Leishmania parasite specific CD4+ synergizes and correlates positively with CD8+ T cells in the production of gamma interferon following immunization of the vervet monkey *(Chlorocebus aethiops)* model

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Abstract. Although there is currently no vaccine against leishmaniasis in routine use anywhere in the world, cases of self cure in cutaneous leishmaniasis, accompanied by solid immunity to reinfection, make vaccine development a feasible control method. Immunity against visceral leishmaniasis is mediated by IFN-γ-inducing parasite specific CD4+ and CD8+ T cells. We assessed the capacity of *Leishmania donovani* sonicate antigen delivered with alum-BCG (AlBCG), monophosphoryl lipid A (MPL) or montanide ISA 720 (MISA) to induce parasite specific CD4+ and CD8+ T cells involved in IFN-γ production following immunization of groups of the vervet monkey model of visceral leishmaniasis. Groups of vervet monkeys were immunized intradermally at three time points on days 0, 28 and 42 and T cell populations involved in the production of IFN-γ measured 21 days after final immunization. Significantly higher CD4+ T cells were induced in the group immunized with MISA+Ag compared to the AlBCG+Ag immunized animals ($P<0.01$) with both groups inducing significantly more CD4+ T cells than other groups ($P<0.0001$). Levels of CD8+ T cells were comparable between AlBCG+Ag and MISA+Ag groups, being significantly higher compared to the MPL+Ag group ($P<0.001$). The CD4+ T cells significantly correlated positively with CD8+ T cells in the studied groups (r=1.00; $P=0.0167$). We conclude that, immunization with MISA+Ag induces robust CD4+ as well as CD8+ T cells involved in the production of IFN-γ indicating stronger ability of this adjuvant over AlBCG in directing cellular immune response in the vervet monkey model.

Introduction

Visceral leishmaniasis or kala-azar is the most dreaded and devastating amongst the various forms of leishmaniasis (Garg and Dube, 2006). The disease, associated with anaemia, fever, weight loss, bone marrow destruction and hepatosplenomegally is fatal in almost all cases if left untreated (Bhowmick and Ravindran, 2008; Mutiso et al., 2011). It may cause epidemic outbreaks with high mortality (WHO, 2007). Although there is currently no vaccine against leishmaniasis in routine use anywhere in the world (Mauricio et al., 2000; Mutiso et al., 2010a; Schroeder and Aebischer, 2011), a vaccine against different forms of leishmaniasis should be feasible considering the wealth of information on genetics and biology of the parasite, clinical and experimental immunology of leishmaniasis, and the availability of vaccines that can protect experimental animals against challenge with different *Leishmania* species (Khamesipour et al., 2006).

Antileishmanial immunity that requires cellular immune responses is mediated by both innate and adaptive immune responses and requires effective activation of macrophages, dendritic cells (DC), and antigen-specific CD4+ and CD8+ T cells (Solbach and Laskay, 2000; Mukbel et al., 2007; Stanley and Engwerda, 2007). Effector CD4+ T cells are responsible for the production of cytokines critical for the activation of macrophages and are required for optimal host response to infection (Kharazmi et al., 1999). Cytotoxic CD8+ T cells also play a host protective role, and are required for the effective clearance of parasites (Stern et al., 1988) and the generation of memory responses (Stager et al., 2000). Although vaccine formulation with killed parasites is still attractive in terms of cost (Garg and Dube, 2006) and safety (Kenney et al., 1999), inactivated vaccines require adjuvants to stimulate an immune response and the choice of adjuvant or immune enhancer determines whether the immune response is effective, ineffective or damaging (Marciani, 2003). Successful vaccine development requires knowing which adjuvants and antigens to use and knowing how to formulate adjuvants and antigens to achieve stable, safe and immunogenic vaccines (Reed et al., 2009).

Furthermore, a mixture of safe *Leishmania* antigens together with an adjuvant that preferentially stimulates specific gamma interferon (IFN-γ)-inducing T cells presents a rational option for a vaccine against leishmaniasis (Aebischer et al., 2000).

