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Immunization with *Leishmania* Vaccine-Alum-BCG and Montanide ISA 720 Adjuvants Induces Low-Grade Type 2 Cytokines and High Levels of IgG2 Subclass Antibodies in the Vervet Monkey (*Chlorocebus aethiops*) Model

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Abstract

The availability of hundreds of adjuvants has prompted a need for identifying rational standards for the selection of adjuvant formulation based on sound immunological principles for human vaccines. As cytokines elaborated by activated T cells are required for the regulation of isotype switch during B-cell development, a study of Th2 cytokines and subclass distribution of the antibodies may shed new light on the processes involved in the polarization of the immune responses during vaccination studies. The aim of this study was to identify an appropriate *Leishmania* vaccine adjuvant based on low Th2 cytokine and high value IgG2 antibody responses. Groups of vervet monkeys were immunized with *Leishmania donovani* sonicate antigen (Ag) alone or in conjunction with alum-BCG (AlBCG), monophosphoryl lipid A (MPL) or montanide ISA 720 (MISA) as adjuvants. Following three time point intradermal injections on days 0, 28 and 42, IL-4, IL-10 and IgG antibody subclasses were quantified by enzyme-linked immunosorbent assay (ELISA) and data analysed by one-way analysis of variance, Tukey–Kramer test and Spearman's rank correlation analysis. Results indicated relatively higher IL-4 and IL-10 cytokine responses following MPL + Ag as compared to AlBCG + Ag or MISA + Ag immunization. There was a positive significant correlation between IL-4 and IL-10 levels (r = 1.000; P = 0.0167). Significantly higher IgG2 antibody responses were associated with either AlBCG + Ag or MISA + Ag as compared to MPL + Ag immunization ($P < 0.05$). The study concludes that both AlBCG and MISA may be used in *Leishmania* vaccine studies that favour low Th2 cytokine and strong IgG2 antibody responses.

Introduction

Human visceral leishmaniasis (VL), kala-azar, is a tropical disease caused by the protozoan parasites of the *Leishmania donovani* complex. The parasites multiply in the macrophages of the spleen, liver, bone marrow and lymph nodes, resulting in a progressive disease which is invariably fatal if untreated. Infection by *L. donovani* in humans induces T cell anergy as assessed by the depression of delayed-type hypersensitivity reaction and failure of peripheral blood T cells to proliferate [1, 2] and to produce gamma interferon (IFN-γ) and interleukin (IL)-2 in response to *Leishmania* antigens [3, 4]. Cytokine analysis reveals enhanced induction of IL-10 and/or IL-4 mRNA in tissues [5, 6], and the enhanced presence of IL-4 in circulation [7] of patients with kala-azar. The disease is also characterized by high levels of *Leishmania*-specific antibodies [8]. Resolution of VL and cure in humans is associated with predominant Thl response, good cell-mediated immunity (CMI), production of interferon-gamma (IFN-γ) and macrophage activation [3]. VL disease (Kala-azar) is associated with a Th2 response, poor

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CMI and hypergammaglobulinaemia. The Th2 response associated with active disease predominantly expresses and produces IL-4 and IL-10 cytokines [5]. Interleukin-4, a predominant Th2 cytokine, has been shown to play an active role in the progression of B-cell activation and switching of the isotype response with a predominant IgGI production in the human and mouse models [9, 10]. On the other hand, IFN-γ (Thl cytokine) down-regulates this activity and enhances IgG2a responses in mice. High levels of IgG1 and IgG3 production have been associated with IL-10 activity and blunting of IFN-γ activity (IgG2) in human VL [11, 12].

The role of IgG4 in parasitic infections is not clear but it has been suggested to play a blocking role in parasitic killing and clearance [13, 14]. This IgG subclass (IgG4) as well as IgG1 and IgG3 have been shown to increase in patients with active VL disease [15]. As cytokines elaborated by activated T cells are required for the regulation of isotype switch during B-cell development [16, 17], a study of the subclass distribution of the antibodies may shed new light on the processes involved in the polarization of the immune responses during vaccination studies and disease. We effectively evaluated the safety [18], Th1 cytokine responses and efficacy of alum-BCG [alone or in conjunction with alum-BCG (AlBCG)], monophosphoryl lipid A (MPL) and montanide ISA 720 (MISA) in the vervet monkey model of VL. These adjuvants were selected based on their promising results in previous vaccination studies [18, 19]. Herein, we report type 2 cytokine responses (IL-4 and IL-10) and IgG subclass distribution following vaccinations of the vervet monkey model against *L. donovani* parasite challenge.

