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Immune response of BALB/c mice against an experimental vaccine of Alum precipitated autoclaved *Leishmania major* (Alum-ALM) mixed with BCG or *Mycobacterium vaccae*

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Abstract. Immune response in BALB/c mice immunized 3 times with different doses (50 µg or 200 µg of protein) of Alum precipitated autoclaved *Leishmania major* (Alum-ALM) mixed with either BCG (1 x10^7 CFU) or different doses of killed *Mycobacterium vaccae* (1 x10^6, 1 x10^7) was assessed. Mice immunized with low dose of Alum-ALM mixed with either BCG or low *M. vaccae* showed a significantly higher IFN-γ production and a lower IL-4 level and a significantly lower parasite burden compared to the control PBS injected group. It seems that immunization with a low dose of Alum-ALM mixed with an adjuvant induces a Th1 type of immune response in susceptible BALB/c mice.

INTRODUCTION

Leishmaniasis represents a group of diseases caused by at least 20 different species of *Leishmania* parasite. Vector and reservoir control measures are costly and not always effective (Murray *et al.*, 2005). Resistance to traditional chemotherapeutic agents is beginning to emerge in endemic areas of VL (Croft *et al.*, 2006) and CL (Palacios *et al.*, 2001; Hadighi *et al.*, 2006; Rojas *et al.*, 2006). It seems that the most practical approach to control leishmaniasis is to develop an affordable vaccine (Khamesipour *et al.*, 2006). Several clinical trials of single and multiple injections of autoclaved *Leishmania major* (ALM) against CL and VL showed a limited efficacy (Sharifi *et al.*, 1998; Momeni *et al.*, 1999; Khalil *et al.*, 2000; Modabber, 2000; Khamesipour *et al.*, 2006). To enhance the immunogenicity of the vaccine, ALM was adsorbed to alum (Aluminum hydroxide), and a new formulation of Alum-ALM was produced. A single injection of Alum-ALM plus IL-12 induced protection against CL in Rhesus monkeys (Kenney *et al.*, 1999). Likewise, Alum-ALM mixed with BCG protected Langur monkeys against VL (Misra *et al.*, 2001). Alum-ALM mixed with BCG showed to be safe and highly immunogenic in phase 1-2 clinical trials in Sudan (Kamil *et al.*, 2003; Khalil *et al.*, 2006; Musa *et al.*, 2008); in addition, promising results in clinical trials of immunotherapy in combination with chemotherapy in Post Kala-azar Dermal Leishmaniasis (PKDL) are seen (Musa *et al.*, 2005; Musa *et al.*, 2008). BCG is the only adjuvant used with first generation *Leishmania* vaccine, new approach in *Leishmania* vaccine development requires using new adjuvant(s) (Modabber, 2000; Khamesipour *et al.*, 2006). BCG (Bacille Calmette-Guérin) showed to induce a Th1 type of response by triggering IL-12 (Matsumoto *et al.*, 1997) and production of IFN-γ, TNF-α and nitric oxide (Marshall *et al.*, 1997; Misra *et al.*, 2001; Kamil *et al.*, 2003)

and was used as an adjuvant or comparator in vaccine studies against leishmaniasis (Convit *et al.*, 1989; Cabrera *et al.*, 2000; Mohebali *et al.*, 2004; Khamesipour *et al.*, 2006).

BCG is a live attenuated preparation and requires strict cold-chain delivery and induces a prolonged lesion and scar in addition to other reported adverse effects (Torisu *et al.*, 1978; Bahar *et al.*, 1996). *Mycobacterium vaccae*, in contrast to BCG is used as a heat-killed preparation in immunotherapy of human diseases (Mwinga *et al.*, 2002; Dlugovitzky *et al.*, 2006; Netto *et al.*, 2006) and some infections in animal models (Hrouda *et al.*, 1998; Ozdemir *et al.*, 2003). Previously it was shown that *M. vaccae* mixed with ALM induce a partial protection against challenge with *L. major* infection in BALB/c and C57BL/6 mice (Keshavarz *et al.*, 2008).

In the current study, the immune response of susceptible BALB/c mice immunized with different doses of first generation whole killed *Leishmania* vaccine (Alum-ALM) mixed with either BCG or *M. vaccae* was evaluated. Alum-ALM used in this experiment is the same preparation which has been used in human clinical trials.