While immunity in murine *L. major* cutaneous leishmaniasis, mediated by parasite-induced production of IFN-γ by CD4+ T cells (Th1 subset) can develop in the absence of CD8+ T cells (Reiner and Locksley, 1995), both CD4+ and CD8+ T cells are required for an effective defense against murine visceral *L. donovani* infection (Sacks et al., 1987; Melby and Anstead, 2001; Dominguez et al., 2002; Das and Ali, 2012). It is therefore important that an appropriate vaccine-adjuvant against visceral leishmaniasis induces both CD4+ T cell (Th1) and CD8+ T cells for effective control of disease. Both types of the T cells produce IFN-γ cytokine critical for the activation of macrophages for effective killing of the intracellular parasites (Melby and Anstead, 2001; Tsagozis et al., 2003). The most recent clinical trials of first generation vaccines have demonstrated a good safety profile but have not conferred significant levels of protection for use as prophylactic vaccines (Lukasz, 2010). The availability of hundreds of adjuvants has prompted a need for identifying rational standards for selection of adjuvant formulation based on safety and sound immunological principles for human vaccines. We previously indicated Montanide ISA 720 as more effective than BCG as an adjuvant for *Leishmania* killed vaccine in the murine system (Mutiso et al., 2010b). Other studies have indicated the successful use of alum plus BCG (Misra et al., 2001) and monophosphoryl lipid A (Coler et al., 2007) in the control of visceral leishmaniasis in the monkey and murine systems respectively. We recently indicated good safety levels of a vaccine comprising *Leishmania donovani* sonicate antigen delivered with either monophosphoryl lipid A or montanide ISA 720 in the vervet monkey model (Mutiso et al., 2012a). However, the study indicated adverse skin reactions in animals that received sonicate antigen in conjunction with alum-BCG. Antibody evaluations indicated high levels of total IgG (Mutiso et al., 2012b) that were associated with high values of IgG2 subclass antibodies and low type 2 cytokines in

animal groups immunized with sonicate antigen delivered with alum-BCG or MISA 720 (Mutiso et al., 2012c). The report also indicated the potential of alum-BCG or MISA 720 in enhancing *L. donovani* sonicate antigen to increase delayed type hypersensitivity (DTH) responses in immunized animals (Mutiso et al., 2012a).

In the present report, we describe levels of parasite specific CD4^{+} and CD8^{+} T cells involved in the production of IFN-γ following *Leishmania donovani* sonicate antigen delivery with montanide ISA 720, alum-BCG or monophosphoryl lipid A in the vervet monkey model of visceral leishmaniasis.

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% fetal bovine serum and 100 μg/ml of gentamycin at 25°C till stationary phase. Stationary phase promastigotes were harvested by centrifugation at 2500 rpm for 15 min at 4°C as described (Mutiso et al., 2012b). The pellet was washed three times in sterile phosphate buffered saline (PBS) by centrifugation. These parasites were used for antigen preparation.

Preparation of soluble Leishmania antigen

Leishmania donovani stationary phase promastigotes were harvested by centrifugation as described (Mutiso et al., 2012b). Harvested promastigotes were washed and sonicated at 18 kHz for five periods of 45 seconds each on ice, separated by intervals of 1 minute. The sonicated material was rapidly frozen and thawed three times in liquid nitrogen for extraction of whole soluble protein as described (Mutiso et al., 2010b). The parasite suspension was centrifuged at 10,000g for 30 minutes at 4°C. Protein concentration of the supernatant was determined using Bio Rad protein assay kit (Bio Rad) and stored at -70°C until use. This antigen was used for coating ELISA plates for antibody assay.

Adjuvants and vaccine preparation

Monophosphoryl lipid A (InvivoGen), Montanide ISA 720 V (Seppic), alum (Rehydragel HPA; Reheis, Berkeley Heights, NJ) and BCG (Serum Institute of India, Hadapsar, India) were used as adjuvants in this study. The vaccination antigen was prepared from *Leishmania donovani* promastigotes. Stationary phase promastigotes were harvested as described before, counted and resuspended in 3 ml PBS at a concentration of 8 x 10^{8} promastigotes making 80 doses of 1 x 10^{7} promastigotes each in a sonicate volume of 37.5 μL per dose. These promastigotes were freeze – thawed three times in liquid nitrogen and sonicated at 18 kHz for five periods of 45 seconds each on ice, separated by intervals of 1 minute. Vaccine dosages included 30 μL of alum (1 mg) precipitated antigen (37.5 μL) plus BCG (50 μL) and sonicate antigen (37.5 μL) mixed with 40 μL of MPL. MISA 720 was used at an adjuvant: antigen ration of 7:3 as per the manufacturer's instructions. All vaccines were reconstituted in sterile PBS to produce a final volume of 120 μL per dose.

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 *Leishmania 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.* Institute of Primate Research Institutional Review Committee (IRC) on Animal Care and Use, approved the study and the Committee's 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. Interferon gamma-producing CD4+ and CD8+ T cell populations were measured on day 21 after last vaccination.

Cell isolation, storage and thawing

Peripheral blood mononuclear cells (PBMCs) were isolated from sodium heparin anticoagulated monkey blood by density centrifugation (Histopaque, Sigma, USA). PBMCs were harvested, washed using Alsever's solution and centrifuged at 400g for 10 minutes. PBMCs were then resuspended in Alsever's solution and counted using Trypan blue exclusion in haemocytometer. After counting, 3 million PBMCs/ml were cryopreserved using 1 ml of 10% Dimethyl Sulfoxide (Sigma, USA) and 90% Fetal Bovine Serum (Sigma, USA) cryopreservation solution. Samples were rate-controlled frozen to -70°C overnight and then transferred into liquid nitrogen until analysis.

