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Role of imiquimod as adjuvant for vaccination against *Leishmania* major infection in BALB/c mice

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Various adjuvants in combination with different antigens have been utilized as a vaccine candidate against leishmaniasis. However, the search for ideal adjuvants is still pursued due to the inefficiencies of current compounds. In the present study, the effect of imiquimod, as an adjuvant, is studied with soluble *Leishmania* antigens (SLA) in BALB/c mice. Four groups of mice were immunized with SLA, SLA plus imiquimod, SLA plus BCG, and PBS as control. Immunized mice received a boosting dose of SLA after 15 days. All groups were challenged with *Leishmania major (L. major)* promastigotes 2 weeks after the booster immunization. Our results showed that strong TH1 responses were induced in groups of SLA plus imiquimod as well as SLA plus BCGafter immunization. These responses included smaller footpad thickness, lower parasite load in lymph node, and higher proliferative response of lymph node cells to SLA, higher levels of interferon γ in culture supernatant of lymph node cells, and higher levels of IgG, and IgG2a in sera. The data supports the possibility of using imiquimod as a suitable adjuvant in *leishmania* vaccination.

Keywords: imiquimod, *Leishmania major*, BCG, adjuvant, vaccination.

INTRODUCTION

Leishmaniasis is caused by different species of the genus *leishmania.* This disease remains a major health problem in endemic countries with approximately 350 million people at risk of infection and the annual incidence of about 2 million. The available drugs for the treatment of the disease are toxic, require multiple doses, have limited efficacies, and their inappropriate use in some cases has led to emergence of drug resistance [1-3]. Over the past decade, co-infection of leishmaniasis and HIV has increasingly been reported; the HIV virus hastens the spread of *Leishmania* and can reawaken latent infections, while *Leishmania* accelerates AIDS onset [4, 5]. Presently, there are no proven vaccines against any form of leishmaniasis, and treatment options are severely limited [3, 6]. Regarding the fact that individuals who recover from leishmaniasis or lesions induced by leishmanization usually illicit long lasting protective immune responses, development of an effective anti-leishmania vaccine is theoretically feasible [1, 3, 7].

In spite of intensive efforts during the past decades, only a few first generation vaccines made from whole killed *Leishmania* parasits alone, or mixed with immunomodulators reached the phase 3 clinical trials. The results of efficacy trials on the first generation *Leishmania* vaccines showed limited efficacies and no improvement were achieved with booster injections. The main reason for this failure seems to be, in some part, due to lack of an appropriate adjuvant [1, 8].

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Moreover, new generation of vaccines against leishmaniasis, particularly those based on recombinant proteins and DNA, are likely to be less immunogenic than first-generation ones [3]. The major steps in developing a successful vaccine against leishmaniasis is to identify appropriate antigens and adjuvants to achieve stable, safe, and immunogenic vaccines, and to acquire knowledge of the type of immune response generated in protected hosts [1, 2]. In leishmaniasis, recovery and protection against further infection mainly depends on induction of TH1-type immune responses, which are associated with the protective immunity [3, 9]; thus, adjuvants that promote TH1 responses are desirable. A wide variety of adjuvants are currently available, but all have their limitations [3, 10]. Therefore, there is a need for development of new and improved adjuvants to enhance the immunogenicity of vaccines, which induce an early, potent and long lasting *Leishmania*-specific immune response against leishmaniasis.

