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Review

New insights to structure and immunological features of *Leishmania* lipophosphoglycan3

Maryam Hosseini[a], Mostafa Haji-Fatahaliha[a], Abolfazl Miahipour[b,**], Mehdi Yousefi[c,*]

[a] Stem Cell and Regenerative Medicine Institute, Tabriz University of Medical Sciences, Tabriz, Iran
[b] Department of Medical Parasitology and Mycology, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran
[c] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

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ABSTRACT

Leishmaniasis is a major public infectious disease caused by the genus *Leishmania*. No effective drug or vaccination strategy for leishmaniasis has been designed yet. Several intracellular *Leishmania* antigens have been recognized to serve in vaccination, ensuring long-lasting protection against *Leishmania* infection. Lipophosphoglican 3 (LPG3) as a member of the heat shock protein 90 family involves in the synthesis of lipophosphoglycan (LPG) and implicates in parasite virulence. Regarding the immunological properties of LPG3 particularly its N-terminal fragment, it would be considered as a favourable adjuvant in *Leishmania* vaccination.

1. Introduction

Leishmaniasis is an important health problem caused by the genus *Leishmania* which is endemic in tropical regions including Southern anzd Southeast Asia, Eastern Mediterranean, South and Central America [1]. *Leishmania* infection is characterized by a wide spectrum of clinical manifestations, from self-healing cutaneous leishmaniasis (CL) to a life-threating visceral leishmaniasis (VL) by the type [2,3]. The parasites replicate as flagellated promastigotes in the midgut of the sand fly vector and infective metacyclic promastigotes are injected into the host through sand fly biting. Promastigotes are rapidly internalized in phagocytes like macrophages and proliferate as non-motile amastigotes within the acidic phagolysosomal compartment [4]. No effective drug or vaccination strategy for leishmaniasis has been designed so far and new strategies for controlling the human disease have not been forthcoming [5]. This may be due to our insufficient knowledge about immune cells participate in the protection against leishmaniasis and important molecules that play a key role in the infection [6]. Furthermore, limited researches on parasite-host interactions have affected the creation of prospective therapeutic drugs or vaccines [7].

Leishmania parasites have been equipped with molecules to infect host cells and survive within them through the interaction of their surface molecules with the cells. The surface of *Leishmania* is covered by family of glycosylphosphatidylinositol (GPI)-anchored glycoconjugates that are structurally different from those found in mammalian cells [8] including lipophosphoglycan (LPG), membrane-bound proteophosphoglycan (mPPG), glycoprotein 63 (GP63) and glycosylinositolphospholipids (GIPLs). Some of these have regulator functions; some are constitutive, while others, like LPG, provide a protective barrier for parasites to persist within the host cells [9–12].

Lipophosphoglycan is one of the most interesting issues that have been studied intensely in *Leishmania* vaccination. LPG expression is up-regulated during *Leishmania* transformation into metacyclic promastigotes upon inoculation by the vector and invasion to host macrophages [13]. LPG is involved in many important parts of the *Leishmania* life cycle and implicated in parasite virulence. Some studies have shown that *Leishmania major* lacking LPG, is weakened to survive in sand fly and infect the host cells [9,14–16]. Since the expression level of LPG is dramatically decreased in the amastigote stage, maybe, the role of LPG is limited to the initial invasion by metacyclic promastigotes [9].

Various researches have focused on *Leishmania* mutants chosen for specific defects in glycoconjugate expression to identify structure-function relationships [17]. These findings make possibility to explain the LPG biosynthetic pathway and its relationship with other glycoconjugates by generating certain knock-out mutants in a virulent background. These studies suggest more research on the biosynthetic genes responsible for glycoconjugate production, especially LPG that may helpful for the development of better specific chemotherapeutic drugs or vaccines against leishmaniasis [18].

In this review, we discuss recent findings about Lipophosphoglycan 3 (LPG3), a gene identified by functional complementation of the *Leishmania donovani* mutant, which plays a critical role in the synthesis

* Corresponding author at: Immunology Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.
** Corresponding author at: Department of Medical Parasitology and Mycology, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran.
E-mail addresses: h_miahipour@yahoo.com (A. Miahipour), Yousefime@tbzmed.ac.ir (M. Yousefi).