Culture medium was prepared by adding 1% penicillin-streptomycin, and either 1%, 10% or 20% heat inactivated fetal bovine serum (Sigma, USA) to RPMI-1640 medium (Sigma, USA) to make R1, R10 and R20 respectively. PBMCs vials were thawed in a 37°C water bath and the samples put into a 50 ml conical tubes. To these samples, 10 ml of R1 (warmed to 37°C) was added drop-wise while swirling the cells. The cells were topped to 25 ml with R1 and centrifuged at 1200 rpm for 10 minutes. The supernatants were decanted and cell pellets resuspended with 500 µl of 0.002% DNAse. Up to 25 ml of R1 was added and tubes centrifuged for 10 minutes at 1200 rpm. The supernatants were decanted and cells resuspended in 1 ml R20 before counting using a counting chamber. Counted cells were resuspended in R20 (RPMI

with 20% FBS) 15 ml tubes and incubated overnight at 37°C, 5% CO_2 atmosphere.

Cell surface staining and measurements

Following overnight rest, 0.5–1 x 10^6 viable PBMC were transferred to individual tubes in 50 µl aliquots and R10 added to each tube followed by centrifugation at 1200 rpm for 10 minutes at 10°C. Supernatants were aspirated and pellet disrupted and resuspended in 200 µl of R10 before addition of 2 µL soluble *Leishmania donovani* antigen (10 µg/ml) to each desired tube. The tubes were placed at 37°C in a humidified CO_2 incubator for 3 hours and Brefeldin A (10 µg/ml final) added to the desired tubes and tubes incubated for 20 hours. The tubes were centrifuged at 1500 rpm for 5 minutes at 10°C and supernatant aspirated and desired cell pellets resuspended in 50 µL PBS wash containing optimally titrated amount of CD3 PerCP, CD8 FITC, and CD4 PE antibodies (BD, Biosciences, Belgium). Tubes were incubated for 20 minutes on ice and 500 µL PBS-wash added to each tube before centrifugation at 1500 rpm for 5 minutes at 10°C. Supernatants were aspirated and tubes agitated to disrupt cell pellets.

To each sample tube, 200 µL of 4% paraformaldehyde was added and tubes vortexed and incubated for 20 minutes on ice. To each sample tube 200 µL permeabilization buffer (Cytoperm) (BD, Biosciences, Belgium) was added and tubes containing sample suspensions were centrifuged at 1500 rpm for 5 minutes at 10°C. Supernatants were aspirated and cell pellet disrupted before addition of 100 µL permeabilization buffer to the sample tubes that were to be stained with anti-cytokine antibody. Tubes were incubated for 5 minutes at room temperature and 10 µL of APC-conjugated anti-IFN-γ cytokine antibody (BD, Biosciences, Belgium) added to the desired sample tubes and contents mixed. Tubes were incubated for 20 minutes at room temperature and 200 µl permeabilization buffer added to each tube and centrifuged at 1500 rpm for 5 minutes at 10°C. Permeabilization steps were done as outlined elsewhere (www.thinkpeptides.com).

Supernatants were aspirated and tubes agitated to disrupt the cell pellets. Cells were then

resuspended in 300 μL fix solution and the tubes vortexed before data acquisition on a fluorescent-activated FACSCalibur cell sorter (Becton Dickinson). Data were analyzed by CellQuest Software (Becton Dickinson) with final events fixed at 6500/sample.

Statistical analysis

Data were initially analyzed by CellQuest Software (Becton Dickinson) before use of GraphPad InStat software, version 3.05, 32 bit for Win 95/NT for further analysis. One-way analysis of variance (ANOVA) was used to compare means of groups. Tukey-Kramer test was used for inter-group statistical analysis. Differences were considered significant where $P<0.05$. Spearman rank correlation was used for correlation analysis.

Results

Parasite-specific gamma-interferon producing CD4+ and CD8+ T cell counts

The actual number of parasite specific CD4+ T cells involved in the production of interferon gamma between the experimental and control groups differed significantly in numbers *(P=0.0001)*. All groups vaccinated with adjuvant plus antigen produced significantly higher IFN-γ inducing CD4+ T cells as compared to either the Ag or the control groups (figures 1a–1e). Vaccinations with MISA+Ag induced the highest numbers of CD4+ T cells involved in IFN-γ production. These cells were significantly more than those induced by AlBCG+Ag vaccinations *(P<0.01)*. Vaccination with MISA+Ag or AlBCG+Ag was more potent than MPL+Ag in inducing Th1 CD4 T cells (*P<0.001*).