Materials and methods

Leishmania parasites. *Leishmania donovani* strain NLB-065 originated from the spleen of an infected patient in Baringo district of Kenya and was maintained by intracardiac hamster-to-hamster passage at the Institute of Primate Research, Nairobi, Kenya. A hamster splenic biopsy was cultured in Schneider's drosophila insect medium supplemented with 20% foetal bovine serum and 100 μg/ml of gentamycin at 25 °C till stationary phase. Stationary phase promastigotes were harvested by centrifugation at 400 g for 15 min at 4 °C as described [18]. The pellet was washed three times in sterile phosphate-buffered saline (PBS) by centrifugation. These parasites were used for antigen preparation and challenge.

Preparation of soluble Leishmania antigen. *Leishmania donovani* stationary phase promastigotes were harvested by centrifugation as described previously. Harvested promastigotes were washed and sonicated at 18 kHz for five periods of 45 s each on ice, separated by intervals of 1 min as described [18]. The sonicated material was rapidly frozen and thawed three times in liquid nitrogen for the extraction of whole soluble protein. The parasite suspension was centrifuged at 10,000 *g* for 30 min at 4 °C. Protein concentration of the supernatant was determined using Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA) and stored at −70 °C until use. This antigen was used for coating ELISA plates for antibody assay.

Preparation of formalin-fixed Leishmania antigens. For *in vitro* lymphocyte proliferation and cytokine secretion assays, *L. donovani* promastigotes were harvested at stationary phase and washed three times in sterile PBS as described before. Parasites were fixed in 1% formal saline for 1 h and then washed three times in PBS as earlier. Parasites were counted in haemocytometer counting chamber and resuspended in a concentration of 5×10^8/ml in sterile PBS and stored at −70 °C until required.

Adjuvants and vaccine preparation. Monophosphoryl lipid A (CAYLA-InvivoGen, Toulouse, France), Montanide ISA 720 V (Seppic, Puteaux, France), alum (Rehydragel HPA; Reheis, Berkeley Heights, NJ, USA) and BCG (Serum Institute of India, Hadapsar, India) were used as adjuvants in this study. The vaccination antigen was prepared from *L. donovani* promastigotes. Stationary phase promastigotes were harvested as described before, counted and resuspended in 3 ml PBS at a concentration of 8×10^8 promastigotes. These promastigotes were freeze-thawed three times in liquid nitrogen and sonicated at 18 kHz for five periods of 45 s each on ice, separated by intervals of 1 min. Each vaccine antigen (sonicate) dose was made from 1×10^7 promastogotes. Vaccine dosages included 1 mg alum precipitated antigen plus BCG (50 μl) and sonicate mixed with 40 μl of MPLA. MISA 720 was used at an adjuvant/antigen ratio of 7:3 as per the manufacturer's instructions. All vaccines were reconstituted in sterile PBS.

Vervet monkeys. Both young and adult vervet monkeys of both sexes were caught in the wild and quarantined for 120 days at the Institute of Primate Research, Karen, Nairobi, Kenya. During the quarantine period, the monkeys were monitored for *Mycobacterium tuberculosis* infection and gastrointestinal and parasitic infections. The animals were tested for antileishmanial antibodies against both *L. donovani* and *L. major* antigen by ELISA and monkeys with no antibody titre were selected for the study. These animals were housed individually in squeeze-back cages and maintained on commercial non-human primate meal, supplemented with weekly fruits and vegetables. Water was provided *ad libitum*. Institutional Animal Care and Use and Institutional Scientific resources and Evaluation Committee guidelines were strictly followed.