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of LPG and other phosphoglycan residues added to extracellular proteins like GP63 and GP46 in all *Leishmania* species [18]

2. The structure of lipophosphoglycan 3

Lipophosphoglycan 3, which belongs to Class II LPG genes was first introduced by *Descoteaux* et al. *(*2002) as a trimmed LPG containing just the Mana1-PO4 residue of the first repeat unit in *L. donovani* [18]. Lipophosphoglycan (LPG) has a complex glycolipid structure on a phosphoglycan context of Gal (b1, 4) Mana1-PO4 repeat units or PG repeats, anchored by GPI molecule [8,19]. LPG has been revealed to play an important role in modulating host immune functions and interrupting different anti-microbial functions of the infected macrophages, including apoptotic and signalling pathways, phagolysosome maturation and nitric oxide (NO) production that eventually lead to establishment of the infection [20].

3. LPG3 as a member of heat shock protein family

Heat-shock proteins (HSPs) are highly conserved proteins involved in every homeostatic events. Most HSPs, but not all, are molecular chaperones participating in folding, assembly, degradation and transporting of proteins across membranes [21,22]. Glycoprotein 96 (gp96) is an endoplasmic reticulum (ER)-resident member of the heat-shock protein 90 (HSP90) family that is designated as glucose-regulated protein 94 (GRP94) [23,24]. GRP94 is a chaperone located in the endoplasmic reticulum with multiple functions, including protein assembly and secretion, and antigen presentation. Earlier studies suggested that LPG3 is homologous with the mammalian GRP94. Based on these studies, LPG3 has a crucial role in the production of LPG and other surface GPI-anchored molecules. The necessity of its role in the synthesis of GPI-anchored proteins is result from researches on lpg3 knockout mice revealed down-regulation of the expression level of GP63 in the absence of LPG3 [18,25,26]. In a study conducted by Larreta and colleagues [27] has been introduced a *L.infantum* GRP94 homologous, to be greatly antigenic like many members of the HSP family, suggesting the effecting role of this protein on infected host immune response [27]. Although *Leishmania* LPG3 shares various properties with GRP94 and localizes in the parasite ER apparatus, some basic differences should be considered. One of them is that LPG3 participates in parasite virulence through its activity for LPG compartment rather than viability compared to GRP94 expressed in other eukaryotes [28,29]. Moreover, the LPG3 mRNA is regulated in a different manner compared to GRP94, since the GRP94 mRNA is usually up-regulated in response to stress situations but studies have stated that heat or stress shocks do not have any effect on LPG3 mRNA expression. Indeed, it appears to be regulated mainly within the development of *Leishmania* lifecycle. The gene of LPG3 is expressed in both two life stages of the parasites (promastigote and amastigote stages), although the expression level of LPG3 declines in the stationary-phase promastigotes and over expression occurs two to seven times in the amastigote stage after macrophage entry of the parasite [30]. Besides, the LPG3 expression preferentially occurs among pathogenic and non-pathogenic species. A study by *Azizi* et al. shows that LPG3 is expressed in *L. tarentolae* (designated as a non-pathogenic form) similar to expression in the amastigote phase of *L. major, L. infantum* and *L. donovani* [26]. Apart from these similarities, findings point to the absence of LPG in *L. tarentolae,* which may be due to the non-pathogenic feature of this species [31]. With these investigations, it has been concluded that the role of LPG3 in *Leishmania* metabolism greatly differs from that in other eukaryotes. In addition, multiple sequence alignment of the LPG3 among four *Leishmania* species (*L.major*, *L. donovani*, *L. infantum* and *L. tarentolae*) showed a highly conservative LPG3 sequence [26], nonetheless, species specific differences between pathogenic forms have been reported [32]. These differences are arise from different gene-regulation in the 3′UTR, which are less conserved between species than the coding sequences [33]. A question that may be asked is, if LPG3 is a virulent factor, why non-pathogenic species express LPG3 too? Maybe, the interaction of LPG3 with other cellular molecules alters at the post-translational level, resulting in changes in parasite virulence [26].