The most effective adjuvant used with *Leishmania* antigens in the murine models has been IL-12, but problems related to its manufacturing, cost, and availability, as well as safety issues, currently preclude this adjuvant from the list of candidates to be used in humans [3]. BCG, an attenuated version of the *Mycobacterium bovis*, has been successfully used for anti-*Leishmania* immunotherapy in some tropical regions [2, 7]. It has clearly shown to be associated with the induction of a TH1 immune response, and is probably the most acceptable TH1-inducing adjuvant presently available for use in humans [2]. However, application of BCG as an adjuvanthas occasionally been associated with some problems, including inflammatory arthritis and autoimmune reactions [2, 11]. In addition, application of a live vaccine as adjuvant may cause further complications, especially in immunocompromised individuals [12]. Thus, these side effects make BCG undesirable as a safe adjuvant candidate for vaccination. Imiquimod and its related compounds are members of the imidazoquinolines, which represent a new class of drugs that are immune-response modifiers [4, 6]. Imiquimod is the active ingredient in a topical cream called Aldara® (3M Pharmaceuticals), which has been approved by the FDA for the topical treatment of genital warts caused by human papilloma viruses. Imiquimod has been utilized as an immunomodulator in cutaneous leishmaniasis therapy and has been shown to induce the release of a number of cytokines, including interferon gamma (IFNγ), tumor necrosis factor (TNF)-α, interleukin (IL)-1b, IL-6, and IL-8 [6].

As imiquimod has been reported as a safe and effective treatment for *Leishmaina*-induced cutaneous lesions [4, 10], we were prompted to investigate its potential role as a vaccine adjuvant with soluble *L. major* antigens in BALB/c mice.

MATERIALS AND METHODS

Animals, parasites and Soluble *Leishmania* Antigen (SLA). Female BALB/c mice, aged 4 to 6 weeks were provided by the animal production facility of Pasteur Institute of Iran. The mice were kept with a 12:12 h light-dark cycle at 25˚C in the animal room of the immunology department and fed with tap water and laboratory pellet chow.

L. major strain (MRHO/IR/75/ER) was cultured in complete RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS) (Biosera, Ringmer, UK), 292 µg/ml L-glutamine, 4.5 mg/ml glucose, 100 µg/ml streptomycin and 100 µg/ml penicillin at 23-25˚C. Virulence of the parasite was maintained by its repeated passage in BALB/c mice. The parasite was harvested at stationary phase of growth, washed three times by Phosphate Buffer Saline (PBS), counted and diluted in PBS to 2×10^7 promastigotes /ml.

SLA was prepared as described by other [3] with minor modifications. Briefly, 2×10^7promastigotes were washed in cold PBS (pH 7.5) for three times. The suspension was freeze-thawed five times, sonicated, and centrifuged at 10,000 *g* for 2 h at 4˚C. The supernatant containing SLA was passed through a 0.22 µm filter and stored at -70˚C until used.

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Immunization of mice. Four different groups of female BALB/c mice (15 mice per group) were immunized through subcutaneous (sc) injection with one of the following procedures: group one received 100 µl of sterile PBS as control, group two was injected with 100 µl SLA alone, group three received a 100 µl SLA injection plus imiquimod cream 5% (Aldara, 3M, Canada) applied topically 1 min to the injection site daily, for 1 week after immunization. Group four received 50 µl SLA plus 50 µl 10^7 CFU BCG. Injection materials were divided into two 50 µl volumes, and each part was injected into one footpad. This was done to prevent any leakage of the injected material from the injection site. Two weeks later all groups, except PBS group, received µl SLA as the booster immunization.

Cell proliferation assay. Two weeks after booster immunization, mice spleen and lymph node cells (3 mice per each group) were removed and a single-cell suspension was prepared and cultured in RPMI 1640 medium (Sigma, Germany) supplemented with 10% FCS (Sigma, Germany), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were added to 96-well plates (Nunc, Denmark) in triplicate at a density of 2×10^5 cells per well and cultured with 5 µg/ml of SLA, or with medium alone. The cultures were incubated for 4 days at 37˚C under 5% CO_2 and pulsed with 0.5 µCi of [^{3}H]-thymidine (Amersham, UK) per well for the last 18 h of incubation. Stimulation index (SI) was obtained by dividing the mean counts per minute (cpm) of the wells stimulated with SLA by the mean cpm of the wells with no stimulation (containing culture medium only).

Cytokine measurements. Mice spleen and lymph node cell suspensions were prepared as described above for cell proliferation assay. Cell culture supernatants from triplicate wells were pooled after 72 h incubation and stored at -70°C for cytokine assay. Cytokines were measured by using commercial IFN-γ, IL-5 and IL-10 capture enzyme-linked immunosorbent assays (ELISAs) (Bendermed system, Austria), as recommended by the manufacture.

