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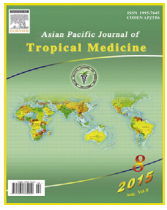

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Analysis of human B cell response to recombinant *Leishmania* LPG3

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ABSTRACT

Objective: To evaluate the capability of recombinant *Leishmania* LPG3 and its fragments in the activation of B cells.

Methods: In the present study, human B cells were purified from peripheral blood of 10 adult healthy subjects using magnetic-activated cell sorting technique. Subsequently, purified B cells were treated with recombinant LPG3, and its N-terminal and C-terminal fragments at different concentrations (2, 10 and 20 μg/mL). B cell activation was assessed through expression of CD69 molecule by flow cytometry and secretion of IL-6, TNF-α and IL-10 cytokines via enzyme-linked immunosorbent assay following treatment with recombinant antigens.

Results: Our results showed that while the recombinant LPG-3 could significantly increase the production of IL-6 and TNF-α ($P < 0.05$) in B cells, it had no effect on the secretion of IL-10 by B cells.

Conclusions: Our study indicated that recombinant LPG-3 and especially its N-terminal fragment could stimulate B cell response as an important immune response component against leishmaniasis. Thus, it seems that it can be considered as an effective adjuvant in vaccine developments against leishmaniasis.

1. Introduction

Leishmaniasis is one of the important infectious diseases caused by an obligate intracellular parasite. It has been demonstrated that over 20 genus of *Leishmania* can induce this disease and their life cycle alternates between two separate developmental stages including promastigotes and amastigotes. The promastigote stage is related to flagellated extracellular stage in which parasite replicate into midgut of the insect vector, whereas amastigotes is referred to intracellular stage in which aflagellate parasite multiply within mammalian host macrophages [1,2]. The epidemiologic studies have been shown that Leishmaniasis is endemic in 88 countries, affecting 12 million people and an estimated 1.5 million to 2 million new cases occur worldwide annually. *Leishmania* infection is prevalent in tropical and subtropical areas of Asia, Africa, southern Europe, the Mediterranean (old world) and central and south America (new world). Leishmaniasis appears in various forms including cutaneous (the most common), visceral which also known as kala-azar (the most severe form of the disease) and mucocutaneous [1–3]. The clinical manifestation of disease may various from self-healing chronic cutaneous lesions to progressive and fatal systemic infection, if left untreated. It has been shown that while the cutaneous Leishmaniasis is caused by *Leishmania aethiopica*, *Leishmania tropica* and *Leishmania major* in the old world, it could be induced by *Leishmania braziliensis*, *Leishmania mexicana*, *Leishmania guyanensis*, and *Leishmania amazonensis* in the new world. CL is the most common in Pakistan, Saudi Arabia, Algeria, Syria, Tunisia, Afghanistan and Iran [4,5].

Paromomycin, pentavalent antimonial, amphotericin B and miltefosine are different chemical drugs used in the control and treatment of *Leishmania* [6,7]. However, application of these drugs was associated with several side effects and drug resistance which led to their limited prescription.

The several vaccination approaches such as whole killed parasites (as a first generation), poly-proteins, recombinant proteins or dendritic cells pulsed with *Leishmania*-derived

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peptides (as second generation) and DNA vaccines (as third generation) have recently been developed, however, none of them was sufficient to protect humans against infection [8,9].

Lipophosphoglycan (LPG) is a highly expressed surface molecule on *Leishmania* promastigotes. Regarding the protective role of LPG for parasite against phagocytosis, it plays an important role in parasite's survival [10,11]. *Leishmania* lipophosphoglycan3 (LPG3) is essential for the synthesis of glycoconjugate molecules, particularly LPG. Thus, it has been thought that LPG3 may has a critical role in parasites infectivity [10,11].

In addition to antibody synthesis, B lymphocytes regulate immune responses and participate in inflammation via secretion of cytokines, growth factors, chemokines and also regulate T cell responses. Several studies showed that B cells can enhance Th1 and Th2 responses [12,13]. Human B lymphocytes are able to secrete IL-6 and TNF-α as pro inflammatory cytokines [14,15]. Some studies demonstrated that antibodies secreted by B cells result in killing of infected macrophages and intracellular parasite [16–18].

In the present study, we evaluated the capability of recombinant *Leishmania* LPG3 and its fragments in the activation of B cells. We assessed activation of B cells through the production of IL-6, TNF-α (as pro-inflammatory cytokines), and IL-10 (as an immune-regulatory cytokine) cytokines and expression of activation CD69 molecule.