4. Innate immunity response to *Leishmania* LPG3

One of the major problems with leishmaniasis is the ability of the parasite to escape and modulate host immune responses allowing parasite persistence and development of chronic infections. Innate immune responses are essential to inhibit parasite establishment and then initiating the adaptive immune responses that is critical for clearance of the infection [7]. Several studies argued that Gp96 have an ability to induce a number of innate immune responses and Th1 promoting cytokines *in-vitro.* Gp96 could stimulate the expression of MHC class II and co-stimulatory molecules and in turn elevation of pro-inflammatory cytokines such as TNF (tumor necrosis factors)-α, IL (interleukin)-1β, IL-6 and IL-12. These studies hypothesis that the immune effect of Gp96 may come from its ability to bind peptide epitopes and introduce them to T cells through antigen presentation pathway. From researches already done, it is concluded that both the N- terminal and C-terminal fragments of Gp96 are able to bind peptides, albeit the N-terminal fragment has a similar effect to the native Gp96 [34,35]. Other studies implied that all immune activities of GRP94 originate in amino acids 1–355 [36]. This tiny chaperone can bind peptides and then be taken up by the receptors such as CD91[37], CD40 [38], scavenger receptor type A and Toll-like receptors (TLR-2 and TLR-4) [39], on macrophages or dendritic cells (DCs) [36]. These findings showed that HSP molecules could induce immune response through complexes with peptides and taken up by antigen-presenting cells (APCs) and help peptide-presenting to T cell in MHC I and II clefts [22,40–43]. However the effect of LPG3 on antigen presenting procedures has not been investigated till now. It would be a good idea to investigate the ability of LPG3 in peptide-binding and participating in T cell induction for supplementary consideration in immunotherapy and vaccine development.

4.1. The interaction of LPG3 with innate immune cells

Two major innate immune cells stimulated against leishmaniasis are neutrophils and macrophages. Neutrophils as the first line of host defense are activated in different manners to kill parasites including phagocytosis, degranulation and neutrophil extracellular traps (NETs). Macrophages are effective cells presenting in the initial and transitory immune responses during leishmaniasis. Indeed, macrophages are the exacting host cells of *Leishmania* species that the parasites imply various evasion strategies to suppress the microbicidal functions of macrophages and persisting infection [7,44,45]. Maybe further studies on the correlation of LPG3 and phagocytic cells benefit to suggest a solution prevent *Leishmania* parasite entry to the cells and establishment of leishmaniasis.

Natural killer (NK) cells are another important components of innate immune system contributed in early response. Activation of NK cells by intracellular pathogens like *Leishmania* induce the production of IFN (interferon)-γ and TNF-α which promote infected phagocytes to kill the parasite by the generation of reactive oxygen or nitrogen intermediates [46,47]. NK cells are the early sources of IFN-γ critical for innate immunity against leishmaniasis [48]. A study demonstrated that NK cells are severely diminished in lesions of patients with diffuse cutaneous leishmaniasis, a progressive form of the disease, and already NK cells are enhanced after *Leishmania* treatment [49].

Recently, a recombinant form of *L. major* LPG3 is cloned in *E. coli* and a hydrophobic and probable transmembrane domain at the beginning of LPG3 protein is determined (Fig. 1). The recombinant LPG3 (rLPG3), rNT-LPG3 and rCT-LPG3 proteins synthesized by the way, were 85, 48.2 and 36.5 kDa, respectively [50]. In the study performed by *Abdian* et al., the antigenicity of rLPG3, rNT-LPG3, and rCT-LPG3