The IFN-γ positive CD4+ T cells did not differ in numbers between the Ag vaccinated and the control groups *(P>0.05)*. As for the IFN-γ+ CD4+ T cell population, the actual number of parasite specific IFN-γ inducing CD8+ T cells also differed significantly in numbers between the experimental-adjuvant and the control groups *(P=0.0001)*. These cells were comparable in the AlBCG+Ag and MISA+Ag groups, being significantly higher than in the MPL+Ag vaccinated group *(P<0.001; figure 2)*. A narrow gap of difference in numbers of the IFN-γ

positive CD8+ T cells was observed between the MPL+Ag and the Ag vaccinated groups *(P<0.05)* with the T cell response being higher in the MPL+Ag group. The IFN-γ positive CD8+ T cells did not differ in numbers between the Ag vaccinated and the control group *(P>0.05)*.

Correlation between IFN-γ-producing CD4+ and CD8+ T cell counts

Spearman rank correlation analysis between IFN-γ positive CD4+ and IFN-γ positive CD8+ T cells indicated a very strong positive correlation between the two T cell populations (r=1.00; P=0.0167). An increase in parasite specific CD4+ T cells involved in IFN-γ induction was accompanied by a corresponding increase in CD8+ T cells involved in IFN-γ production (figure 3).

Discussion

Although there is currently no vaccine against leishmaniasis in routine use anywhere in the world (Mauricio et al., 2000; Mutiso et al., 2010a; Schroeder and Aebischer, 2011), a vaccine against different forms of leishmaniasis should be feasible considering the wealth of information on genetics and biology of the parasite, clinical and experimental immunology of leishmaniasis, and the availability of vaccines that can protect experimental animals against challenge with different *Leishmania* species (Khamesipour et al., 2006). The present study describes some attempts aimed to understand the relative expression of IFN-γ-producing CD4+ and CD8+ T cells induced upon immunization with selected adjuvants-antigen combinations against visceral leishmaniasis. The study explores the relationships between levels of CD4+ and CD8+ T cells induced following immunization. Protection against *Leishmania* infection relies on cell mediated immune response, which implies that a successful immunization protocol should be able to activate cell-mediated immunity in the immunized animals (Melby et al., 1998). Acquired immunity in murine *L. major* cutaneous leishmaniasis is mediated by parasite-induced production of IFN-γ by CD4 T cells (Th1 subset), and can develop in the absence of CD8 T cells (Reiner and Locksley, 1995). However, both CD4 and CD8 T cells are

required for an effective defense against visceral *L. donovani* infection (Melby and Anstead, 2001). Furthermore, cure for all forms of leishmaniasis is affected through cellular immune response capable of activating host macrophages to eliminate the parasite (Tripathi et al., 2007). Effector CD4+ T cells are responsible for the production of cytokines critical for the activation of macrophages and are required for optimal host response to infection. Cytotoxic CD8+ T cells also play a host protective role, and are required for the effective clearance of parasites (Stern et al., 1988) and the generation of memory responses (Stager et al., 2000). Induction of apoptosis of parasitized macrophages, as well as direct, performing-mediated cytotoxicity, are candidate mechanisms employed by CD8 cells in their effort to restrain parasite multiplication. One common role of both CD4 and CD8 T cells in the control of leishmaniasis is the induction of IFN-γ (Roberts, 2006; Pereira et al., 2008; Nateghi et al., 2010). Interferon gamma is a crucial cytokine that enables macrophages to clear intracellular amastigotes in a nitric oxide (NO)-dependent manner (Murray et al., 1985). It has been indicated that the most important role of CD8 lymphocytes during infection with *Leishmania* spp. is IFN-γ secretion (Reiner and Locksley, 1995).

Considering the central role played by both CD4+ T cell (Th1) and CD8+ T cells in the effective control of leishmaniasis, it is therefore important that an appropriate vaccine-adjuvant against visceral leishmaniasis induces high levels of these lymphocytes. The present study evaluated the production of IFN-γ by CD4+ and CD8+ cells in vervet monkeys following immunization with *Leishmania donovani* sonicate antigen delivered with Alum plus BCG, monophosphoryl lipid A or montanide ISA 720 as adjuvants. Successful vaccine development requires knowing which adjuvants to use and knowing how to formulate adjuvants and antigens to achieve stable, safe and immunologic vaccines (Reed et al., 2009). The availability of hundreds of adjuvants has prompted a need for identifying rational standards for selection of adjuvant formulation based on sound immunological principles for human vaccines. We previously indicated Montanide ISA 720 as more effective than BCG

as an adjuvant for *Leishmania* killed vaccine in the murine system (Mutiso et al., 2010b). Other studies have indicated the successful use of alum plus BCG (Misra et al., 2001) and monophosphoryl lipid A (Coler et al., 2007) in the control of visceral leishmaniasis in the monkey and murine systems respectively.