MATERIALS AND METHODS

Mice

Female BALB/c mice 8-10 week-old were purchased from Pasture Institute of Iran and maintained in animal house of the Center and fed with tap water and laboratory pellet chow. Animals were housed in a colony room 12/12 h light/dark cycle at 21°C with free access to water and food. Experiments were carried out according to Tehran University of Medical Sciences, Ethical Committee Acts.

Leishmania lysate preparation

Leishmania major strain (MRHO/IR/75/ER) was used in this study is the same isolate which has been used in mass leishmanization, preparation of Old World *Leishmania* vaccine and Leishmanin (Khamesipour *et al.*, 2006). *Leishmania* lysate was prepared from *L. major* harvested at stationary phase, washed 3 times with PBS (pH 7.2), and then the pellet of promastigotes was freeze-thawed 10 times and microscopically checked to ensure the uniform lysis. Protein concentration of the lysate was determined using Bradford method (Bradford, 1976).

Immunization schedule and challenge

Nine groups of BALB/c mice, 10 mice per group, were immunized subcutaneously (SC) on the back 3 times, 21 days interval, with 2 doses of Alum-ALM (50 µg or 200 µg protein) mixed with either *M. vaccae* (2 doses; 1 x 10^6 or 1 x 10^7) or BCG (1 x 10^7 CFU equals to 400 µg protein) in a volume of 100 µL. A group of mice was injected with PBS and used as a control of immunization (Table 1). The site of SC immunization was monitored and nodule formation was recorded for each group of mice.

At day 35, after the last booster injection, the immunized and PBS control groups of mice were challenged SC into the left footpad with 50 µL of 1 x 10^6 *L. major* promastigotes harvested at stationary phase (day 4-5 of culture). Footpad swellings were monitored weekly, the thickness measured using a caliper and recorded for every injected mouse.

The number of mice that succumbed to leishmaniasis was recorded for each group of injected mice.

Serum antibody titration

Blood samples were collected from different groups of mice at week 3 after the booster injection and sera of each group were pooled. Sera were used for analysis of anti-*Leishmania* IgG1 and IgG2a subclasses using ELISA method (mAbs from Sigma, USA) (Badiee *et al.*, 2007). OD of samples was read at 450 nm and reported as the ratio of IgG1 to IgG2a.

Spleen and lymph node cells culture and cytokine assay

At day 35 after the last booster injection, 3-4 mice from each group (same number of mice as in other studies: Su *et al.*, 2000; Daneshvar *et al.*, 2003) were sacrificed and the spleen

Table 1. The details of components used for immunization of BALB/c mice

Group	Components used for immunization
1	Alum-ALM (H: 200 µg) + M.v (H: 1 x 10^7)
2	Alum-ALM (H: 200 µg) + M.v (L: 1 x 10^6)
3	Alum-ALM (H: 200 µg)
4	Alum-ALM (H: 200 µg) + BCG (1 x 10^7 CFU = 400 µg protein)
5	Alum-ALM (L: 50 µg) + M.v (H: 1 x 10^7)
6	Alum-ALM (L: 50 µg) + M.v (L: 1 x 10^6)
7	Alum-ALM (L: 50 µg)
8	Alum-ALM (L: 50 µg) + BCG (1 x 10^7 CFU = 400 µg protein)
9	Phosphate Buffered Saline (PBS)

H = High dose, L = Low dose

and draining popliteal, inguinal, superficial inguinal, axillary and mesenteric lymph nodes were removed, a single-cell suspension from pooled cells of individual mice was prepared in triplicate (2 x 10^5 cells/well) and cultured in RPMI 1640 (Sigma, USA) supplemented with 15% FCS (Gibco, USA), 2 mM L-glutamin, 0.05 mM 2-mercaptoethanol, 100 U penicillin/ml and 100 µg streptomycin/ml in a final volume of 200 µL per well in U-bottom 96 well plates (Nunc, Denmark). Cell cultures were stimulated either with 1 µg/ml of Concanavalin A (Con-A; Sigma, USA) or 25 µg/ml of *L. major* lysate, or unstimulated as control. The plates were incubated at 37°C and 10% CO_2. Supernatants were harvested at 72 hours and cytokines (IL-4 and IFN-γ) were titrated by double sandwich ELISA method using specific monoclonal antibodies (Biosource, USA) according to the manufacturer's instruction.

