDC1s), Clec9a-DTR BAC strain for depletion of cDC1s, Xcr1-DTR venus and Karma strains for complete depletion of cDC1s, and Clec4a4-DTR strain that allows for ablation of cDC2s [57]. Similarly, several DTR mouse strains have been generated for depletion of pDCs, monocytes and macrophages, allowing functional study of specific cell subsets (reviewed in [58]).

In the early phase of murine Leishmania infection, three types of DCs including epidermal LCs, dermal DCs (dDC) and inflammatory DCs, are localized at the site of infection and mediate APC function. DCs take up parasites at the site of infection and then migrate to the dLN to present antigens to T lymphocytes, initiating an adaptive immune response [59], but there is a discrepancy about the extent to which each subset of DCs is involved in the immune response generation against Leishmania [60]. This discrepancy seems to be mainly due to differences in the parasite species, the dosage, and the route of administration [61]. A timing schedule is proposed in establishment of a protective immune response to L. major infection in murine model, in which dDCs and LCs play a role early in infection, but later the cells are replaced by inflammatory monocyte-derived DCs and lymph node-resident DCs (reviewed in [61]).

Another study showed that CD8α−Langerin−DCs migrate to dLNs to present antigens to specific T cells to induce protective immune response against L. major infection [62], later a functional dichotomy has been suggested for two subsets of dDCs, where Langerin−dDC population mediates a CD4+ T-cell response, but Langerin + dDC subset is involved in early priming of CD8+ T cells [63].

Experimental data indicate a suppressive role for skin LCs in low dose L. major infection by expansion of parasite-specific regulatory T cells, whereas both murine and human data suggest that dermal inflammatory DC is associated with enhanced induction of Th1 response and promoting protection [61, 64].

**Leishmania persistent infection and immune evasion mechanisms**

*Leishmania* parasite uses several immune evading strategies [65–67] which might need involvement of cell surface molecules, particularly gp63 and LPG [68]. Metacyclic *Leishmania* promastigotes avoid complement-mediated lysis via surface LPG by deactivation of the classical and alternative pathways [69].

*Leishmania* amastigotes inhibit the assembly of NADPH complex which generates ROS [70], and interfere with several phosphorylation signaling pathways of the cells [71–73]. Down-regulation of Toll-like receptors (TLR) and the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathways genes are suggested in NK cells from DCL patients caused by *L. mexicana* [74]. Similarly, *L. donovani* infected macrophages are defective in the ability to phosphorylate downstream molecules of JAK2/STAT1 signaling pathways including STAT-1, JAK1, and JAK2 in response to IFN-γ [71, 75].

It was shown that inhibition of PKC-dependent activity contributes to the survival of *L. donovani* inside the macrophages [76] and inhibition of a mitogen-activated protein kinase (MAP kinase) of host cells following *Leishmania* infection is confirmed by several studies [77, 78]. Induction of ceramide synthesis in *L. donovani*-infected murine macrophages mediates inactivation of ERK1/2 MAP kinases which results in inhibition of transcription factors AP-1 and NF-κB, NO generation, and a lower parasite burden [79].

*Leishmania* also activates various molecules that inhibit intracellular signaling cascades. An important negative regulatory molecule is PTP SHP-1 which is involved in limiting the activation of the JAK/STAT pathways following *L. donovani* infection [75]. Induction of SHP-1 is vital for inhibition of NO generation which occurs through the inactivation of JAK2 and ERK1/2, and transcription factors NF-κB and AP-1 [80]. Another survival strategy used by *Leishmania* parasites is detoxification of important antimicrobial molecules that are secreted into the phagolysosome including superoxide radicals and nitrite derivatives, such as peroxidoxins LcPxn1/2 [81] and a superoxide dismutase [82].

Within macrophages, *Leishmania* promastigotes transform into amastigotes and replicate continuously until causing cell death and rupture. Microbicidal mechanisms of macrophages later on infection eliminate the intracellular parasites through NO production [83]. The fate of *Leishmania* parasites within DC is less clear, in both human CL and murine *Leishmania* infection, parasites
Persistence has been reported [84–86], but the main host cells for long-term persistence is not clearly defined. In the lymph nodes of mouse after cure of *L. major* infection, both macrophages and dendritic cells which derived from the skin, showed to harbour viable parasites [84]. LCs containing parasite are also detected in the skin lesion infiltrate from *L. major*-infected mouse [87].