The expresssion of the highest numbers of IFN-γ-inducing CD4 T cells in animals vaccinated with MISA+Ag is a confirmation that montanide ISA 720 is superior adjuvant associated with induction of Th1 immune responses. This is also confirmed by the the expression of the highest numbers of IFN-γ-producing CD8 cells in the same adjuvant vaccinated animals as compared to other adjuvant+Ag vaccinated groups. Reports on montanide trials done with HIV- and malaria-derived antigens as well as in a cancer vaccine have reached a general concesus that it is highly immunogenic inducing both Th1 type cellular and humoral responses (Mutiso et al., 2010a; Kenney and Edelman, 2003; Myriam et al., 2005; Oliveira et al., 2005; Collins et al., 2006).

Our present results indicate alum-BCG as a lesser adjuvant than MISA 720 in the production of IFN-γ-inducing CD4 T cells as well as in the expression of IFN-γ-inducing CD8 despite the response being comparable in the latter lymphocyte expression. However alum plus BCG has been evaluated in *Leishmania* vaccine studies and has been associated with strong Th1 immune responses including production of IFN-γ (Misra et al., 2001; Kamil et al., 2003). Both alum-BCG and MISA can equally be used to induce effective Th1 immune responses against visceral *L. donovani* infection. Our present results failed to establish MPL as a competitive adjuvant in the expression of IFN-γ-inducing CD4 and CD8 T cells as compared to alum-BCG and MISA adjuvants. Monophosphoryl lipid A used with *Leishmania*-derived recombinant polyprotein Leish-111f antigen was shown to be highly immunogenic in a vaccine against murine *L. infantum* leishmaniasis (Coler et al., 2007). Failure of the MPLA+Ag used in this study to induce high levels of FN-γ-producing CD4+ and CD8+ T cell levels comparable to other antigen-adjuvant groups may be attributed to the formulation of this adjuvant (Mutiso et al., 2012b).
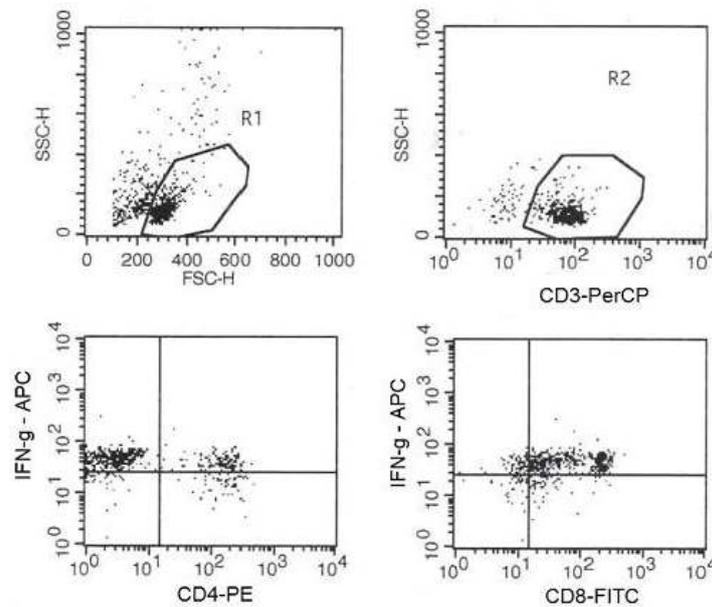
Figure 1(a). Flow cytometer dot graphs indicating gating strategy for CD3+ T cells (R1 and R2) and separation of parasite specific CD4+ and CD8+ T cells involved in the production of IFN-γ (upper left compartment for all dot graphs) from PBMCs obtained from animals following immunizations. Animals were immunized at three time points with sonicate antigen (Ag) plus alum-BCG (AlBCG+Ag). Peripheral blood mononuclear cells (PBMCs) harvested 21 days after the third immunization were stimulated *in vitro* with *Leishmania donovani* antigen before cells were stained for measurement of interferon gamma-producing CD4+ and CD8+ T cell populations in flow cytometer. Data shown indicate dots representing individual CD4 and CD8 T cells measured from PBMCs from experimental animal groups
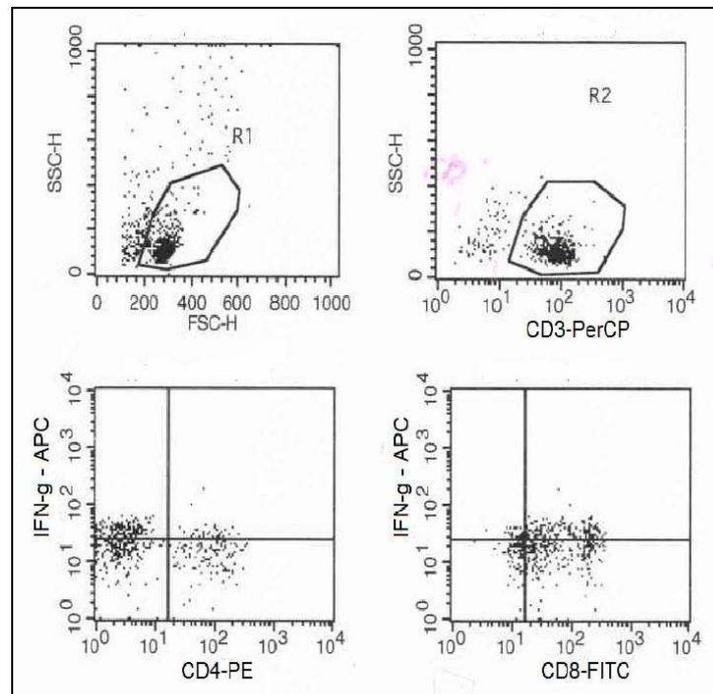