Delayed Type Hypersensitivity (DTH)

DTH was assessed in experimental and PBS control mice at day 35 after the last booster injection exactly before parasite burden analysis. *Leishmania major* promastigotes were harvested at day 5 of culture, washed in cold PBS (pH 7.2) and freez-thawed 6 times and resuspended in PBS for inoculation (Alimohammadian *et al.*, 1993). DTH was assessed by SC inoculation of 1x10^7 promastigotes into palm of left hand in a volume of 40 µL and as a control the right palm was injected with 40 µL of PBS. The thickness of injected palms was measured at 24, 48 and 72 hours using a caliper. The data collected at 72 hours were more apparent and were presented.

Evaluation of parasite burden

At week 7 after challenge, number of live parasites in infected draining lymph nodes was quantified by Limiting Dilution Analysis (LDA) (Titus *et al.*, 1985). Briefly, each pooled cells of lymph nodes from individual mouse was weighed before homogenization and then different serial dilutions of lymph node cells were prepared in Schneider's medium (Sigma, USA) supplemented with 10% FCS, 2% normal human urine, 1% penicillin/streptomycin solution, 1% of 1 M HEPES solution (pH 7.3) and 0.1% of 50 mg gentamicin/ml solution and distributed in 96-well flat-bottom microtiter plates (Nunc, Denmark). Number of viable parasite per mg of tissue was determined from the highest dilution at which growing promastigotes were identified after 10 days of incubation at 25°C with humidity using ELIDA software (Taswell, 1987).

Statistics

Statistical analysis was performed by using SPSS 11.5 software (SPSS Inc., USA). Significance of differences between different groups of mice was determined by repeated measure or one way ANOVA test or *t*-test. *P* value of < 0.05 was considered as significant.

RESULTS

Safety analysis

Nodule formation at the site of immunization was checked as a safety criterion. The size of nodule in group of mice that received BCG was larger than the other groups (data not shown). The healing process of nodules was monitored and showed that Alum-ALM mixed with BCG leads to ulceration of the nodules which healed eventually.

Footpad swelling, death rate, and parasite burden

The results of footpad measurement showed that there was no significant difference in footpad swelling size between immunized and control group of mice during 12 weeks after challenge with *L. major* (except for group 1 after week 9 which shows significant increase comparing other groups with $P<0.05$) (Figure 1). The number of mice succumbed to leishmaniasis compared between different groups. There was no significant difference between the death rate in different groups up to week 12 after challenge when all the mice were sacrificed and the study was ended due to ethical considerations.

Parasite load of pooled draining lymph nodes of different groups was quantified at 7 weeks post challenge. Parasite burden showed a significantly ($P < 0.05$) lower parasite number in one mouse from either groups of mice received different doses of Alum-ALM mixed with BCG and 1 mouse received a low dose of Alum-ALM plus low dose of *M. vaccae* (Table 2); however, the mean parasite burden of these groups did not show any significant difference comparing with the control group. On the other hand, the mean of parasite burden in mice received high dose of Alum-ALM mixed with high dose of *M. vaccae* was significantly higher than the PBS control group ($P < 0.05$).

DTH response

The results of Leishmanin Skin Test (LST) which was performed on hands of mice showed that groups of mice received Alum-ALM mixed with BCG induced a significantly ($P < 0.05$) stronger LST response than PBS control group (Figure 2). No significant difference was seen between other groups