Bogdan et al. showed that fibroblasts are responsible for about 40% of the persisting parasites in the draining lymph nodes of mouse after healing of cutaneous lesion due to *L. major* infection. The infected fibroblasts did not eliminate the parasites comparable to the infected macrophages, thus fibroblasts are proposed as safe host cells for the parasites in latent infection [88].

Owing to limited capability of de novo synthesis, *Leishmania* amastigotes require essential nutritional elements, such as amino acids, purines, lipids, and other metabolites which must be available within the parasitophorous vacuole (PV) to support amastigotes growth (metabolic pathways reviewed in [89]). Upon phagocytosis, metacyclic promastigotes transform into amastigotes in the host cell where they encounter a limitation in the availability of several nutrients (reviewed in [89, 90]). *Leishmania* parasites constitutively express genes involved in core pathways of carbon metabolism throughout the life cycle [91]. Oligosaccharides such as mannose and galactose are integrated into the structure of LPG [92] which play a significant role in survival of the parasites within phagolysosomes by involvement in macrophage oxidative responses [93]. Additionally, lipid bodies are organelles in the macrophage consist of neutral lipids mainly triacylglycerol and sterol esters which partially support parasite’s nutrient requirements and are involved in phagosome maturation and production of eicosanoids molecules which regulate immunity by either promoting or modulating inflammatory responses [94].

On the other hand, parasite ferric iron reductase (LFR1), ferrous iron transporter (LIT1) and heme transporter (LHR1) contribute to provide iron sources for *Leishmania* parasites and as such these molecules are essential for *Leishmania* viability and intracellular survival [95]. Up regulation of iron exporters including natural resistance-associated macrophage protein (NRAMP-1) and Ferropor- tin (Fpn-1) which restrict the availability of iron to the parasite are recently shown in monocytes isolated from PKDL patients [96]. A secretory peroxidase of *L. donovani* down regulates NRAMP1 expression in peritoneal macrophages and allows iron access to *Leishmania* inside PV [97] and another iron regulator, hepcidin, facilitates iron sequestration within macrophages by mediating cell surface degradation of the iron exporter ferroportin [98]. For more detailed survival factors see reference [99].

**Interleukin 12 (IL-12) production**

*Leishmania* induces IL-12 production at early hours of infection which leads to NK cell activation and IFN-γ production [100, 101]. A central role is assumed for both IL-12 and IFN-γ to drive CD4+ T-cell differentiation and subsequent induction of protective immune response to *L. major* infection in mouse [102]. In normally resistant mouse strains, in vivo neutralization of IL-12 results in inhibition of IFN-γ production by NK cells in lymph nodes [101]. IL-12 is also necessary for down-regulation of Th2 type responses during *L. major* infection in vivo [103]. Nevertheless, there is evidence showing that the early IFN-γ production following *L. major* infection is IL-12 independent [104].

In vitro and ex vivo studies showed that IL-12 deficient mice from resistant strain mount a strong Th2 type response with a high level of IL-4 and a low level of IFN-γ expressions and develop progressive uncontrolled lesions similar to genetically susceptible BALB/c mice [105]. The role of IL-12 in the development of protective CD4+ T-cell-mediated immunity in *Leishmania* infection has been shown in several other studies through neutralization of IL-12 using monoclonal antibody or deletion of IL-12 gene in resistant mice strains [106, 107] or through rIL-12 treatment in susceptible mice strains [100, 108, 109]. These findings implicate that IL-12 is essential for the development of effective Th1 type of response in leishmaniasis [110].

As mentioned in the previous sections, *L. major* has the ability to block IL-12 production in macrophages [19, 111] and DCs remain the major source of IL-12 in *Leishmania* infection. IL-1α acts in conjunction with IL-12 and promotes Th1 differentiation and prevents disease progression in *L. major* susceptible BALB/c mice [48, 112]. Experimental studies suggested that sustained IL-12 is required for the maintenance of Th1 response in *Leishmania* infection [106, 109, 113].

Although, IL-12 seems to be essential in the dichotomy of immune responses to *L. major* infection in susceptible vs. resistant mice strains [107, 110], the role of IL-12 in human leishmaniasis is not fully clear. PBMCs culture from CL patients of Old World indicates a higher level of IFN-γ and IL-12 expression and a lower level of IL-4 and IL-10 expression in the healing compared to non-healing CL patients [114]. In contrast, in CL caused by *L. mexicana*, in situ expression of IL-12 mRNA was found to be higher in non-healing lesions coincide with high expression of IL-10, indicating that IL-12 alone could not induce lesion healing [115, 116]. Unresponsiveness of T cells to IL-12 activation is associated with persistence of parasite and active lesion due to *L. guyanensis* [117].