Figure 1(b). Flow cytometer dot graphs indicating gating strategy for CD3+ T cells (R1 and R2) and separation of parasite specific CD4+ and CD8+ T cells involved in the production of IFN-γ (upper left compartment for all dot graphs) from PBMCs obtained from animals following immunizations. Animals were immunized at three time points with sonicate Ag plus monophosphoryl lipid A (MPL+Ag). Peripheral blood mononuclear cells (PBMCs) harvested 21 days after the third immunization were stimulated *in vitro* with *Leishmania donovani* antigen before cells were stained for measurement of interferon gamma-producing CD4+ and CD8+ T cell populations in flow cytometer. Data shown indicate dots representing individual CD4 and CD8 T cells measured from PBMCs from experimental animal groups.
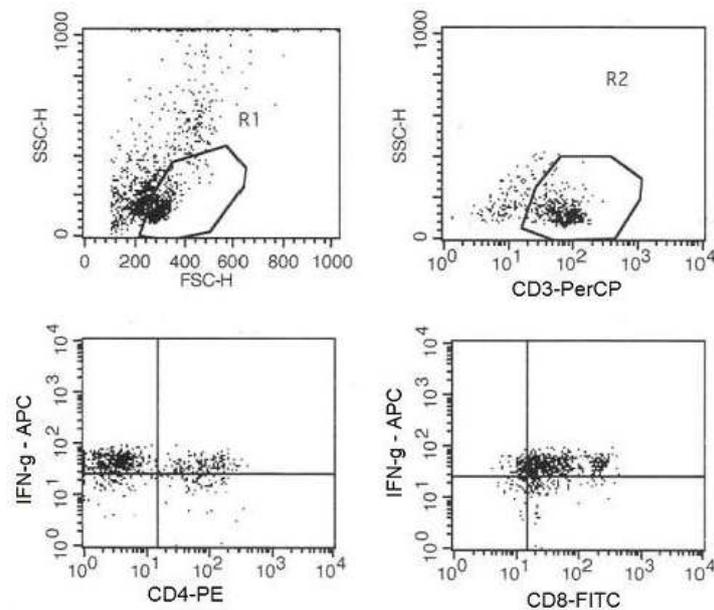
Figure 1(c). Flow cytometer dot graphs indicating gating strategy for CD3+ T cells (R1 and R2) and separation of parasite specific CD4+ and CD8+ T cells involved in the production of IFN-γ (upper left compartment for all dot graphs) from PBMCs obtained from animals following immunizations. Animals were immunized at three time points with sonicate Ag plus montanide ISA 720 (MISA+Ag). Peripheral blood mononuclear cells (PBMCs) harvested 21 days after the third immunization were stimulated *in vitro* with *Leishmania donovani* antigen before cells were stained for measurement of interferon gamma-producing CD4+ and CD8+ T cell populations in flow cytometer. Data shown indicate dots representing individual CD4 and CD8 T cells measured from PBMCs from experimental animal groups
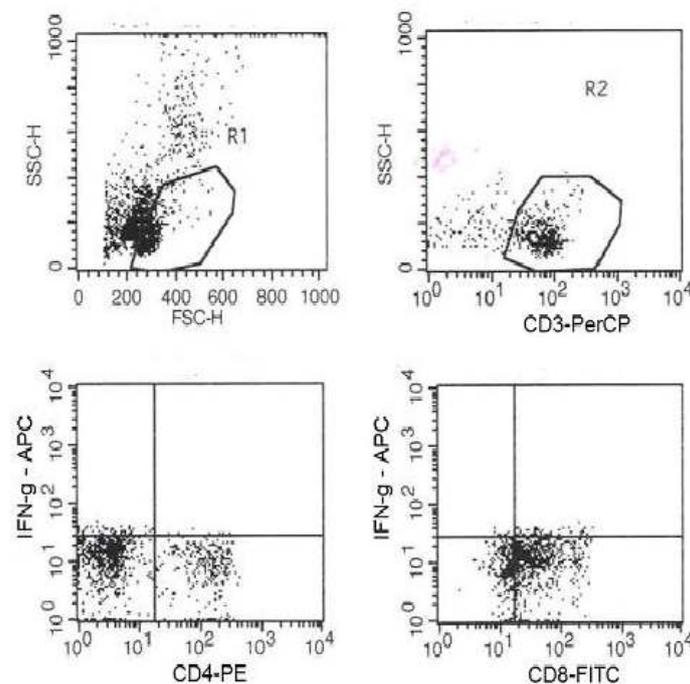