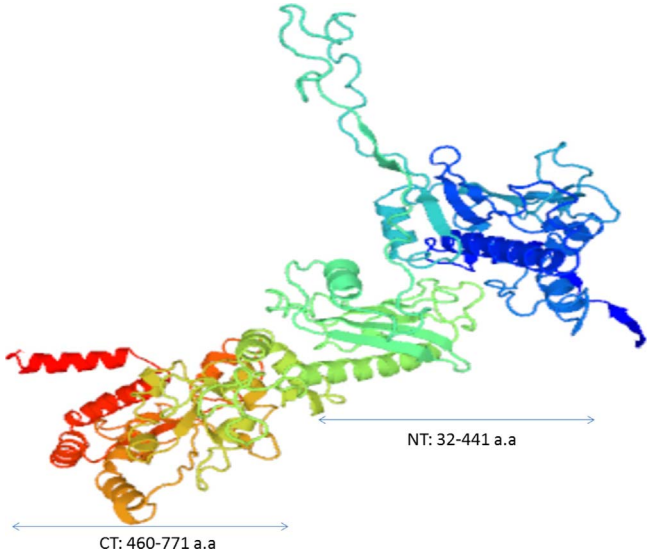

Fig. 1. The structure of Leishmania major LPG3 The LPG3 structure is designed using SWISSPROT software and N terminal (from 32 to 441 a.a) and C terminal (from 460 to 771 a.a) segments are determined based on studies described in the text. The N terminal fragment of LPG3 is a hydrophobic and probable transmembrane domain that may responsible for the immunological functions of the whole protein. NT: N terminal, CT: C terminal.

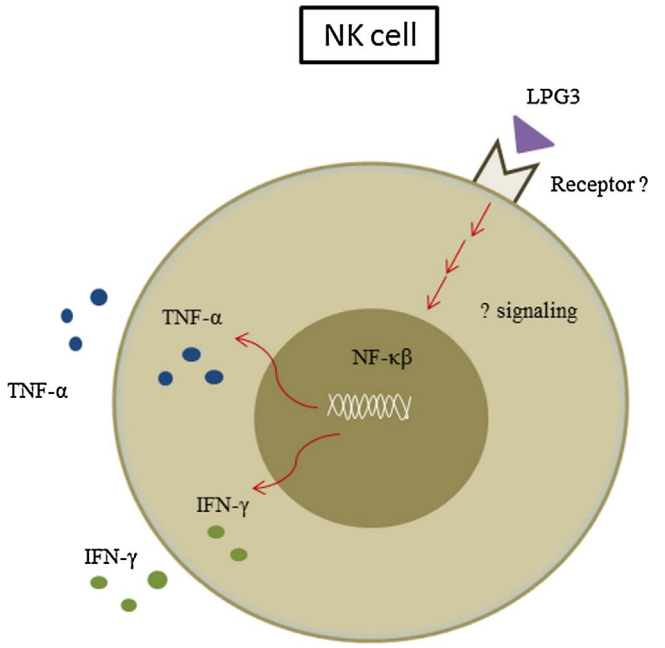

Fig. 2. The stimulation of natural killer (NK) cells by recombinant Leishmania LPG3 NK cells are stimulated by rLPG3 through rLPG3 binding to unknown receptor and the secretion of IFN-γ and TNF-α is result at the end. Recombinant LPG3 is not a suitable ligand for TLR 2 as shown in the figure. Maybe other receptors are responsible for NK cell stimulation by LPG3.

was examined for the first time using sera reactivity assessment in patients suffering from leishmaniasis. Sera reactivity against LPG3 and NT-LPG3 in visceral leishmaniasis (VL) was significantly higher than in cutaneous leishmaniasis (CL). However, CT-LPG3 was less antigenic in both CL and VL [50].

In a study reported by our group, the properties of LPG3 and its fragments in the induction of innate immunity were investigated using NK cells isolated from healthy individuals by magnetic-activated cell sorting (MACS) technique. NK cells were cultured by different concentrations of rLPG3, rNT-LPG3 and rCT-LPG3 for 48 h. Our result showed that rLPG3 and its fragments have a stimulatory effect on IFN-γ production by NK cells. We also found that rLPG3, but not NT and CT fragments could induce NK cells to produce TNF-α, indicating the necessity of whole LPG3 for TNF-α production by the cells [51].