IL-12 was initially used as an adjuvant with soluble *Leishmania* antigen (SLA) against *L. major* challenge in murine model [118], recombinant human IL-12 was used.
as adjuvant with alum plus heat-killed *L. amazonensis* antigen [119] or killed *L. major* in primate models [120] however, the use of rhIL-12 in human leishmaniasis has not been verified, mainly due to safety issues and significant toxicity explored during clinical trials on other diseases (reviewed in [121]).

Neutralization of IL-12, both at early or late stage of *L. donovani* infection caused increased parasite load, reduced IFN-γ, IL-4, TNF and iNOS production, resulting in inhibition of tissue granuloma formation in the liver of susceptible BALB/c mice [122, 123]. IL-12 is crucial for induction of IFN-γ producing T cells and protective host responses in the liver. Although in experimental model of *L. donovani* infection, IL-12 has an anti-Leishmania activity even in the absence of IFN-γ, which appears to be dependent on TNF production [124]. DCs in the spleen are the critical source of early IL-12 production following *L. donovani* infection and activation of DCs is crucial for optimal induction of immunity in the liver during the early phase of VL infection [122, 125, 126]. Study on *L. infantum* infected mice demonstrated that myeloid DCs, TLR9, and IL-12 are functionally linked to the activation of NK cells to produce IFN-γ [127].

In human VL, CD4+ T cells mediate a protective immune response by production of various cytokines and chemokines that contribute in granuloma formation and parasite killing, such as IL-2, IL-12, IFN-γ, TNF, lymphotoksin (LT) and granulocyte/macrophage colony-stimulating factor (GM-CSF), which have been measured in serum samples [128, 129]. PBMCs from active VL patients failed to produce IL-12 or IFN-γ in response to in vitro stimulation with *L. donovani* antigens, however, the addition of exogenous rhIL-12 to PBMCs from the same patients resulted in the expansion of IFN-γ production [130]. Similarly, addition of IL-12 to the PBMCs cultures from American VL patients restored cellular immune responses showed by proliferative response and IFN-γ production [131].

Cytokines of Th1/Th2 types

It is well known that *L. major* infection in susceptible BALB/c mice is associated with generation of Th2 response with a high level of IL-4, progression of the disease and death, whereas almost in all other strains of mice, resistance is associated with generation of Th1 type of response with production of high IFN-γ level that induce healing lesion and protection against further lesion development [83, 132–134]. Although there are well established explanation in regard to immune response in murine model of leishmaniasis, but regardless of tremendous studies on Th1/Th2 cytokine responses (as reviewed in [135]), the mechanisms of cure and protection in human leishmaniasis is not well defined yet [4, 136].

In anthroponotic CL (ACL) caused by *L. tropica*, correlation of a high expression level of Th2 cytokines including IL-4 and IL-10 with antimonial unresponsiveness, and upregulation of Th1 cytokines including IL-1β, IL-12 P40 and IFN-γ genes with response to treatment is shown [137]. In New World, individuals cured from CL showed a significant increase in the frequency of cells expressing Th1-type cytotoxic production profile (IFN-γ+/granzyme B+/perforin+) which is an indicative of imbalance toward a cytotoxic response [138]. In some clinical forms of human leishmaniasis such as American CL, non-healing ACL and PKDL a mixed Th1/Th2 response is seen in vitro and in situ [139–142]. Furthermore, it is well established that human VL displays a Th2 response at early stage of the disease which shifts toward a mixed Th1/Th2 patterns, with high levels of IFN-γ as well as IL-4/IL-13 secretion [143–145].

Several studies have been completed over the last decades to explore the role of Th1/Th2 responses in human leishmaniasis [146–149] and phenotype of Th1/Th2 cells or their polarised cytokines in lesion, cell culture or plasma have been characterized in leishmaniasis patients [136, 150–154]. However, several *Leishmania* antigens predominantly stimulate Th1 responses *in vitro*, that are not necessarily associated with protection [155]. In some cases, antigens which are associated with an early Th2 response such as *Leishmania*-Activated C-Kinase Antigen (LACK) or cysteine protease CPB2.8 are found to be protective if administered with an appropriate adjuvant [155–158].