Experimental protocol. *Leishmania donovani* antibody-free adult vervet monkeys with a mean body weight of 3.4 kg were selected and divided into five groups of three monkeys each as follows: group 1, alum precipitated sonicate plus BCG (AlBCG + Ag); group 2, sonicate plus monophosphoryl lipid A (MPLA + Ag); group 3,

sonicate plus montanide ISA 720 (MISA + Ag); group 4, sonicate (Ag) alone and group 5 non-vaccinated control (naïve control). The experimental groups were vaccinated three times intradermally at days 0, 28 and 42. On day 21 after last vaccination, total IgG and IgG subclasses and type 2 cytokine (IL-4 and IL-10) responses were determined.

Enzyme-linked immunosorbent assay for antibodies (ELISA). Quantification of total IgG: Polystyrene micro-ELISA plates (Dynatech Laboratories, Sussex, UK) were coated overnight with 100 μl of soluble *L. donovani* antigen (10 μg/ml) diluted in bicarbonate buffer, pH 9.6. Excess coating buffer was flicked off and non-specific binding sites were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. Unbound BSA was washed off six times with 0.05% Tween 20 in PBS. One hundred microlitres of diluted serum (1/125 in 1% BSA in PBS-Tween) samples was dispensed into the wells and incubated for 1 h at 37 °C. Unbound serum was washed off six times as above and 100 μl of 1/2000 horse radish peroxidase conjugated polyclonal goat antimonkey IgG (Rockland Immunochemicals, Philadelphia, PA, USA) was added and followed by incubation for 1 h at 37 °C. Unbound conjugate was washed off as above before adding 100 μl of orthophenyldiamine substrate (OPD; Sigma, Gillingham, UK, final concentration 0.4 μg/ml) in citrate buffer. The plates were incubated at 37 °C in the dark for 30 min and optical density was read at 450 nm using a microplate reader (Dynatech Laboratories).

Quantification of IgG subclasses: Parasite-specific total IgG antibody responses were measured by enzyme-linked immunosorbent assay as described [18]. Briefly, polystyrene micro-ELISA plates (Dynatech Laboratories) were coated overnight with 100 μl of soluble *L. donovani* antigen (10 μg/ml) diluted in bicarbonate buffer, pH 9.6. Excess coating buffer was flicked off and non-specific binding sites were blocked with 3% BSA in PBS for 1 h at 37 °C. Unbound BSA was washed off six times with 0.05% Tween 20 in PBS. One hundred microlitres of diluted serum (1/125 in 1% BSA in PBS-Tween) samples was dispensed into the wells and incubated for 1 h at 37 °C. Unbound serum was washed off six times as above and 100 μl of sheep anti-human IgG1, IgG2, IgG3 and IgG4 (Binding Site, Birmingham, UK) antibodies were added to individual well plates at a concentration of 10 μg/well added and followed by incubation for 1 h at 37 °C. Unbound primary antibodies were washed off as above before adding Donkey anti-sheep horseraddish peroxidase conjugate diluted at 1/5000. The plates were incubated for 1 h at 37 °C before washing to remove unbound secondary antibody. Tetramethylbenzidine substrate was added and plates incubated at 37 °C in the dark for 30 min and optical density read at 630 nm using a microplate reader (Dynatech Laboratories).

Quantification of type 2 cytokines (IL-4 and IL-10): Purified PBMCs were adjusted to 3×10^6/ml in complete RPMI 1640 medium (Gibco, Langley, OK, USA) which consisted of 10% foetal bovine serum (Flow Laboratories, Rockville, ML, USA) and stimulated with *L. donovani* promastigotes as described previously [18]. Culture supernatants were collected from triplicate wells after 72 h of stimulation, and the concentrations of IL-4 and IL-10 in the supernatant was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, polysterene micro-ELISA plates (Dynatech Laboratories) were coated overnight with 50 μl of a 2-μg/ml concentration of capture monoclonal antibody to human IL-4 or IL-10 (MabTech, Sweden) diluted in bicarbonate buffer pH 9.6. Excess coating buffer was removed, and non-specific binding sites were blocked with 3% BSA (Sigma) in PBS for 1 h at 37 °C. The plates were washed four times with 0.05% Tween 20 in PBS, and 50 μl of culture supernatant was dispensed to appropriate wells. Human IL-4 or IL-10 diluted (1–600 U/ml) in 1% BSA in PBS-Tween was used as a standard. The plate was incubated at 37 °C for 1 h and then washed four times. Biotinylated secondary monoclonal antibody to human IL-4 or IL-10 (50 μl of a 1/2000 dilution) was added, followed by incubation at 37 °C for 1 h. The plate was washed four times as before, 50 μl of 1/300-diluted alkaline phosphatase-conjugated streptavidin was added, and the mixtures were incubated for 1 h as described previously. The plate was washed 10 times in PBS-Tween, and 50 μl of nitrophenyl phosphate substrate (1 mg/ml) in diethanolamine buffer was added. The plate was incubated at 37 °C in the dark for 45 min, and absorbance was read at 405 nm. Cytokine levels were assessed by comparison with the standard curve generated with human IL-4 or IL-10.