Antigen-specific antibody assay. Two weeks after booster immunization, mice were retro-orbitally bled for serum preparation. Blood samples were collected, allowed clotting and sera were separated and pooled in each group of mice. ELISA plates (Greiner Bio-One, Frickenhausen, Germany) were coated overnight with 15 µg/ml of SLA at 4˚C, blocked with PBS plus 1% bovine serum albumin (BSA-PBS). Serial dilutions of serum samples were added and plates were incubated for 2 h at 37˚C. For detection of specific total IgG, peroxidase-conjugated goat anti-mouse antibody (Sigma, Germany) was added to each well and the plate was incubated for 1 h at 37˚C before addition of tetramethylbenzidine (TMB) substrate (Sigma, Germany). For detection of IgG subclass, goat anti-mouse IgG2a antibody (Sigma, Germany) was added and the plate was incubated for 1 h at 37˚C. Peroxidase-conjugated rabbit anti-goat antibody (Sigma, Germany) was added to each well and the plate was incubated for 1 h at 37˚C, before the addition of TMB. The reactions were stopped by addition of 1N HCl and the optical density was read at 450 nm.

Challenge with *L. major* promastigotes. The immunized animals (three mice per group) were challenged subcutaneously in the left footpad with *L. major* promastigotes (MRHO/IR/75/ER) harvested at stationary phase (2×10^6 in a 50µl volume) 2 weeks after the booster immunization. The same volume of PBS was injected to right footpads as control. The development of lesions was recorded by weekly measurement of footpad thickness using a metric caliper (sensitivity, 0.05 mm). Grading of lesion size was done by subtracting the thickness of the uninfected contra lateral footpad from that of the infected one.

Quantitative parasite burden after challenge. The numbers of viable *L. major* parasites in popliteal lymphnodes of mice were enumerated by a limiting dilution assay as described elsewhere [13]. Briefly, popliteal lymph nodes were removed under

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sterile conditions, homogenized and resuspended in culture media composed of RPMI1640 plus 15% heat-inactivated FCS (Biosera), penicillin (100 U/ml), and streptomycin (50 mg/ml). Cell suspensions were serially diluted (10 fold) in a 96-well plates and kept at 24˚C for 1 week. Eight repeats were used for each dilution. After 1 week of incubation, plates were examined with an inverted microscope. The presence or absence of mobile promastigotes was recorded in each well. The final titer was the last dilution for which the well contained at least one parasite. Parasite load for each sample was calculated based on ELIDA program.

Statistical analysis. A one-way analysis of variance statistical test (ANOVA) was used to assess the significance of the differences among various groups. In cases of significant F values, the multiple-comparison Tukey test was used to compare the means of different treatment groups. *P* values of <0.05 were considered statistically significant.

RESULTS

The results of proliferative response showed that SLA induced proliferative response in splenocytes and lymph node cells from all immunized mice (Fig. 1). Comparison of stimulation indices 2 weeks after booster immunization indicated that the proliferative responses decreased significantly between experimental groups in the following order: SLA plus BCG, SLA plus imiquimod, SLA, and PBS.

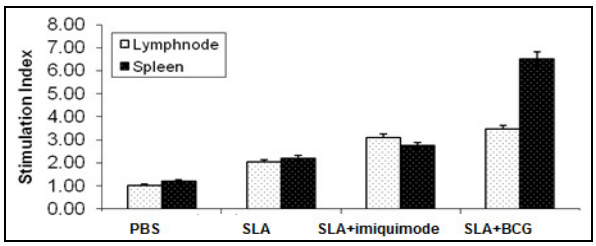

Fig. 1. Proliferative responses of lymph node and spleen cells shown as stimulation index (SI)+SD in different groups of mice two weeks after booster vaccination. The data was obtained from triplicate cultures of pooled cells from 3 mice per group.