Interferon-γ (IFN-γ)

At early stage of *Leishmania* infection, IFN-γ participates in the control parasite growth and lesion development. IFN-γ activates effector mechanisms/signaling pathways of macrophages to eliminate intracellular pathogens primarily through NO production. Characterization of immune response in CL patients shows an upregulation of IFN-γ production around the lesions [114, 159–161] and production of a significant high level of IFN-γ but a low level of IL-10 from T cells in culture after healing [147, 148, 162], indicating the possible involvement of IFN-γ in healing process of CL lesions. In *L. braziliensis* infection a long-lasting Th1 response with elevated level of IFN-γ and down regulation of IL-4 and IL-10 production is shown in vitro and in situ which is apparently associated with healing of the skin lesion(s) [140, 141, 163].

In our studies on CL patients, a role for both CD4+ and CD8+ T lymphocytes as the main source of IFN-γ production is shown [151, 152, 164], but prior to the development of adaptive immune response, IFN-γ is primarily produced by NK cells [165], the role for NK cells in innate immune response is shown in different forms of human leishmaniasis [163, 166] (reviewed in [167]).
It should be noted that exacerbated Th1-cell-mediated immune response during CL, accompanies with excessive secretion of pro-inflammatory cytokines including IFN-γ, could cause tissue damage and contribute to the lesion progress [168].

VL is associated with T-cell suppression, which is characterized by lack of proliferation and IFN-γ production by PBMCs in response to *Leishmania* antigens in vitro [169, 170]. While it is assumed that this in vitro unresponsiveness of VL patients might be due to the defects in immune system, whole blood cells of active VL patients maintain the capacity to secrete significant levels of antigen specific IFN-γ and IL-10 [171] and CD4+ T cells are found as the main source of IFN-γ production [172]. Individuals cured of VL usually mount antigen-specific IFN-γ response in vitro and convert to leishmanin skin-test positive [173–175].

In experimental VL, IFN-γ plays a critical role in the early immune response leading to control of parasite burden and eventual resolution of *L. donovani* infection which occurs within well-formed tissue granulomas in the liver of mice [176, 177]. Treatment of *L. donovani* infected nude BALB/c mice with IFN-γ activated macrophages in mice but requires the presence of T cells for anti-*Leishmania* activity [178]. Experiments in mice showed that administration of IFN-γ increased the efficacy of antimony chemotherapy [179] and IFN-γ is used as an adjunct therapy for severe or refractory cases of VL [180].

IFN-γ is a key cytokine of the immune system that involves in regulation of various cellular events through transcriptional control over different genes [181]. Up-regulation of class I and class II MHC expression [182], activation of microbialid mechanisms including induction of the NADPH-dependent oxidase system, priming NO production, tryptophan depletion, up-regulation of lysosomal enzymes [182], augmenting surface expression of FcγRI on mononuclear phagocytes, thereby promoting antibody-dependent cell-mediated cytotoxicity, and stimulation of complement-mediated phagocytosis are among the most important functions of IFN-γ.

In addition, IFN-γ orchestrates the trafficking of immune cells to the sites of inflammation through regulating the expression of adhesion molecules (e.g., ICAM-1, VCAM-1) and chemokines (e.g., IP-10, MCP-1, MIG, MIP-1α, RANTES) [182]. Also, IFN-γ synergizes or antagonizes the effects of many cytokines through involvement in the cell signaling pathways. IFN-γ exerts its effector anti-microbial functions in macrophages through inducible transcription factor Stat1 [183], a cytosolic latent transcription factor that participate in regulation of target genes and transmit the immunological effects of IFN-γ [184]. Stat1 induces expression of iNOS and cytokines such as IL-12, TNF, and IL-1β. Findings from *L. major* infection in resistant C57Bl/6 mice lacking the Stat1 gene demonstrated that Stat1-mediated IFN-γ induction is indispensable for the development of protective immunity against *Leishmania* infection [185]. Furthermore, a novel function of constitutive Stat1 in modulation of phagosomal acidification is shown, which contributes in intracellular *Leishmania* growth in macrophage [186] (Table 1).