Statistical analysis. Non-parametric one-way analysis of variance (ANOVA) was used to compare means of groups. Tukey–Kramer test was used for intergroup statistical analysis. Differences were considered significant where $P < 0.05$. Where applicable, Spearman's rank correlation was used for correlation analysis.

Results

Type 2 cytokines (IL-4 and IL-10)

Type 2 (Th2) cytokine levels or inhibitory cytokines, IL-4 and IL-10 were generally low but prominent in the MPL + Ag vaccinated group. Significantly higher levels of IL-4 were observed in the vaccinated animal groups as compared to the control group ($F = 8.506$; $P = 0.0029$; Fig. 1). Low levels of the cytokine were induced in all the vaccinated animal groups and these ranged from 27.56 pg/ml in the MPLA + Ag group to 5.0006 pg/ml in the Ag group. The baseline mean level of IL-4 in the

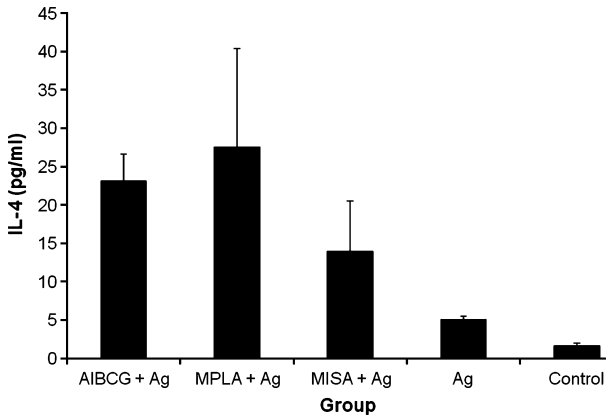

Figure 1 Interleukin-4 cytokine responses *Leishmania donovani* antigen in vaccinated animals. Animals were vaccinated at three different time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AlBCG + Ag), monophosphoryl lipid A (MPLA + Ag) or montanide ISA 720 V (MISA + Ag) and IL-4 cytokine responses determined on day 21 after the third vaccination. Data shown indicate mean IL-4 level ±SD in each vaccination group.

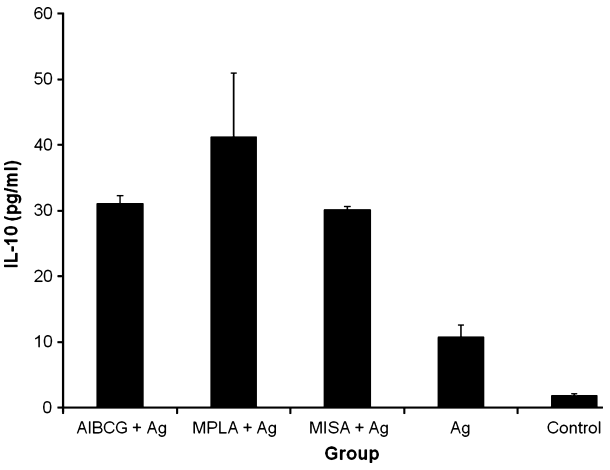

Figure 2 Interleukin-10 cytokine responses *Leishmania donovani* antigen in vaccinated animals. Animals were vaccinated at three different time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AlBCG + Ag), monophosphoryl lipid A (MPLA + Ag) or montanide ISA 720 V (MISA + Ag) and IL-10 cytokine responses determined on day 21 after the third vaccination. Data shown indicate mean IL-10 level ±SD in each vaccination group.

control group was 1.66 pg/ml. Levels of induced IL-4 did not differ between the antigen–adjuvant vaccinated groups ($P > 0.05$). Interleukin-4 levels induced in the AlBCG + Ag and MPL + Ag were significantly higher than those induced in the Ag group. Among the experimental groups, the MPL + Ag group induced the highest IL-4 cytokine levels, followed by AlBCG + Ag, then MISA + Ag, while the Ag group recorded the least IL-4 amounts. Although the adjuvant + Ag vaccinated groups induced statistically comparable IL-4 levels, the IL-4 cytokine levels induced following vaccination with MISA + Ag were comparable to the baseline values induced by the control group.