Additional studies were conducted to find how LPG3 activates NK cells to secret inflammatory cytokines. Preceding literatures showed that *L. major* LPG is an acceptable ligand for Toll-like receptor (TLR)2 on the surface of macrophages and NK cells and stimulates the secretion of TNF-α and IFN-γ cytokines [52–54]. Toll-like receptors (TLRs) are important members of the family of pattern recognition receptors expressed on the cell surface or intracellular endosomes of various immune cells. According to studies, heat-shock proteins can activate DCs via the TLR2/4 signalling pathway. TLR2 and TLR4, alone or in combination with other surface receptors of DCs, mediate uptake and processing of HSP-peptide complexes [22,55–58]. Together with these findings, and due to a wide range of ligands that is recognized by TLR2, [59] we supposed LPG3 could stimulate NK cells through TLR2 signalling as well. Conversely, our results showed that rLPG3 could upregulate the expression of the activation marker (CD 69) and some key inflammatory cytokines (IFN-γ and TNF-α) in human purified NK cells through TLR2-independent manner (Fig. 2) [49]. Our results were accepted by treatment of human purified T and B cells with rLPG3 that indicated activation of T and B lymphocytes by rLPG3 molecule is independent of TLR2 signalling; similar to activation of NK cells [51,60,61]. It is possible that other receptors have been activated by LPG3. Forthcoming studies should be done to determine this latter hypothesis and consider in clinical therapies.

5. Adaptive immune response to LPG3

5.1. The induction of t cell mediated immune response against LPG3

Generally, cell-mediated immune responses are the main protective response against leishmaniasis; through activation of macrophages by IFN-γ, leading to destruction of phagocytised parasites in hydrogen peroxide (H_2O_2) or nitrogen oxide (NO)-dependent manners [62]. Although T helper (Th) 1 type responses control disease burden and cure infection, Th2 type responses exacerbate disease progression. Thus, the most important thing to be considered is the induction of Th1 mediated immune responses in development of effective *Leishmania* vaccines [63]. Th17 cells are identified by the production of inflammatory cytokines such as IL-17A, IL-17F, and IL-22 [64]. Some studies revealed that Th17 cells mediate several pathological tissue damages in human muco-cutaneous leishmaniasis, but a protective effect in human VL [65,66].

Based on studies, HSPs have capability to elicit a specific, powerful and cellular adaptive immune response, which seems to arise from their function as a chaperone for various peptides [22,67,68]. We investigated the ability of recombinant LPG3 (rLPG3) to activate isolated human T cells in different concentrations. The results showed that rLPG3 enhances the expression of CD69 as an activation marker on the surface of T cells and promoted differentiation of $CD4^+$ T lymphocytes toward a T helper 1 (Th1) phenotype through up-regulation of Th1 lineage-specific transcription factor (T-bet) and IFN-γ expression in moderate (10 μg/ml) and high (20 μg/ml) concentrations of rLPG3 (Fig. 3) [61,69]. These results were followed by an enhancement of IFN-γ: IL-4 ratios, revealed a significant shift to Th1 phenotype in T cells treated with moderate and high doses of rLPG3. However, no significant effect of rLPG3 on Th2 and Th17 lineage cells was observed even in rLPG3 high concentration. Our researches on LPG3 fragments revealed that NT-LPG3 had a stimulatory effect on CD69 expression and induced IFN-γ secretion in moderate and high doses. In contrast, CT-LPG3 had no significant effect on T cell activation even by high concentration [61,69]. These results indicated the immune-stimulatory