The levels of IFN-γ, IL-5, and IL-10 were measured in culture supernatant of lymph node or spleen cells from different experimental groups two weeks after booster immunization. Spleen and lymphnode cells from all groups released little or no IFN-γ, IL-5 and IL-10 in the absence of antigen stimulation. However, upon SLA stimulation IFN-γ, IL-5 and IL-10 production increased significantly. As shown in Fig. 2, the highest IFN-γ level was seen in mice immunized with SLA plus BCG followed by those immunized with SLA plus imiquimod cream and the difference observed between these two groups was significant just for lymph node cells (P <0.02). The difference between IFN-γ level in the group of SLA plus imiquimod and SLA alone was also significant (P <0.05) (Fig. 2a). On the other hand, SLA alone produced the highest level of IL-5 and IL-10 in both lymph node cells and splenocytes and the difference between this group and three other groups was significant (P <0.05) (Fig. 2 b and c).

Specific serum IgG and IgG2a responses were assayed by ELISA 2 weeks after booster immunization (Fig. 3). The IgG level in mice immunized with SLA plus BCG was significantly higher than in the mice received SLA plus topical imiquimod cream (P <0.01), and the latter group produced significantly higher IgG than the groups of SLA alone and PBS (P <0.01) (Fig. 3a).

The same results were obtained for IgG2a subclass; the mice immunized with SLA plus BCG produced significantly higher IgG2a than the mice received SLA plus topical imiquimod cream, and the two other groups. The difference between groups of SLA plus imiquimod and SLA alone was also significant (P <0.01) (Fig. 3 b).

In this experiment, footpad thickness was used as a continuous surrogate for infection levels as the mice can be monitored for several weeks without being sacrificed. Mice were challenged 2 weeks after booster immunization by injection of 2×10^6 *L. major* promastigotes in stationary phase to left footpad and the same volume (50 µl) of PBS to right footpad.

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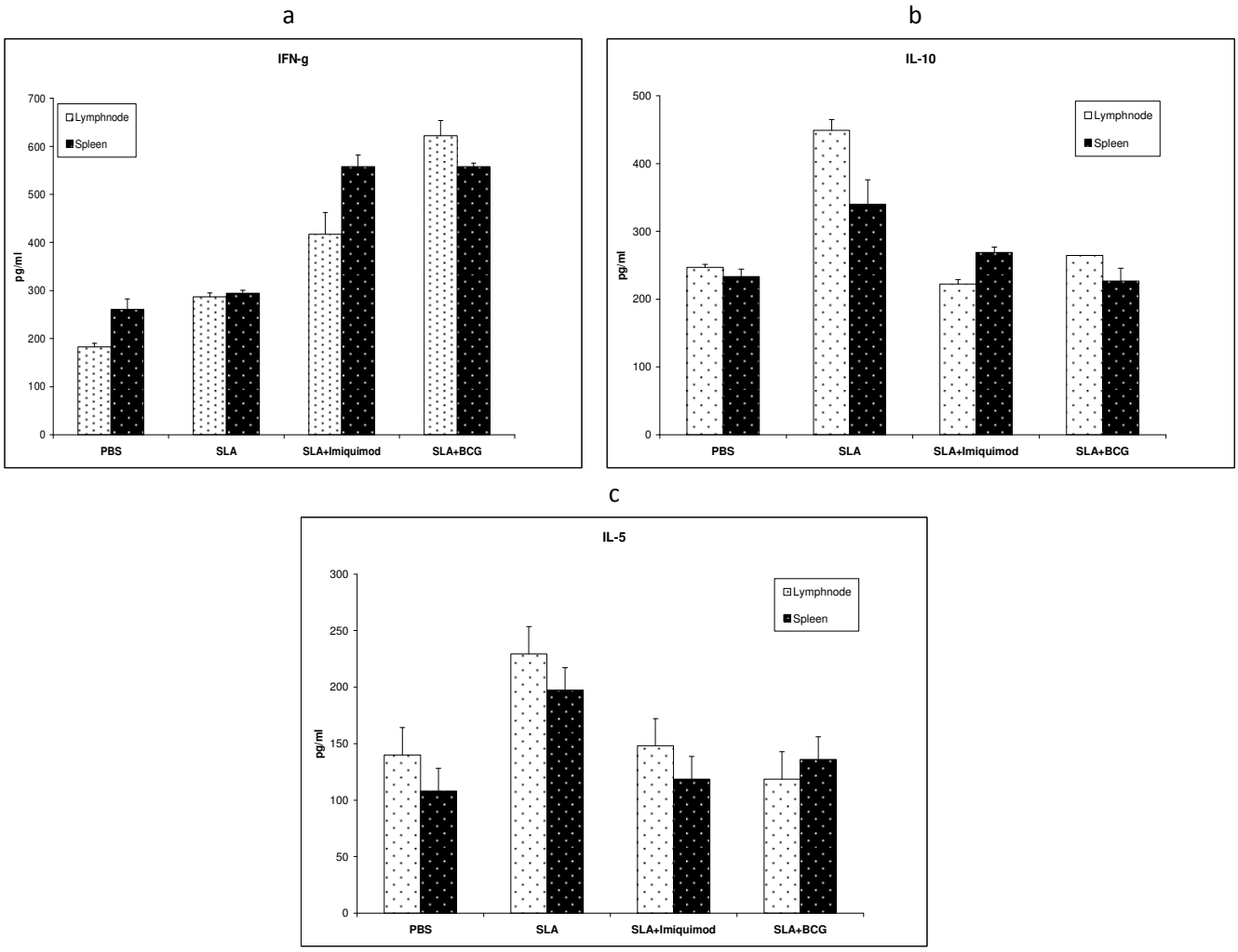