Figure 1(d). Flow cytometer dot graphs indicating gating strategy for CD3+ T cells (R1 and R2) and separation of parasite specific CD4+ and CD8+ T cells involved in the production of IFN-γ (upper left compartment for all dot graphs) from PBMCs obtained from animals following immunizations. Animals were immunized at three time points with sonicate Ag alone (Ag). Peripheral blood mononuclear cells (PBMCs) harvested 21 days after the third immunization were stimulated *in vitro* with *Leishmania donovani* antigen before cells were stained for measurement of interferon gamma-producing CD4+ and CD8+ T cell populations in flow cytometer. Data shown indicate dots representing individual CD4 and CD8 T cells measured from PBMCs from experimental animal groups
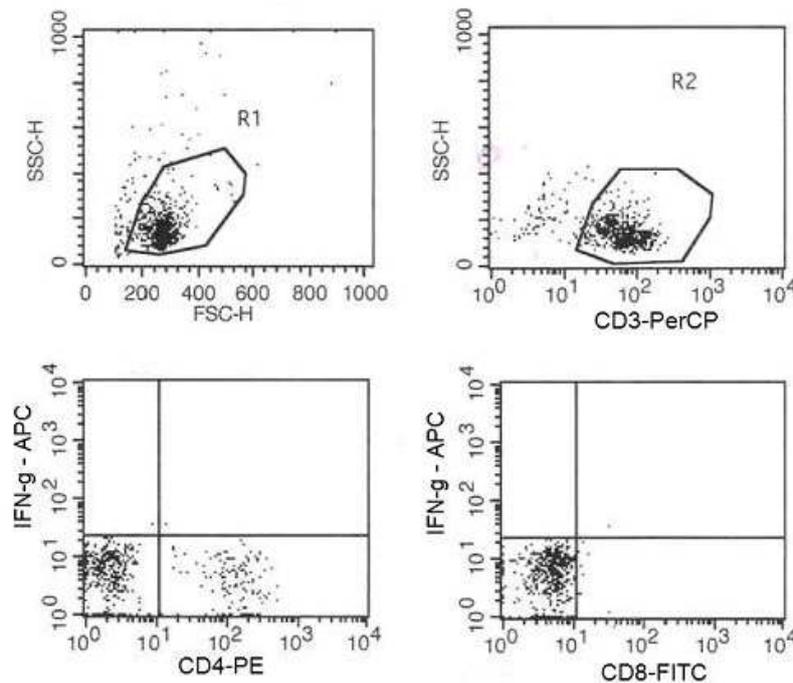
Figure 1(e). Flow cytometer dot graphs indicating gating strategy for CD3+ T cells (R1 and R2) and separation of parasite specific CD4+ and CD8+ T cells involved in the production of IFN-γ (upper left compartment for all dot graphs) from PBMCs obtained from animals following immunizations. This figure indicate the control (unvaccinated) group. Peripheral blood mononuclear cells (PBMCs) harvested 21 days after the third immunization were stimulated *in vitro* with *Leishmania donovani* antigen before cells were stained for measurement of interferon gamma-producing CD4+ and CD8+ T cell populations in flow cytometer. Data shown indicate dots representing individual CD4 and CD8 T cells measured from PBMCs from experimental animal groups.
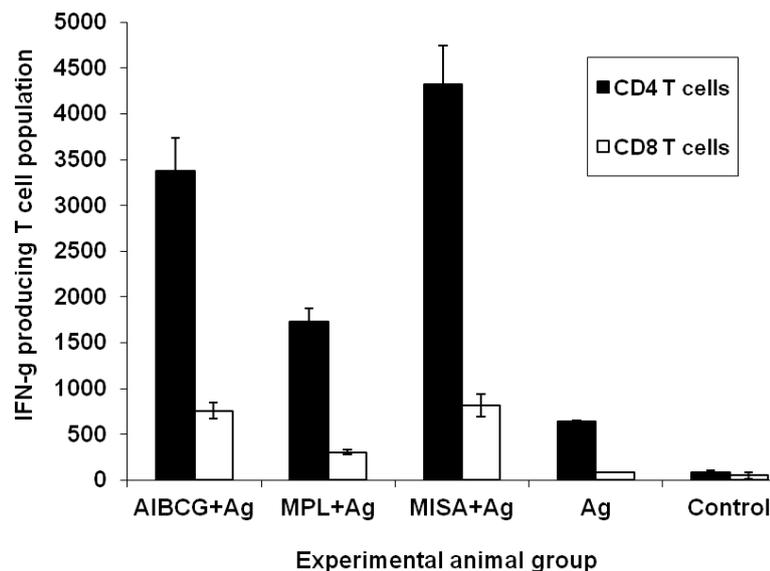