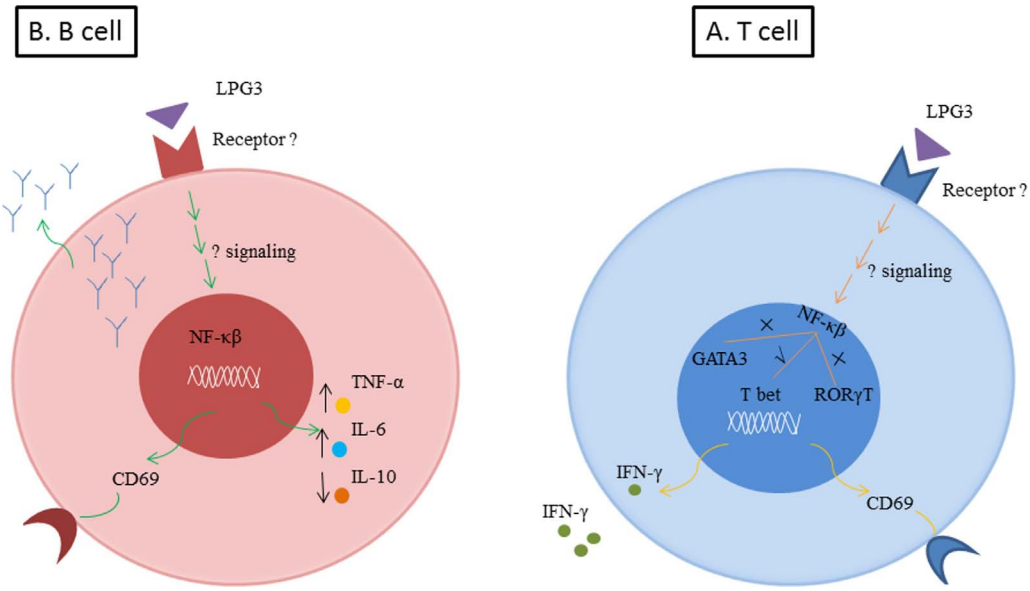

Fig. 3. The stimulation of T lymphocyte and B lymphocyte by recombinant Leishmania LPG3 A. T cells are stimulated by rLPG3 through recognition of unknown receptor that induced up-regulation of CD69 expression on T cell surfaces. Recombinant LPG3 are prompted differentiation of Th1 cells through enhancement of T-bet transcription factor and secretion of IFN-γ. B. B lymphocytes are induced by rLPG3 by mysterious signalling pathway that increasing in CD69 expression is happened following rLPG3 binding. Recombinant LPG3 has also effect on up-regulation of inflammatory cytokines such as IL-6 and TNF-α in the B cells but depression of IL-10 in the cells. Based on studies argued in the text, LPG3 could affect antibody secretion from mouse B cells that is in favor to Th1 phenotype responses.

effects of LPG3 (particularly NT-LPG3) as a potent immune-component of *leishmania* in vaccination against leishmaniasis and suggested that the major functions of LPG3 may have been elicited from its N-terminal fragment like Gp96. More studies are needed to investigate adjuvant properties of rLPG3 for *leishmania* therapy.

5.2. The induction of B cell mediated immune response against LPG3

B lymphocytes are the exact source of antibody production and regulate immune responses. B cells participate in inflammation through secretion of cytokines, growth factors, chemokines, and also regulate T cell responses. Several studies showed that B cells could enhance Th1 and Th2 responses by producing several pro-inflammatory and anti-inflammatory cytokines [70,71]. According to studies, antibodies facilitate the phagocytosis of *L. major* and a higher parasite burden has been observed in mice lacking antibodies following a reduction in T cell response and IFN-γ synthesis [72]. Furthermore, reduced chemotaxy of monocytes and lymphocytes at the site of cutaneous lesions has been detected in B lymphocyte-deficient mice compared to wild type [20]. In an investigation by our group, we evaluated the capability of recombinant LPG3 and its fragments to activate human purified B cells. Results showed that rLPG3 could activate B lymphocytes via up-regulation of CD69 and the production of IL-6 and TNF-α (as pro-inflammatory cytokines) but not IL-10 (as an immune-regulatory cytokine). Moreover, rNT-LPG3 was able to induce CD69 expression and enhance inflammatory cytokines just in a high (20 μg/ml) concentration of rLPG3 (Fig. 1) [60].

6. The role of LPG3 in vaccination strategy

Along with failures in treatment and various problems such as long-lasting scars or expensive drugs, it seems effective vaccines and immune-preventive agents may lessen these complications [5]. Therefore, many vaccination strategies have been developed, those involving killed or live attenuated *Leishman* parasites, salivary antigen-based vaccines, recombinant proteins, and DNA vaccines. However, no effective vaccine is yet available and those investigated till now have been unsatisfactory in clinical studies [73].