Fig. 2. The mean+SD production of IFN-γ (a), IL-5 (b), and IL-10 (c) (pg/ml) by spleen and lymph node cells of mice in different groups. The data was obtained from three mice per group.

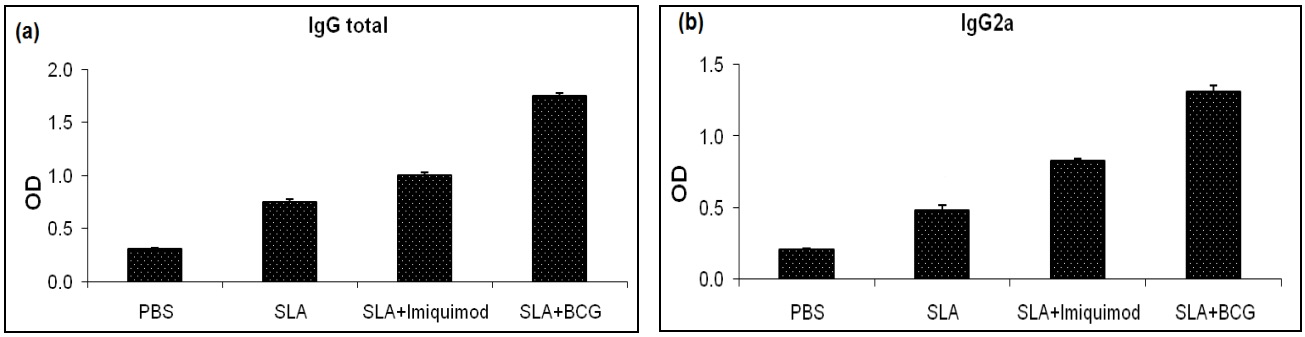

Fig. 3. The mean+SDanti-SLA specific IgG (a) and IgG2a (b) in mice sera 2 weeks after booster immunization. The data was obtained from three mice per group.

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The footpad swelling was measured by subtracting the diameter of the left footpad by the diameter of the right one. As shown in Fig. 4, the mice immunized with SLA plus BCG, in contrary to those immunized with SLA plus imiquimod cream, SLA alone, and PBS, developed strong protection against infection with no swelling detected in footpads (P <0.01).