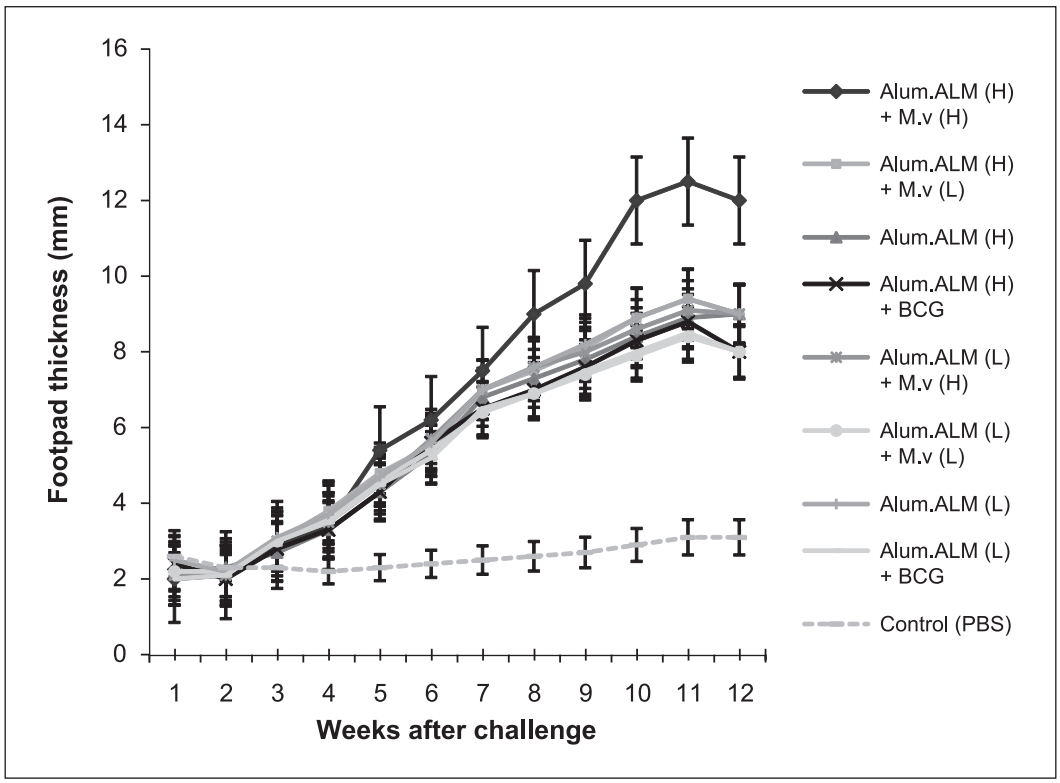

Figure 1. Footpad swelling after challenge with live *L. major* in immunized BALB/c mice.
At day 35, after the last booster injection, the immunized and PBS control groups of mice were challenged SC into the left footpad with 50 µL of 1 x 10^6 stationary phase *L. major* promastigotes. Footpad thickness was monitored weekly, measured using a caliper and recorded for every injected mouse.

Table 2. Parasite burden in lymph nodes of BALB/c mice immunized with different components

Group	Vaccine	Mean No. of parasite/mgtissue ± SE
1	Alum.ALM (H) + M.v (H)	17966.6 ± 4481.5x 10^{2*}
2	Alum.ALM (H) + M.v (L)	4433.2 ± 1823.8x 10^{2}
3	Alum.ALM (H)	5960.2 ± 2540.4x 10^{2}
4	Alum.ALM (H) + BCG	2705.2 ± 1181.8x 10^{2} $^{\$}$
5	Alum.ALM (L) + M.v (H)	4311.1 ± 1877.0x 10^{2}
6	Alum.ALM (L) + M.v (L)	3656.0 ± 1487.0x 10^{2} $^{\&}$
7	Alum.ALM (L)	4785.5 ± 1922.0x 10^{2}
8	Alum.ALM (L) + BCG	3011.6 ± 1256.8x 10^{2} §
9	Control (PBS)	5591.2 ± 2334.0x 10^{2}

$^{\$}$ One mouse showed a lower number of 905.5±433.0 x 10^{2} parasite/mg tissue
$^{\&}$ One mouse showed a lower number of 807.0±485.0 x 10^{2} parasite/mg tissue
§ One mouse showed a lower number of 1009.5±518.0 x 10^{2} parasite/mg tissue
* Shows statistically significant difference comparing with PBS injected control group.