Results on IL-10 cytokine production indicated a significant difference between the experimental and the control groups ($F = 38.67$; $P < 0.0001$). Slightly higher amounts of IL-10 were produced in the corresponding experimental animal groups as compared to IL-4 levels. However, as in the case for IL-4, the IL-10 values were low and they ranged from a low of 10.79 pg/ml in the Ag vaccinated group to a high of 41.208 pg/ml in the MPL + Ag vaccinated animals. Baseline mean value was 1.802 pg/ml in the control group. Adjuvant-Ag vaccinated groups produced significantly higher IL-10 than the Ag group ($P < 0.01$; Fig. 2). As in the case for IL-4, there was no statistical difference in production of IL-10 among the adjuvant vaccinated groups. Levels of IL-10 induced by Ag vaccination were no different from those in the control group. There was a positive and significant correlation between IL-4 and IL-10 levels in the corresponding experimental and control groups ($r = 1.000$; $P = 0.0167$).

Parasite-specific IgG subclass responses

Production of antigen-specific IgG subclasses 1–4 was compared within and between groups. There was no significant difference in the production of IgG1 between all study groups ($F = 3.163$; $P = 0.0636$). The levels of IgG2 indicated a high significant difference between the experimental and control groups ($F = 34.283$; $P < 0.0001$; Fig. 3). Vaccination with AlBCG + Ag induced the highest IgG2 levels as compared to other vaccinated groups. The MISA + Ag vaccinated group produced significantly higher ($P < 0.05$) levels of IgG2 than the MPL group. The IgG2 levels induced by the MPL group did not differ with levels induced by the Ag or control group ($P > 0.05$). Experimental groups produced higher IgG3 levels as compared to the control group ($F = 16.482$; $P = 0.0002$). Vaccination with AlBCG + Ag induced IgG3 levels comparable to those induced by the MISA group ($P > 0.05$) but significantly higher than in the MPL + Ag vaccinated group ($P < 0.01$). The IgG3 subclass levels induced by vaccination with MPL + Ag were not different from the levels observed in the Ag vaccinated or control group ($P > 0.05$). Although there was significant difference in the production of IgG4 subclass antibodies between experimental and control groups ($F = 4.715$; $P = 0.0213$), only the AlBCG + Ag and control intergroup analysis concluded a significant differences ($P < 0.05$). Other intergroup computations did not indicate any significant differences.

Intragroup analysis indicated a significant difference in the production of IgG subclasses 1–4 following vaccination with AlBCG + Ag ($F = 31.614$; $P < 0.0001$). The absorbance values for subclasses were 0.105 (IgG1),

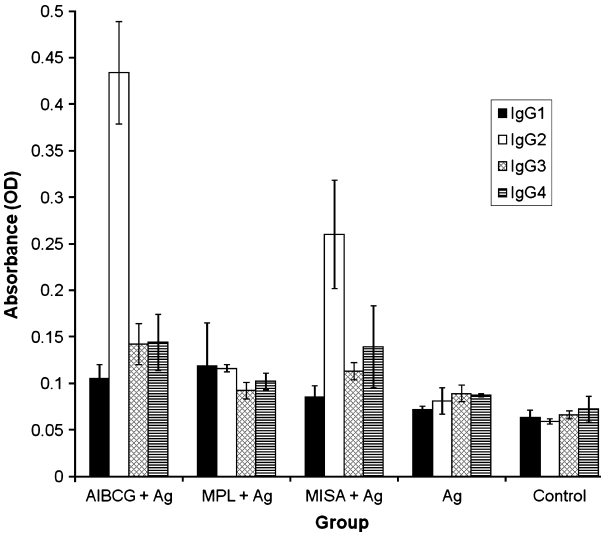

Figure 3 Antileishmania IgG subclass antibody responses to *Leishmania donovani* antigen in vaccinated animals. Animals were vaccinated at three different time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AlBCG + Ag), monophosphoryl lipid A (MPLA + Ag) or montanide ISA 720 V (MISA + Ag) and IgG1–4 subclass antibody levels determined on day 21 after the third vaccination. Data shown indicate mean IgG subclass level ±SD in each vaccination group.