2. Material and methods

2.1. B cells isolation by magnetic-activated cell sorting (MACS)

The heparinized peripheral blood samples were collected from 10 adult healthy volunteers with no history of *Leishmania* infection after obtaining their informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Ficoll–Paque (Sigma, St. Louis, MO) from whole blood, as described previously [19]. Negative selection (depletion of unwanted cells) were used to isolate B cells from PBMC (B cell isolation kit II, Miltenyi Biotech, Bergisch-Gladbach, Germany), according to the manufacturers instruction. For magnetic labeling, PBMCs were washed twice with MACS buffer reagent (300 ×*g* for 10 min) and supernatant was completely removed. PBMCs were incubated with 10 μL of B cell biotin-antibody cocktail per 10^7 total cells for 5 min in the refrigerator (2 °C–8 °C). Subsequently, the cells were resuspended in 40 μL of MACS buffer per 10^7 total cells. After incubation, 30 μL of MACS buffer and 20 μL of B cell microbead cocktail were added per 10^7 total cells. Cells suspension incubated for 10 min in the refrigerator (2 °C–8 °C). Subsequently, MACS buffer (500 μL) was added to the cell suspension and B cells were isolated using magnetic separation.

2.2. B cell purity assessment

Purity of B cells was evaluated by flow cytometry using FITC-conjugated mouse anti-human CD19 (eBiosciences, San Diego, CA) monoclonal antibody (mAb). Briefly, isolated B cells were washed twice with washing buffer (PBS 0.15 M, 0.5%BSA, 0.1% NaN_3) and incubated with FITC-conjugated anti CD19 for 40 min at 4 °C in the dark place. After incubation, the cells were washed with washing buffer. Percent of CD19 positive population was determined using flow cytometer (BD FACSCalibur). Data were analyzed using the FlowJo software version 7.2.5 (Tree Star Inc., USA). The purity of B cells was usually greater than 90% (Figure 1).

2.3. Treatment of purified B cells with different concentration of recombinant LPG3, NT-LPG3 and CT-LPG3 fragments

Purified B cells were co-incubated with recombinant LPG3, NT-LPG3 and CT-LPG3 fragments that were produced at Pasteur institute, Iran [20] and were kindly gifted from Professor Rafati. Briefly, 1 × 10^6 B cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, USA), penicillin (100 IU) and streptomycin (100 μg/mL) (Biosera, UK) The cells were treated with different concentrations of recombinant LPG3, NT and CT fragments (2, 10 and 20 μg/mL) for 24 and 48 h at 37 °C in a humidified 5% CO_2 incubator.

2.4. Cytokine assay

To evaluate the effects of LPG3 and its fragments on the production of IL-6, TNF-α and IL-10 by B cells, cell free supernatants of the cultured cells were harvested and the concentrations of IL-6, TNF-α and IL-10 were measured by standard sandwich ELISA kits

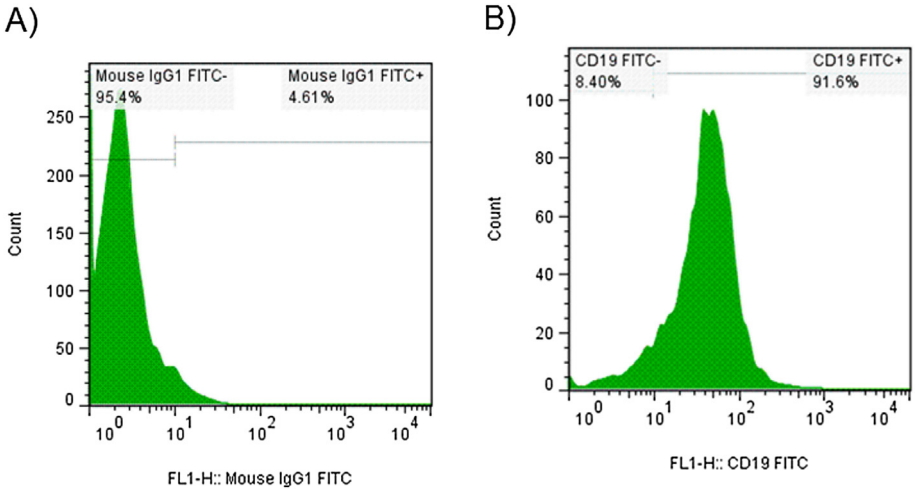

Figure 1. Histogram graphs of purified B cells by MACS method.
The isolated cells were stained with FITC-anti-CD19 and isotype control antibody. (A) Unstained cells. (B) B cell enriched fraction.