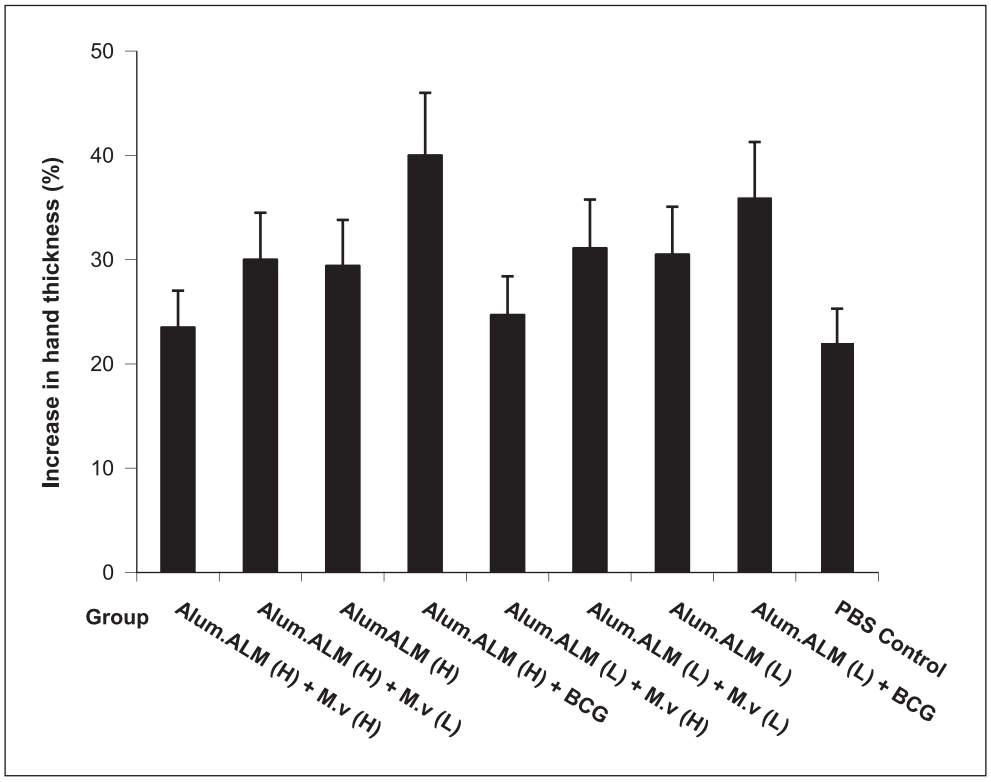

Figure 2. DTH response to *Leishmania* antigens (LST) in BALB/c mice immunized with different components.
At day 35 after the last booster injection 1x10^{7} freeze/thawed *L. major* promastigotes injected subcutaneously on the left palm in a volume of 40 µL and as a control the right palm was injected with 40 µL of PBS. DTH was assessed at 72 hrs using a caliper.

of mice. A weaker but not significant LST response was seen in group of mice received high dose of Alum-ALM mixed with high dose of *M. vaccae*.

Cytokine production analysis

Cytokine titration (IL-4 and IFN-γ) was done on supernatants of lymph node cells and spleen cells co-cultured with *Leishmania* lysate antigen or mitogen (Figure 3 and Figure 4). The results showed a significantly ($P < 0.001$) higher IFN-γ production in groups of mice received different doses of Alum-ALM mixed with BCG compared to the PBS injected control group (Figure 4a-b). A significantly ($P < 0.001$) higher IFN-γ production was seen in group of mice received low dose of Alum-ALM mixed with different doses of *M. vaccae* in comparison with the group of mice received PBS or group of mice received low dose of Alum-ALM alone. Immunization of mice with high dose of Alum-ALM mixed with high dose of *M. vaccae* induced a significantly ($P < 0.001$) higher IL-4 level compared to PBS control group (Figure 3a-b). The amount of IL-4 in group of mice received low dose of Alum-ALM alone was significantly ($P < 0.05$) higher than the PBS control group, the amount of IL-4 in the former group (high dose of Alum-ALM + high dose of *M. vaccae*) was significantly higher than the latter group received low dose of Alum-ALM alone ($P < 0.05$).

Antibody assay

Study of humoral immune response indicated that IgG1/IgG2a antibody titer was significantly ($P < 0.05$) lower in group of mice received low dose of Alum-ALM mixed with BCG or *M. vaccae* (Figure 5). On the other hand, IgG1/IgG2a antibody ratio was significantly ($P < 0.05$) higher in groups of mice received high Alum-ALM alone or mixed with *M. vaccae*.

DISCUSSION

It is at least theoretically possible to develop an effective vaccine against different forms of leishmaniasis; recovery of CL induces a long lasting protection and generates strong immune response indicated by *in vivo* LST conversion and *in vitro* high IFN-γ production (Mahmoodi *et al.*, 2003). Leishmanization (LZ) induces strong immune response and protection against further infection (Khamesipour *et al.*, 2005).

In this study the safety and immunogenicity of an experimental *Leishmania* vaccine (Alum-ALM) which is currently in human clinical trials of prophylaxis (Khalil *et al.*, 2006; Khamesipour *et al.*, 2006) and immunotherapy of leishmaniasis (Musa *et al.*, 2008) was assessed. In this study similar to human clinical trials, BCG was used as an adjuvant, and moreover the possibility of using *M. vaccae* as an adjuvant was investigated. BCG is live attenuated and induces an ulcer and scar at the site of inoculation and in addition BCG requires cold chain transportation which is difficult in some endemic foci; instead *M. vaccae* is heat killed and does not need strict cold chain delivery.