**Interleukin 4 (IL-4)/interleukin 13 (IL-13)**

Early evidence showed that expansion of IL-4 induces Th2 response in murine *L. major* infection and results in exacerbation of the lesion and generalization of the disease which eventually kills the animals [187, 188], neutralization of IL-4 using anti-IL-4 antibodies significantly but not completely attenuated the progression of infection in BALB/c mice [189, 190]. IL-13 plays a role in chronicity of non-healing infection in mice [191]. Evidence provided from experiments on the IFN-γ/IL-4 genetically engineered mice showed that IL-4 is a key player in susceptibility to *L. major* infection and magnitude of IL-4 response determines the severity of the disease in BALB/c mice [192]. Down-regulation of Th1 response through inhibition of IL-12 receptor (IL-12Rβ2) expression is mediated by IL-4 in *L. major* [193–195], by IL-13 in *L. amazonensis* [191] or independent of IL-4/IL-13 in *L. mexicana* infection [196].

Despite these evidence, the role of IL-4 as a major factor which contributes in susceptibility is controversial. Although IL-4/IL-13 mediate susceptibility to *Leishmania* infection in murine model [192, 197] effector Th2 immune response is also evidenced in the absence of IL-4/IL-13 cytokines, where IL-4−/− and IL-4Ra−/− mice were highly susceptible to *L. major* parasite [198]. These data suggested the possibility of involvement of other cytokines in the development of Th1/Th2 immune response during *Leishmania* infection. Furthermore, in contrast to the general consensus, a role for IL-4 cytokine in promoting a Th1 immune response has also been suggested [199]. Based on this report the time at which IL-4 is presented is determinative, during the initial activation of DCs, IL-4 induces production of IL-12 and promotes a Th1 response which is associated with resistance to *L. major* infection in susceptible BALB/c mice, but later during the period of T cell priming, IL-4 induces a Th2 response associated with progressive infection in resistant mice [199]. An experiment on the influence of recombinant IL-4/IFN-γ on murine macrophage showed that IL-4 synergizes with IFN-γ to activate macrophages and provides a strong stimulus to kill *L. major* amastigotes at low concentrations of IFN-γ [200].