(R&D, Minneapolis, MN) according to manufacturer's instructions. Briefly, 96-well microtiter plates were coated with anti-IL-6, anti-TNF-α and anti-IL-10 coating Abs in 100 mM Na_2HPO_4, pH 9.0, incubated for 12 h at 4 °C, and blocked with blocking buffer containing 0.05% Tween20 and 10% FBS. Collected supernatants and recombinant IL-6, TNF-α and IL-10 standards were added (R&D Systems) and incubated at room temperature for 2 h. Following washing, biotinylated anti IL-6, TNF-α and IL-10 antibodies were added and incubated for 60 min at room temperature. Following washing, microtiter plates were incubated with streptavidine-polyHRP conjugate with p-nitrophenyl phosphate (4 mg/mL) as substrate. The absorbance at 405 nm was measured using a microtiter plate reader and the concentrations of IL-6, TNF-α and IL-10 were calculated from a standard curve of recombinant human IL-6, TNF-α and IL-10 standards, respectively. The concentrations of IL-6, TNF-α and IL-10 for each sample was calculated by regression analysis using the mean absorbance (average of triplicate readings of the samples added).

2.5. Assessment of B cell activation markers by flow cytometry

Expressions of CD69 were evaluated by flow cytometry to demonstrate activation state of B cells treated with rLPG3, CT and NT fragments. Briefly, 10^6 cells were washed with PBS and stained with anti-CD69 PE and anti-CD19 FITC monoclonal antibodies (eBiosciences, San Diego, CA). Following 45 min incubation at 4 °C in dark, the cells were washed and analyzed by flow cytometer and by FlowJo software.

2.6. Statistical analysis

For analysis of cytokine production, an intergroup comparison was performed by *Kruskal–Wallis* non-parametric *ANOVA* test. *P*-values below 0.05 were regarded as statistically significant.

3. Results

3.1. Recombinant LPG3 and its fragments stimulate the expression of CD69 on B cells

The representative dot plots illustrating the analysis method used for assessment of CD69 expressing B cells are shown in Figure 2. As shown in Figure 3, the stimulation of B cells with LPG-3 and NT fragment (but not CT fragment) could significantly increase the expression of CD69 on B cells ($P < 0.05$).

3.2. Recombinant LPG3 induced IL-6 production in B cells

The treatment of B cells with 10 and 20 μg/ml concentrations of recombinant LPG3 led to significant increase in secretion of

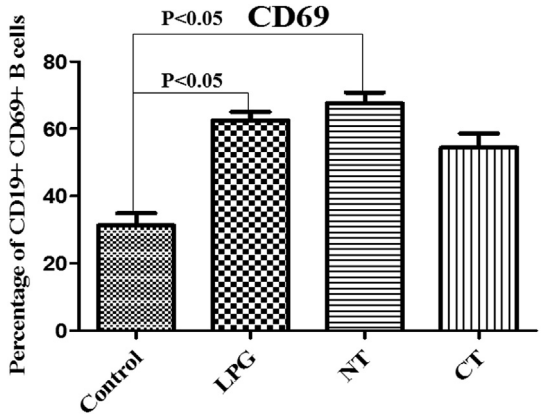

Figure 2. Expression of CD69 by B cells treated with 10 μg/mL rLPG3 and its fragments for 24–48 h.
LPG3 and NT-LPG3 has the most stimulatory effect on CD69 expression. Data represents mean ± SD (*P* value < 0.05).