Figure 2. *Leishmania donovani* specific CD4+ and CD8+ T cell numbers involved in the production of IFN-γ. Animals were immunized at three time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AlBCG+Ag), monophosphoryl lipid A (MPLA+Ag) or montanide ISA 720 V (MISA+Ag). Peripheral blood mononuclear cells (PBMCs) harvested 21 days after the third immunization were stimulated *in vitro* with *Leishmania donovani* antigen and cells stained for measurement of interferon gamma-producing CD4+ and CD8+ T cell numbers in flow cytometer. Data shown indicate mean numbers of CD4 or CD8 ± SD in each group.
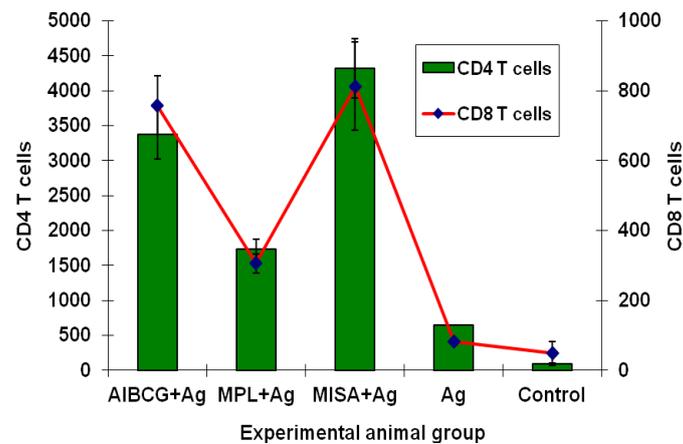
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Figure 3. Association between numbers of *L. donovani* parasite specific CD4^{+} and CD8^{+} T cells involved in the production of IFN-γ. Animals were immunized at three time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AlBCG+Ag), monophosphoryl lipid A (MPLA+Ag) or montanide ISA 720 V (MISA+Ag) and peripheral blood mononuclear cells (PBMCs) harvested 21 days after the third immunization were stimulated *in vitro* with *Leishmania donovani* antigen before staining cells for measurement of actual numbers of specific CD4^{+} and CD8^{+} T cells involved in the production of IFN-γ. Cells were acquired using BD FACSCalibur. Data shown indicate relationship between mean numbers of CD4 or CD8 ± SD in each group

In the study carried out by Coler et al. (2007), the monophosphoryl lipid A was in a stable emulsion while our present study used monophosphoryl lipid A formulated in water. It may appear that, the aqueous formulation of monophosphoryl lipid A may be considered less effective than the emulsion-based formulation (Reed et al., 2009). However, in a different study using pneumocococcal-CRM197 conjugate vaccine in health toddlers, MPLA in aqueous formulation was associated with high cellular immune responses (Vernacchio et al., 2002). The difference in the toddler studies with our results may be attributable to batch to batch disparities or due to the antigens used. The almost baseline IFN-γ-inducing CD4 or CD8 T cells numbers associated with vaccinations with sonicate antigen alone indicates the importance of the adjuvants used in this study as inducers of Th1 immune responses.

On the basis of the results presented in the present study, we conclude that, immunization with montanide ISA 720 as an adjuvant is associated with high levels of CD4 and CD8 T lymphocytes involved in the production of IFN-γ crucial for effective control against visceral leishmaniasis. Furthermore, this response is higher than that indicated following immunization with either alum-BCG or monophosphoryl lipid A. Montanide ISA 720 has the additional advantage that it has been used in human vaccine trials (Toledo et al., 2001; Oliveira et al., 2005; Pierce et al., 2010; Herrera et al., 2011) and is strongly recommended by the manufacturer for clinical trials in humans (Gomez et al., 1999). The adjuvant has also been shown to have good safety levels as well as strong cellular immune responses associated with high efficacy levels (Mutiso et al., 2012a). The vervet monkey model has been well documented by us (Olobo et al., 1992; Gicheru et al., 1995; Gicheru et al., 1997; Gicheru et al., 2001; Masina et al., 2003) as a suitable animal model for human leishmaniasis vaccine studies and therefore these results may be evaluated in humans.

Acknowledgements

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