Safety analysis of injected preparation showed that although a local reaction such as nodule and ulcer was seen in mice received a new formulation of Alum-ALM mixed with either BCG or *M. vaccae* but the Alum-ALM is tolerable and safe. Most of the nodules induced at the site of vaccination in group of mice received BCG, eventually were ulcerated and then healed. In contrast, nodules at the site of vaccination in groups of mice received *M. vaccae* were not ulcerated. The lesion induced by Alum-ALM mixed with BCG was stronger than the one induced by Alum-ALM mixed with *M. vaccae* or when ALM (without Alum) mixed with BCG (Keshavarz *et al.*, 2008). No other major local adverse reaction related to the absorption of ALM to alum was seen in the immunized animals.

Footpad swelling at the site of challenge and the death rate due to challenge were used as criteria of progression and severity of the disease (Badiee *et al.*, 2007; Keshavarz *et al.*, 2008). In this study, there was no significant difference in death rate and footpad thickness between various groups of mice up to 12 weeks after challenge when the study was terminated due to ethical

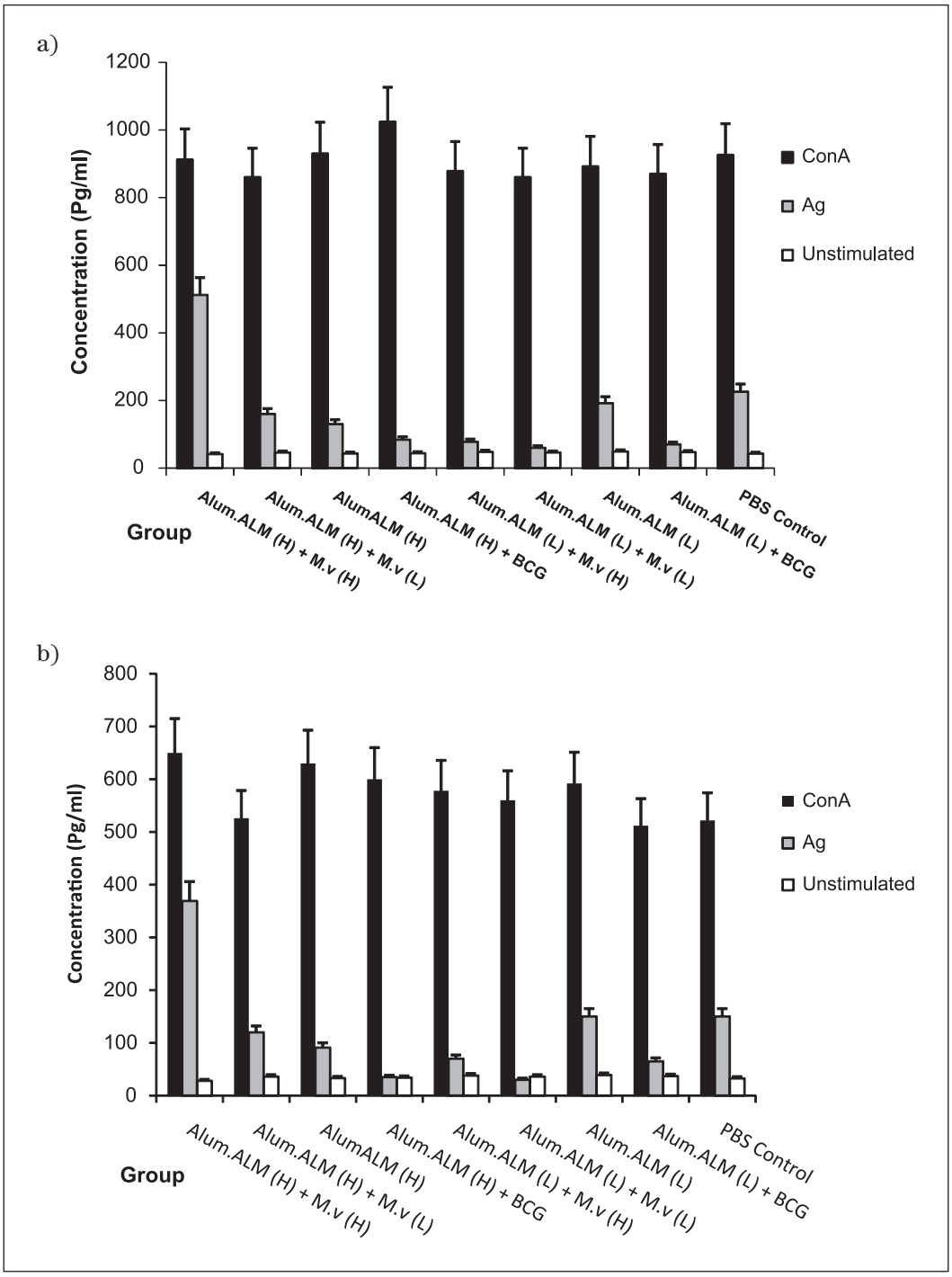

Figure 3(a-b). IL-4 cytokine levels of lymph nodes and spleen cell culture of BALB/c mice immunized with different components.

At day 35 after the last booster injection, a single-cell suspension of the pooled draining lymph nodes and spleen cells from each group was prepared in triplicate (2 x 10^5 cells/well) in complete RPMI 1640 stimulated with 1 µg of Concanavalin A /ml or 25 µg/ml of *L. major* lysate or without stimulation in U-bottom 96 well plates. The plates were incubated at 37°C and 10% CO_2 at humidity. IL-4 was titrated by double sandwich ELISA method using specific monoclonal antibodies using supernatants of 72 hours culture. Data shows the results of lymphnodes (3a) and spleen (3b) cells culture.