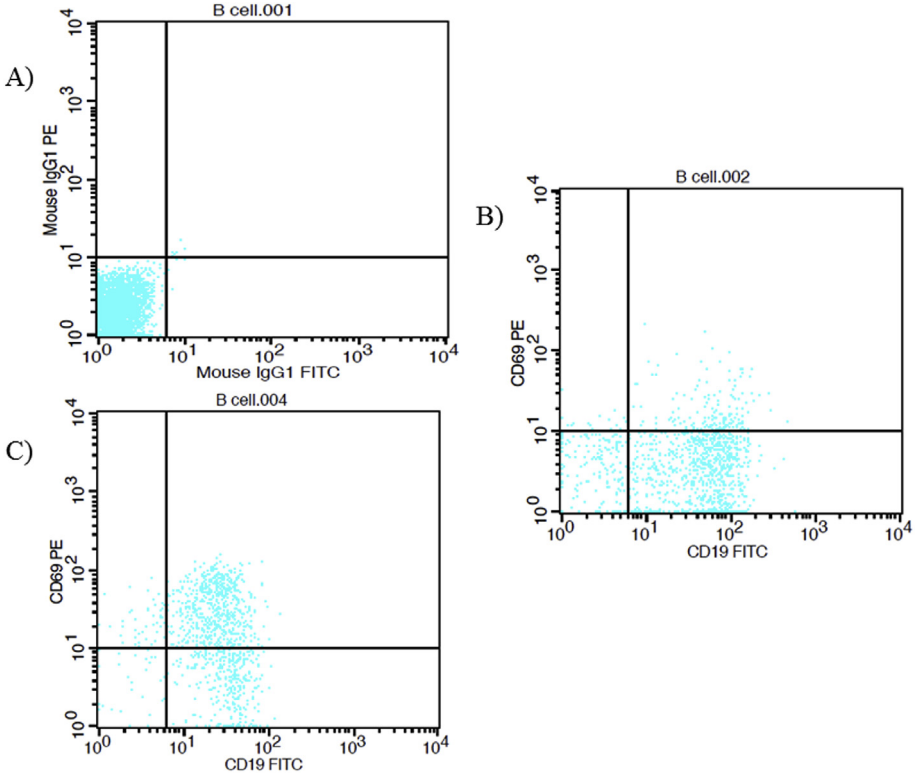

Figure 3. Dot plots demonstrating analysis method used for assessment of CD69 expressing B cells (A) isotype control (B) untreated B cells (C) B cells treated with rLPG3.

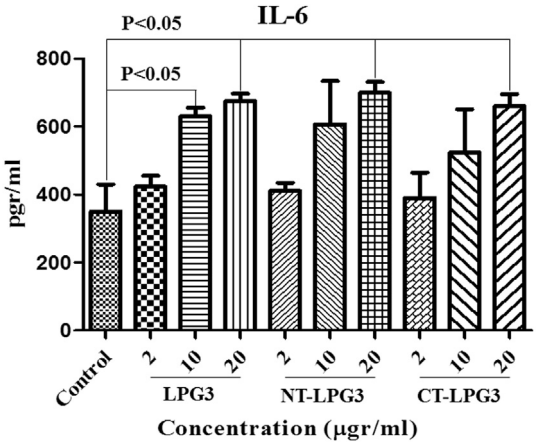

Figure 4. IL-6 secretion by B cells treated with different concentrations (2, 10 and 20 µg/mL) of rLPG3 and its fragments for 24–48 h.
Secretion of IL-6 in 10 and 20 µg/mL of rLPG3 and 20 µg/mL of NT-LPG3 and CT-LPG3 was statistically significant (P value < 0.05).

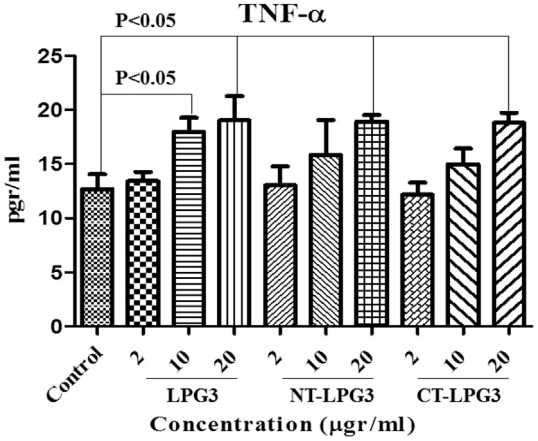

Figure 5. TNF-α secretion by B cells treated with rLPG3 and its fragments for 24–48 h.
Secretion of TNF-α in 10 and 20 µg/mL of rLPG3 and 20 µg/mL of NT-LPG3 and CT-LPG3 was statistically significant (P value < 0.05).

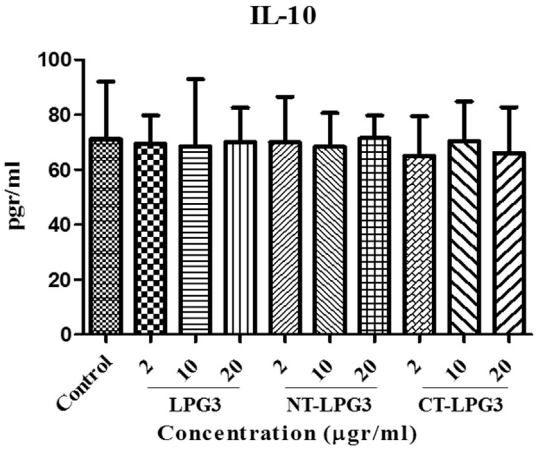

Figure 6. IL-10 secretion by B cells treated with rLPG3 and its fragments for 24–48 h.
LPG3 and its fragments had no stimulatory effect on IL-10 secretion by B cells.

IL-6 by B cells (P < 0.05) (Figure 4). Moreover, the 20 µg/mL (but not 2 and 10 µg/mL) concentration of recombinant NT and CT fragments of LPG3 could significantly increase the secretion of IL-6 by B cells.

3.3. Recombinant LPG3 induced TNF-α production in B cells

Our results showed that the high concentrations (10 and 20 µg/mL) of recombinant LPG3 could significantly increase the production of TNF-α in B cells. On the other hand, only the highest concentration (20 µg/mL) of NT and CT fragments had significant stimulatory effects on IL-6 secretion by B cells (Figure 5).

3.4. Effect of recombinant LPG3 and its fragments on IL-10 production in B cells

Our results showed that recombinant LPG3 and its fragments had no effects on the production of IL-10 by B cells (Figure 6).

4. Discussion

Active immunotherapy is the novel promising approach for eliminating and controlling of *Leishmania* infection. The presence of potent adjuvant is essential component of effective active immunotherapy.

In the present study, we were evaluated the B cell stimulating potential of different concentrations of LPG3, NT-LPG3 and CT-LPG3 fragments. CD69 is an important activation marker that expresses on the surface of mature lymphocytes [21]. We found that rLPG3 and its NT fragment have a significant effect on upregulation of CD69 on the surface of purified B cells.

It has been demonstrated that B cell-derived antibodies facilitate the phagocytosis of *Leishmania major*. Moreover, it observed that the higher parasite burden was associated with a reduction of T cell response and IFN-γ production in mice lacking antibodies following infection [22]. Furthermore, reduced chemotaxy of monocytes and lymphocytes at the site of cutaneous lesion has been observed in B lymphocytes deficient mice compared to wild type [23]. B cells also produce several pro-inflammatory and anti-inflammatory cytokines which regulate the immune response during *Leishmania* infection [24]. Our results indicate that recombinant LPG3 could activate B lymphocytes to produce and secrete IL-6 and TNF-α but not IL-10. Moreover, NT and CT fragments are able to induce IL-6 and TNF-α secretion.

IL-6 as a multifunctional cytokine promotes inflammatory responses against infection. Moreover, IL-6 participates in the development of Th1 and Th2 immune responses in mammals [25,26]. However, the function of IL-6 as a pro-inflammatory cytokines in cell-mediated immunity against leishmaniasis has not been clearly understood [27]. Akuffo *et al*. [28] demonstrated that *Leishmania aethiopica* antigen enhances the production of IL-6 from PBMCs of localized cutaneous leishmaniasis patients more than in adults healthy volunteers.

IL-10 is an immune-modulatory cytokine and mediates a variety of both immunosuppressive and immunostimulatory activities in mouse and human immune cells [29]. IL-10 is the one of important anti-inflammatory cytokines [30], countered as a

suppressor of macrophage functions [31,32]. It has been demonstrated that IL-10 can proceed parasite persistence [23]. Moreover, the immune suppressor activity of IL-10 has been indicated both in human [33] and mice visceral leishmaniasis [34]. A recent study demonstrated that IL-10-deficient BALB/c mice are highly resistant to *Leishmania donovani* infection [34]. However, our results showed that none of the LPG-3 and its fragments had effects on the production of IL-10 by B cells.

It has been demonstrated that TNF-α has a protective effect in experimental mouse cutaneous leishmaniasis [35]. Consistently, it is reported that treatment of mice with TNF-α could decrease parasitic burden and lesion size [36,37].

Our data were consistent with previous report [20] regarding the immunogenic nature of recombinant LPG3 in BALB/c mice. Moreover, Larreta *et al*. [38] reported that 84% of the dogs visceral leishmaniasis sera reacted with *Leishmania infantum* LPG3.

In conclusion, recombinant LPG3 stimulates human B cells to secrete pro-inflammatory IL-6 and TNF-α cytokines and inhibits production of regulatory IL-10 cytokine that may be considered as a promising candidate for *Leishmania* vaccine design in near future.

Conflict of interest statement

We declare that we have no conflict of interest.

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