In human leishmaniasis, usually IL-4 level is negligible and hard to measure on culture supernatant of stimulated PBMCs in vitro [201, 202]. It was shown that in chronic and destructive MCL a mix Th1/Th2 type cytokines exist, with prominent upregulation of IL-4 mRNA expression in...
| Biomarkers | Mice studies | Human studies | Ref. |
|------------|--------------|---------------|------|
| IFN-γ | IFN-γ participate in the control of intracellular parasite growth and lesion development | Possible role in healing process is supposed | [176, 177] |
| | A critical role in the early immune responses of VL leading to resolution of L. d. infection within granulomas of liver | Upregulation of IFN-γ in the lesions and PBMC culture after healing of CL patients is shown | |
| | | Excessive secretion may cause immunopathological effects | |
| | | Active VL is associated with lack of immune response in PBMCs culture | |
| | | Cured VL mount antigen-specific IFN-γ responses in vitro | |
| IL-12 | Early IL-12 production leads to NK activation and IFN-γ production leading to protective T cell response to L. m. infection | Not fully clear | [48, 100, 101, 102, 103, 105, 106, 109, 105, 110, 113] |
| | The absence of IL-12 reverts Th1 response to Th2 against L.m. infection | High levels of IL-12 and IFN-γ in PBMC culture of healing CL cases | |
| | Activate effector mechanisms of MQ including NO production | Used as an adjuvant to killed L.m. vaccine in vervet monkey | |
| | Essential role for development of effective Th1 response and induction of IFN-γ production from T and NK cells | Exogenous rIL-12 to PBMCs from VL patients resulted in the expansion of IFN-γ production in response to L. donovani stimulation | |
| | Role in the granuloma formation in the liver of L. d. infection | Not fully clear | |
| TNF | Synergism with IFN-γ in activation of MQ to produce iNOS | High production in in active CL, reduced in healed CL | [209, 210, 213, 214] |
| | Essential for the granuloma formation in liver and induction of protective immunity against VL | Increased levels of TNF and frequency of TNF-producing T cells correlated with severity of disease in CL cases | |
| | Progressive parasite burden and death of mice lacking TNF | Elevated levels in lesions of treatment non-responder CL cases | |
| | | Possible role in early lesion development in CL | |
| | | Clinical use of TNF inhibitor reduces TNF pathology | [136, 150, 207, 211, 208] |
| Biomarkers | Mice studies | Ref. | Human studies | Ref. |
|------------|--------------|------|---------------|------|
| IL-10      | Natural CD25+FoxP3+Treg cells responsible for suppressing immune response in infection site CD25-FoxP3- IL-10 producing Treg cells prevent sterile cure and delay healing A deteriorating role in experimental VL, impaired Th1 response | [216, 217, 226, 230] | Elevated levels of IL-10 in human VL Antigen-specific production of IL-10 in whole blood culture of patients with active VL The absence of IL-10 with increased levels of proinflammatory cytokines cause exacerbating lesion development. (L.b.) | [171, 227-229] |
| IL-17      | High levels of IL-17 in BALB/c mice infected with L.m IL-17 deficiency associated with better control of disease | [232, 233] | No significant difference between active vs. healed CL cases in production of IL-17 IL-17 levels correlate with inflammatory response in CL and ML lesions | [136, 234, 235] |
| IL-4/IL-13 | Polarizing immune response toward Th2 type associated with non-healing disease in BALB/c mice IL-13 plays a role in maintaining a chronic non-healing infection May downregulate IL-12 production | [187-190, 193-196] | IL-4 levels are hard to measure in blood culture IL-13 expression in lesions and blood culture are measured as indicator of Th2 response IL-4 levels are hard to measure in blood culture IL-13 expression in lesions and blood culture are measured as indicator of Th2 response | [149, 164, 202, 204] |
| iNOS/NO/ROS| Role of ROS in infection control varies and depends on the parasite species NO is essential in controlling Leishmania infection In the absence of iNOS, L. m. is disseminated in the body of mice | [41, 43] | The function of iNOS and of NO in human leishmaniasis is less known ROS important in control of L. b. infection, is shown to be involved in killing of L. b. by human macrophages Antileishmanial function iNOS is shown in skin biopsies from American CL patients due to L. mex Controversy on the role of NO | [39, 42, 46] |
| Arginase 1 | Induction of arginase 1 promotes uncontrolled growth of Leishmania in vivo, leading to nonhealing infection Inhibition of arginase 1 is associated with reduced parasites and delays in disease outcome in BALB/c mice L. d. infected hamster spleen showed low NOS2 but high Arg1 activity and expression and increased polyamine synthesis | [29, 30] | Increased levels of arginase 1 is found in PBMCs and plasma of VL and VL-HIV coinfected patients High levels of arginase 1 activity in lesions and PMNs of patients with active and chronic CL due to L. m./L. t Significant plasma levels and lesion expression of arginase 1 in L. am. DCL patients | [30, 31, 32] |
the lesions [203]. In both Old World [149, 164] and New World [204] leishmaniasis, IL-13 production in the lesions and peripheral blood have been measured as an indicator of Th2 response.

**Tumor necrosis factor (TNF)**

Existing data on the role of TNF in human leishmaniasis development are controversial, but most of the reports implicate that unregulated production of TNF contributes to the clinical outcome of leishmaniasis at early stage of infection [205, 206]. In two sequential studies of Zoonotic CL (ZCL) caused by *L. major*, we have shown that the mean level of TNF in plasma and supernatant of stimulated cells in culture is significantly higher in active CL patients than in healthy volunteers and significantly reduces after treatment of the lesion(s) [136, 150]. Similar reports from New World are exist showing elevated levels of TNF production in CL lesions of who are nonresponsive to antimonial treatment [207]. Investigation of the immune response of American CL patients revealed a significant upregulation of gene expression of TNF and IFN-γ cytokines within 24 h of in vitro stimulation of the cells which shifted to a dominant IL-10 and IL-4 production after 48 h [208], showing a possible role for pro-inflammatory cytokines in early phases of CL lesion development.

IFN-γ and TNF act synergistically in the activation of macrophages to produce iNOS/NO during murine *Leishmania* infection [209, 210]. In leishmaniasis, cytokine balance is important in T-cell homeostasis and maintenance of protective immunity and imbalanced cytokines might induce pathogenesis. TNF shows a reciprocal role in the outcome of human leishmaniasis and an increased level of TNF correlates with severity of the lesion [211].

In mice, TNF antagonizes alternative activation of macrophages and dendritic cells by IL-4 and TNF has a restricting effect on arg1 expression leading to the production of NO by iNOS and parasite control [212].

In experimental VL, TNF produced by *Leishmania* infected Kupffer cells is essential for the granuloma formation and induction of protective immunity in liver [213]. Parasite burden is progressively increased in mice lacking TNF which leads to death [214]. Therefore, TNF appears to be a critical cytokine in resolution of experimental visceral infection [215].