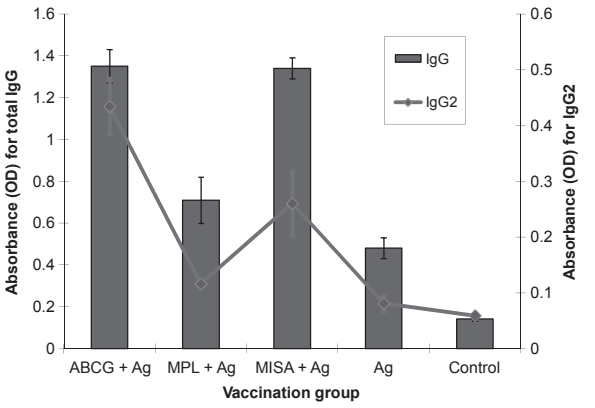

Figure 4 Relationship between total IgG and IgG2 antibody responses to *Leishmania donovani* antigen in vaccinated animals. Animals were vaccinated at three different time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AlBCG + Ag), monophosphoryl lipid A (MPLA + Ag) or montanide ISA 720 V (MISA + Ag) and total IgG and IgG2 antibody responses determined on day 21 after the third vaccination. Data shown indicate mean antibody level ±SD in each vaccination group.

0.434 (IgG2), 0.142 (IgG3) and 0.144 (IgG4). Parasite-specific IgG2 was significantly produced in large amounts as compared to all other subclasses ($P < 0.001$). The amounts of IgG1, 3 and 4 produced in this group were comparable ($P > 0.05$). As in the AlBCG group, there were significant differences in the production of IgG1–4 subclasses in the MISA group ($F = 12.945$; $P = 0.0019$). Absorbance values were 0.085 (IgG1), 0.26 (IgG2), 0.113 (IgG3) and 0.139 (IgG4). The subclass, IgG2 was significantly higher than any other subclass ($P < 0.01$) with no difference between levels IG1, IgG3 and IgG4 subclasses ($P > 0.05$). There was no difference in the levels of IgG1–4 subclasses produced following vaccinations with MPL + Ag ($F = 1.33$; $P = 0.3105$) with subclass absorbance values being 0.119 (IgG1), 0.116 (IgG2), 0.092 (IgG3) and 0.102 (IgG4). Absorbance values for subclasses in the Ag ranged from 0.072 to 0.089, while those in the control group were from 0.059 to 0.072. Analysis of levels of IgG1–4 subclasses in the Ag vaccinated ($F = 2.835$; $P = 0.1061$) and control ($F = 1.257$; $P = 0.3522$) groups did not indicate any statistical difference between values within each group.

Correlation between total IgG and IgG2 subclass

Parasite-specific total IgG antibody responses were quantified and results [18] correlated with the most prominent IgG subclass (IgG2). Spearman rank correlation analysis concluded a positive and significant correlation between total IgG and IgG2 antibody responses ($r = 1.000$; $P = 0.0167$; Fig. 4).

Discussion

Leishmania donovani sonicate antigen-specific IL-10 production, which is reported to be important for the cellular immune depression that accompanies active VL disease, as well as that of IL-4 promoting susceptibility to *Leishmania* infections, was low and did not cause vaccine failure. It is likely that the high Th1 responses in both AlBCG + Ag and MISA + Ag vaccinated groups may have depressed the polarization of Th2 immune responses, thereby driving the response to predominantly Th1 with high IFN-γ production [18]. Furthermore, on presentation of *Leishmania* antigens to $CD4^+$ T cells, the concomitant secretion of IL-12 drives the proliferation of (IFN-γ secreting) Th1 cells and NK cells, which activate macrophages and inhibit Th2 responses [20]. There is limited data on evaluation of type 2 immune responses in *Leishmania* vaccine studies using alum-BCG and this is the first study to measure type 2 cytokine responses in vaccine trials in animal models of leishmaniasis using MISA 720 as an adjuvant. However, in a murine *L. Infantum* vaccine study, MPLA delivered with Leish-111f antigen induced only marginal Th2 cytokines and strong Th1 immune responses [21]. In the present study, MPL induced relatively higher Th2 cytokine responses than other adjuvant-Ag groups leading to failure of Th1/Th2 cytokine responses to correlate within the study animal groups.