Seven weeks after the last challenge, the number of living parasite in lymph nodes of the groups SLA plus BCG, and SLA plus imiquimod cream was significantly lower than compared the group SLA alone the and the control group (P <0.01). The parasite burden in the mice immunized with SLA plus BCG was not significantly higher than the mice received SLA plus imiquimodcream (Fig. 5).

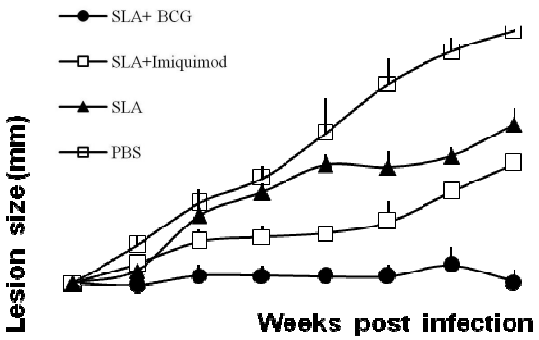

Fig. 4. The mean+SD footpad thicknesses of the mice, challenged with 2×10^6*L. major*promastigotes, 2 weeks after the booster immunization. The data was obtained from three mice per group.

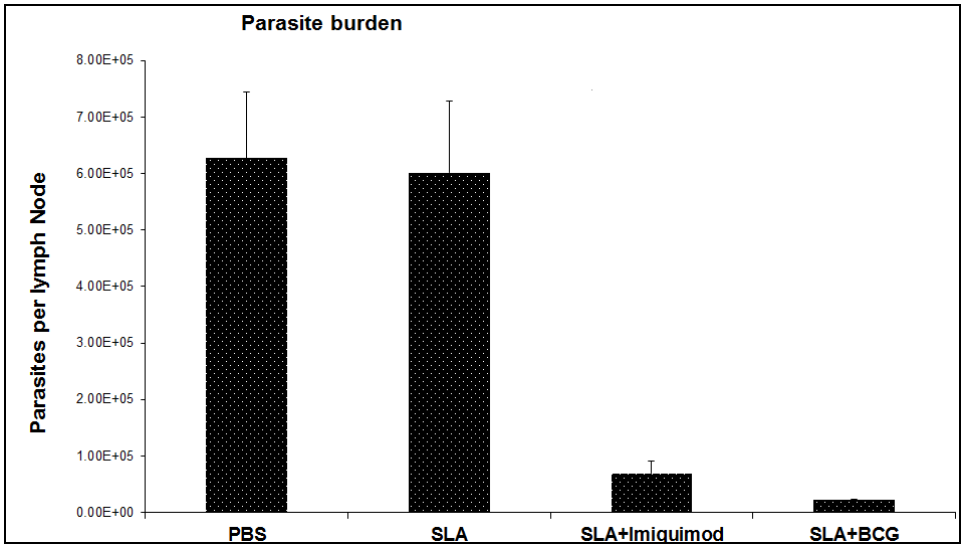

Fig. 5. The mean+SD parasite load in lymph node of immunized mice 7 weeks after challenge with 2×10^6 stationary phase living Promastigote. The data was obtained from three mice per group.

DISCUSSION

The choice of antigen and adjuvant are of paramount importance when developing a new vaccine for end-point use in humans. Successful vaccine development requires knowledge of which adjuvants to use and how to formulate adjuvants and antigens to achieve safe and immunogenic vaccines.

It is well known that *L.major* induce lesions develop in presence of TH2 mediated immunity, while the TH1 type mediates long life protection against reinfection [9]. In previous studies, vaccination of mice with heat-killed *L. major* did not provide protection against infection unless an appropriate adjuvant was included [10, 14]. Therefore, adjuvants that promote TH1 responses are particularly desira-