**Interleukin 10 (IL-10)**

Following resolution of *Leishmania* infection in mice, a population of IL-10 producing CD25⁺Foxp3⁺ Treg cells prevent sterile cure and establish a chronic infection, allowing memory generation for a long-lasting protection (reviewed in [216]). Similarly, a population of antigen-induced
CD25−Foxp3− regulatory T cells that produce IL-10 is expanded following *Leishmania* infection [217] which modulates immune response to control immunopathological effects leading to delay in lesion healing.

The deteriorating role of IL-10 is shown in IL-10 deficient BALB/c mice which were able to control the progressive *L. major* infection with 1000-fold lower parasite burden [25]. Function of IL-10 as an inhibitory cytokine is well described; in vitro, IL-10 inhibits antigen-specific T-cell proliferation and type 1 cytokine production [218, 219] and renders macrophages refractory to activation by IFN-γ for intracellular killing [25, 220, 221]. Recombinant mouse IL-10 showed a potent suppressing effect on the ability of mouse peritoneal macrophages to release TNF, reactive oxygen intermediates (ROI) and to a lesser extent reactive nitrogen intermediates (RNI) [222]. In resistant C57BL/6 mouse peritoneal macrophages to release TNF, IL-10 showed a potent suppressing effect on the ability of mouse peritoneal macrophages to release TNF, reactive oxygen intermediates (ROI) and to a lesser extent reactive nitrogen intermediates (RNI) [222].

A deteriorating role of IL-10 in experimental VL is seen, in which IL-10 overexpressed mice showed an increased parasite replication and impaired Th1 type responses. Despite subsequent granuloma formation, infection persisted, and antimony-treatment failed [226]. In human leishmaniasis due to *L. braziliensis*, when increased production of IFN-γ and TNF coincides with the absence of IL-10 in situ, a strong inflammatory reaction is promoted leading to destructive lesion development especially in ML [227–229]. Elevated level of IL-10 has been frequently reported in clinical studies of human VL and seems to contribute in pathogenesis of VL [230]. IL-10 is the key immunosuppressive cytokine in VL patients which is hard to detect in cultures of PBMCs collected from VL patients [170], but antigen-driven production of IL-10 is observed in whole blood of patients with active disease [171].

**Interleukin 17 (IL-17) and interleukin 22 (IL-22)**

Th17 population homing in skin and mucosal sites, produce cytokines such as IL-17, IL-22, and IL-23. IL-17 and IL-22 which are involved in the rapid response to infections, both by recruiting neutrophils and inducing production of antimicrobial peptides [231].

High levels of IL-17 have been found in BALB/c mice following infection with *L. major*, and IL-17 deficiency is associated with control of the disease [232]. In mouse model of leishmaniasis, in the absence of IL-10 modulation, both IFN-γ and IL-17 production levels are increased and cause more severe disease following high doses of *L. major*, which is reversed by neutralization of IL-17 [233]. IFN-γ and IL-17 levels correlate with the inflammatory response in the skin of patients with CL and ML [234, 235] indicating possible involvement of Th17 population in pathogenesis. However, in our study on human leishmaniasis, no significant difference was seen between active and cured CL individuals in the production of IL-17 from stimulated PBMCs [136]. Furthermore, we found that the mean level of IL-22 production in plasma and in SLA stimulated PBMCs of active VL patients was significantly higher than healthy controls and was significantly decreased in the same patients after healing of VL due to *L. infantum* [136, 150]. The results suggested that the level of IL-22 production is conversely related to VL cure. It is claimed that IL-17 and IL-22 may have a synergistic role with Th1 cytokines in protection against human VL due to *L. donovani* [236].