One of the main issues in vaccine development that should be considered is implementation of new adjuvants to improve the antigen presentation and protective immune response. Among various peptides, HSPs are natural adjuvants that stimulate the innate and adaptive immune response against infectious diseases [74]. The adjuvant effect of GRP94 has been investigated for many pathogens such as *Listeria monocytogenes* [75], Hepatitis B virus [35] and human papillomavirus (HPV) infection [74]. Various studies depicted the protective effect of DNA vaccine with Gp96-proteins against *Listeria monocytogenes* (an intracellular pathogen) in a mouse model [75]. Analyses of the cellular immune response have shown significant epitope-specific IFN-γ and CTL responses. These observations introduced DNA vaccination with Gp96 fusion proteins as a beneficial strategy to elicit protective immunity against intracellular pathogens [75]. As LPG3 homology with Gp96 it is plausible designing effective vaccines that LPG3 induces CTL and Th1 responses in a same manner [18].

Recently, the protective properties of *L. major* LPG3 was examined as a vaccine candidate in two regimens, DNA/DNA and DNA/Protein (prime-boost), against *L. major* infection in BALB/c mice model. It has been shown that rLPG3 and rNT-LPG3 are highly immunogenic and stimulate the production of both IgG1 and IgG2a with a delayed lesion development or a smaller lesion when compared with control. In the prime-boost immunization regimen, the level of antibody response against rLPG3 and rNT-LPG3 was higher than DNA/DNA immunization with a higher level of IgG2a/IgG1 in the former strategy. The level of IFN-γ in the supernatant of splenocytes from mice immunized with DNA/DNA and prime-boost regimens were significantly high in comparison with control. According to a direct correlation between IgG2a titers, IFN-γ production and lesion size, all together support the notion that vaccination, especially in prime-boost way, induce the immune response. Moreover, immunization with prime-boost vaccination has a higher ratio (two times) of IFN-γ/IL-5, suggesting a Th1 phenotype tendency [50].

Another study evaluated the immunogenicity of *L. infantum* LPG3 as a DNA vaccine against murine VL. The results indicated a mixed Th1/Th2 response following immunization, consistency with the production of both IFN-γ and IL-10 by splenocytes in comparison to control. However, no significant reduction parasite burden was reported in the splenic immunized mice. Moreover, serum levels of IgG antibody showed no significant difference between the LPG3 DNA and the empty vector. It has been also shown that the co-administration of rHSP70 with the DNA vaccine have no additive protective advantage on experimental infectious challenge [62]. In another study, the properties of recombinant LPG3 expressed by *L. tarentolae* were assessed in combination with a Th1-promoting adjuvant, CpG oligodeoxynucleotides (CpG-ODN), against *L. infantum* infection in a BALB/c mice model. The results showed a significant enhancement of IFN-γ production in co-administration of rLPG3 with CpG-ODN that led to a shift towards a Th1

response against rLPG3 and the predominant presence of IgG2a antibodies in the sera. But immunization with rLPG3 conjugated by CpG-ODN induced partial protection against infectious challenge in a mice model [76].

7. Conclusion

Lipophosphoglycan 3 (LPG3) is a new *Leishmania* immunogenic protein introduced as a promising adjuvant component in vaccine development against leishmaniasis. Although LPG3 is one member of the HSPfamilies, the actual functions of the protein is unknown, especially its role in virulence. Regarding studies, the N terminal fragment of LPG3 may be responsible for the immunological functions of the whole protein. Some ambiguous aspects of LPG3 that should been cleared in further investigations are declaration of signalling pathway implicated by LPG3 to induce the immune cells, identification of an exact receptor that bind to LPG3 and elicit signals into the cells, the effect of LPG3 on the other immune cells like neutrophils and macrophages, the potential role of LPG3 in antigen presenting procedures and many other considerations that help us to implicate in vaccine strategies against leishmaniasis.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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