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ble. A wide variety of adjuvants to achieve this goal are currently available, but all have limitations. Hydrous sulfate of aluminum (Alum) has been used as adjuvant for vaccine formulations in human with FDA approval. However, Alum adjuvants often lack potency and may be less effective in activating cellular responses [2]. The strongest adjuvant, Freund's adjuvant, cannot be used in human because of severe local toxicity [2]. Dendritic cells are another potent adjuvant, but their application is limited by the cost and the time required for preparation [10]. Topical application of Imiquimod as a cream is licensed for treatment of HPV and basal cell carcinoma; this compound has demonstrated an acceptable efficacy in human clinical trials for leishmania vaccines [15, 16]. The exact mechanism of action imiquimod action is unknown, but it is thought that its activity as a TLR-7 agonist mimics a microbial antigen that induces expression of different cytokines such as IL-1, IL-6, IL-12, IFN-γ and TNF-α These stimulate both the innate immune system and the cell-mediated immune response, enhances migration of Langerhans cells from the dermis to regional lymph nodes, which finally result to diminished pathology associated with the *Leishmania* infection [10, 14, 16-18]. Imiquimod has shown to stimulate leishmanicidal activity via nitric oxide synthesis in infected macrophages using both in-vitro and in-vivo in mouse models [4, 6, 19]. In the current study, the immune responses of BALB/c mice induced by sc injection of SLA pretreated with imiquimod as a topical cream and the SLA in combination with BCG compared with those induced by sc injection of SLA alone. Use of imiquimod or BCG as adjuvants for SLA resulted in significantly more protective immune responses as compared to SLA alone. This protective immune response included higher lymphocyte proliferative response, higher specific IgG titer, lower parasite burden, higher levels of IFN-γ, lower levels of IL-5 and IL-10. The cytokine analysis suggests that the sc SLA in combination with both adjuvants (imiquimod and BCG) prime the TH1 response in immunized mice (Fig. 2). Topical application of imiquimod and its related compound R848 subcutaneously (which are both TLR7 and/or TLR8 agonists) in previous studies has been shown to increase concentration of IFN-γ and inhibit production of the TH2 cytokine IL-5 in mouse; however, mice treated with topical imiquimod alone or sc R848 alone were not protected against subsequent challenge infections [17, 14]. It has been shown that mice immunized with sc Ovalbumin (OVA) alone induced no significant anti-OVA cellular immune responses; however, by imiquimod pretreatment, OVA-immunized mice produced a strong cellular immune response [10]. It is noteworthy that the proliferative response level in SLA plus BCG group was higher in comparison with the group of SLA plus topical imiquimod. BCG has been reported to promote better antigen presentation and stimulate a cellular immune response via secretion of IL-2 and IFN-γ, leading to macrophage activation and intracellular parasite killing [12]. Antigenic cross-reactivity between *mycobacteria* and *Leishmania* is known [20]. In previous studies, vaccines composed of promastigote antigens and BCG induced strong cellular immune responses evidenced by a high index of conversion of the intra dermal skin reaction to *Leishmania* antigens [7, 11].

The IgG2a subclass of IgG has shown to be associated with TH1 response [21]. In this study, to provide further evidence for TH1 response induction following sc vaccination with SLA plus imiquimod or BCG, the IgG2a levels in immune mice were determined. Assessment of anti-SLA IgG and IgG2a isotype showed a preferential TH1 type response with both adjuvants. In a study on topical application effect of imiquimod on production of anti-OVA IgG and IgG2a in mice, it was shown that much higher amounts of these antibodies are released in sera of mice pretreated with imiquimod compared to those received OVA alone [10]. Imiquimod treatment of murine B-cells has shown to change the immunoglobulin response to antigens, i.e., increase in the level of the TH1 associated im-

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munoglobulin (IgG2a), and decrease in levels of the TH2 associated immunoglobulin (IgG1 and IgE) [10, 14, 22]. The findings of this study is in accordance with previous studies, which showed that use of imiquimod as an adjuvant for a pure SLA prototype vaccine markedly augments TH1 immune responses and resulted in a significant protection against *L. major* challenge in BALB/c mice, while the same vaccine alone failed to achieve these goals. Further studies are needed to optimize imiquimod as an adjuvant for vaccines against leishmaniasis in humans.

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