**Serum antibodies**

There are studies that conceive a deteriorating role for B cells in experimental models of leishmaniasis by producing antibodies [237, 238] or cytokines such as IL-10 [226, 239, 240]. Although antibody response is induced in leishmaniasis especially VL, but antibody response does not play any significant role in protection. There are studies which indicate that IgG antibodies may be crucial in suppressing the host immune response by generating a high IL-10 response. *L. major* amastigotes opsonized with host IgG antibodies may ligate FcγR on murine macrophages to induce production of IL-10 [25]. In vivo studies found that Fc-deficient mice infected with *L. mexicana* produce less IL-10 and are less susceptible to infection [241, 242]. In human or mouse infection with *L. mexicana*, antibodies are raised against surface glycoinositol phospholipids of the parasite which induce production of IL-10 from monocytes [243]. The role of antibodies is not completely clear, humoral immune response does not have a protective role in CL and antibody response in CL of Old World is very low and sometimes difficult to detect (Khamesipour A, unpublished data); therefore, antibody titration is not applicable as a marker of cure or protection (reviewed in [244]). Nevertheless, humoral immune responses have been measured as a diagnostic approach in New World CL [245] and high level of anti-*Leishmania* antibodies are seen in VL patients which is used as diagnostic tool [246, 247]. It is shown that upon recovery of kala-azar, different antibody titers decline [248].

It is shown that human or canine reservoirs that exposed to sand fly saliva induce a high antibody response which is used as marker of exposure in surveillance studies [249, 250], reviewed in [251]. The yellow proteins LJM11 and LJM17 from saliva of *Lutzomyia longipalpis* are recognized by sera from humans living in VL endemic areas and animal reservoirs [252, 253]. PpSP32 is the immunodominant target for the serum antibody raised in humans naturally exposed...
to Phlebotomus papatasi saliva [254]. The apyrase rSP01B and rSP01 and the yellow protein rSP03B from saliva of Ph. perniciosus, a principal vector of L. infantum in the Mediterranean Basin, are promising markers of canine exposure [255, 256].

**Leishmanin (LST)/Montenegro (MST) skin test**

Delayed type hypersensitivity (DTH), is used to evaluate cell mediated immune response in a few diseases, Leishmanin (LST) or Montenegro (MST) skin test is a DTH test similar to Mantoux test which is in use since 1929. In 1990s, at Pasteur Institute of Iran with full support of TDR/WHO a standard leishmanin was produced under GMP condition, in the recent one, the same L. major which was applied to mass leishmanization of more than 2 million people, was used. Leishmanin was harvested at early stationary phase, washed and were killed using thimerosal (0.1%), then the parasites were washed and treated with thimerosal, the number of parasite adjusted to 1 × 10^7 Leishmania per mL, aliquoted and then each batch goes through control measures including toxicity, potency etc. tests. About 0.1 mL of leishmanin is inoculated intradermally using fine needle into ventral forearm [257, 258]. LST is used in epidemiological studies and for investigation of past exposure and is almost the unique tool to evaluate efficacy of experimental vaccine efficacy [259]. Usually, a portion of the residents of endemic areas are leishmanin positive. In Iran, the percentage of recovered persons with LST-positive results (≥ 5 mm indurations) was 99%, 94%, and 70% for areas with ZCL, ACL, and ZVL, respectively [260]. LST positivity is not an indication of protection, in studies completed in Iran, LST positive individuals are as sensitive as LST negatives in regard to develop CL lesion [261].

In New World, of healthy individuals without a history of CL living in endemic areas, 10–15% have a positive MST result [262, 163, 263]. Both LCL and MCL present positive response to MST, indicating a cell-mediated immunity against the parasite and MST is reported to be positive in more than 90% of American tegumentary leishmaniasis [264, 265].

In relation to various clinical forms, one evidence showed that most of the strongly positive responses are seen in lupoid, and most of the negative LSTs are seen in sporotrichoid type of CL [266].

**Concluding remarks**

Leishmaniasis, a neglected disease with strong links with poverty, has long been a major public health problem in many developing countries with high morbidity and mortality rates. It seems necessary to implement effective measures as diagnostics, prophylactics and therapeutics to control this infection [267]. Advances in the understanding of the biology of Leishmania have not yet been translated into the development of vaccine or new therapeutic measures. During recent years, large-scale genomic and proteomic analyses have allowed characterization of the network pathways involving in the pathogenesis of Leishmania parasite. Combining these data offers a more comprehensive body of information that could be used to identify specific biomarker(s) of immunity against leishmaniasis. The potential biomarker(s) would be used as new target for development of vaccine and/or drug against leishmaniasis.

Most of our current understanding of the role of different markers of immune response in leishmaniasis has been obtained by works performed in experimental animal models. Although results from these studies provide important insights into Leishmania immunity, but cannot always be extrapolated to humans as there seem to be significant differences between human and murine immune response against Leishmania infection. Hence, limited numbers of biomarkers have been investigated and so far none of which could be used as a definitive outstanding surrogate of protection against human leishmaniasis.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

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