The relatively higher Th2 cytokine responses in the Ag and MPL + Ag vaccinated groups as opposed to other experimental groups may have depressed the development of type 1 responses in the two animal groups. Studies have indicated that the secretion of IL-4 during antigen presentation to $CD4^+$ T cells drives Th2 cell development that inhibits Th1 responses and promotes B lymphocyte growth and development [22]. This may clearly point out that although these adjuvants are believed to induce both strong humoral and cell-mediated immune responses [18, 23, 24], the response is biased towards cellular which consequently may inhibit the development of strong type 2 responses. With regard to specific T-helper-type 1 or 2 (Th1 or Th2)-derived cell-mediated immunity, studies have shown clearly that MPL has greater Th1 than Th2 stimulating potential [25].

The three adjuvants evaluated in this study have been associated with both cellular and humoral immune responses and it would be expected that these adjuvants induce high antibody responses following vaccinations. The predominant production of IgG2 and IgG4 following vaccination with AlBCG + Ag may indicate this adjuvant as a potent inducer of mixed type 1 (IgG2) and type 2 (IgG4) immune response–associated antibodies. The high production of IgG2 and low production of all other subclasses upon vaccination with MISA + Ag clearly indicates this adjuvant as a superior Th1 antibody inducer. Vaccination against murine malaria using MISA 720 related adjuvant, MISA 51 was predominantly associated with type 1 (IgG2a and IgG2b) antibody responses and low IgG1 and IgG3 subclass responses. In the present study, it was interesting to note that only the two adjuvant groups, AlBCG and MISA, inducing the highest IgG2 subclass antibodies were associated with protection of challenged monkeys against visceral disease [18].

It has been shown that murine IgG2a and IgG2b equivalent to human IgG2 have been implicated in conferring protection against various forms of infection [26]. The failure to induce type 1 antibody responses by MPL in the present study may be a matter of the formulation of the adjuvant used (aqueous). In a murine *L. infantum* study, immunization with MPL in stable emulsion with Leish-111f antigen produced a robust antibody IgG1 and IgG2a response [21]. In the present study, IgG antibody subclass (IgG1–4) responses upon immunization with MPL was comparable to responses in the Ag group. The selective stimulation for IgG2 production coupled with depressed secretion of IgG1, IgG3 and IgG4 in both the AlBCG and MISA adjuvant groups may add new knowledge on immunogenicity of these adjuvants in vaccine studies. Furthermore, this is the first study to try to characterize IgG subclass responses in *Leishmania* vaccine studies. It may therefore be right to suggest that, although both adjuvants are associated with cellular as well as humoral immune responses, the antibody response is selective for type 1 immune response associated IgG subclass (IgG2) antibody response. In any case, high levels of IgG1 and IgG3 production have been associated with IL-10 activity and blunting of IFN-γ activity (IgG2) in VL [11, 12]. The role of IgG4 in parasitic infections is not clear but it has been suggested to play a blocking role in parasitic killing and clearance [13, 14]. This IgG4 subclass as well as IgG1 and IgG3 have been shown to increase in patients with active VL disease [15]. In the present study, comparable IgG1–4 subclasses responses observed both between and within the MPL + Ag and Ag vaccinated groups, may indicate a failure of the MPL used in this study to immunopotentiate the sonicate antigen.

In the light of these findings, it should be possible to conclude that vaccination of the vervet monkey model with alum-BCG or montanide ISA 720 delivered with *L. donovani* whole cell sonicate antigen induces moderate amounts of IL-4 or IL-10 and upregulates levels of IgG2 as compared to vaccination with MPLA which was associated with higher levels of Th2 cytokines and low values of IgG2 antibody responses. We may therefore clearly conclude that alum-BG or montanide ISA 720 has greater Th1 than Th2 stimulating potential.

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