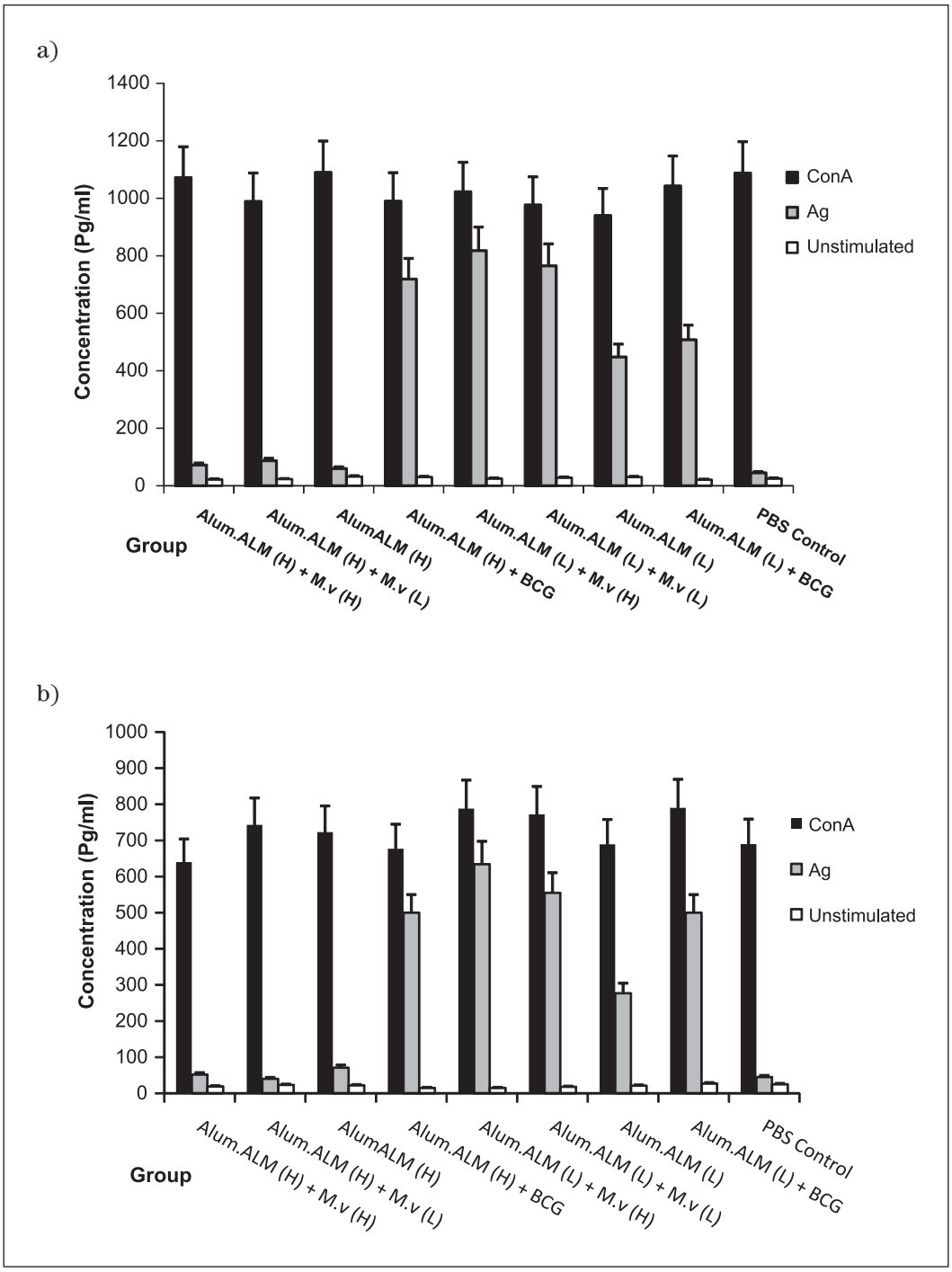

Figure 4(a-b). IFN-γ cytokine levels of lymph nodes and spleen cell culture of BALB/c mice immunized with different components

At day 35 after the last booster injection, a single-cell suspension of the pooled draining lymph nodes and spleen cells from each group was prepared in triplicate (2 x 10^5 cells/well) in complete RPMI 1640 stimulated with 1 µg of Concanavalin A /ml or 25 µg/ml of *L. major* lysate or without stimulation in U-bottom 96 well plates. The plates were incubated at 37°C and 10% CO_2 at humidity. IFN-γ was titrated by double sandwich ELISA method using specific monoclonal antibodies using supernatants of 72 hours culture. Data shows the results of lymphnodes (4a) and spleen (4b) cells culture.

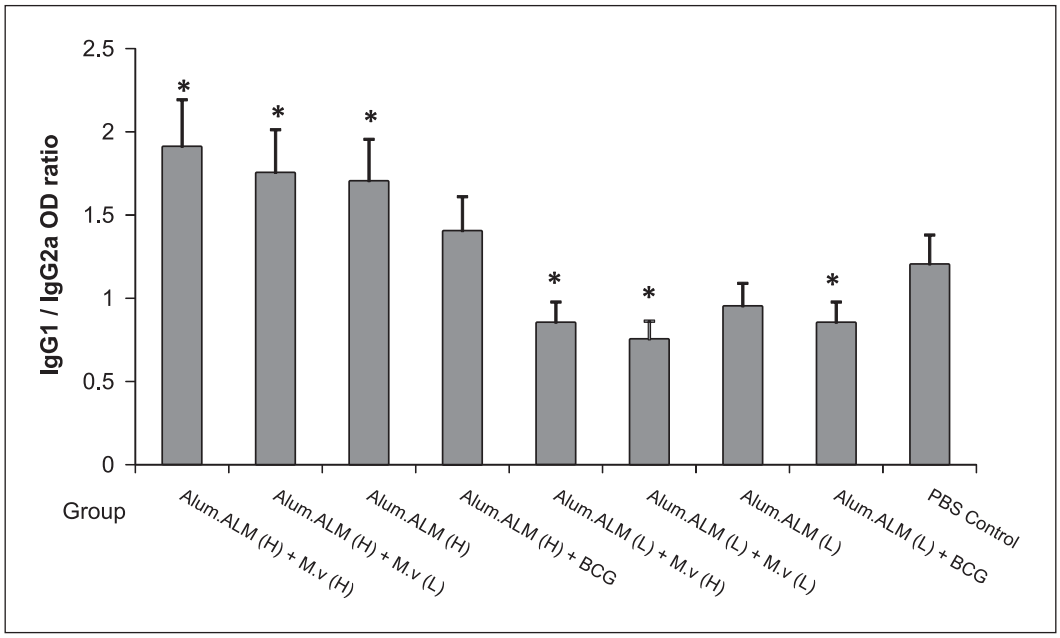

Figure 5. Serum absorbance of IgG1 and IgG2a antibodies in BALB/c mice immunized with different components.
At week 3 after the last booster injection sera from blood of each group of mice were pooled. Anti-*Leishmania* IgG1 and IgG2a subclasses were quantitated using ELISA method. ODs were read on 450 nm and reported as the ratio of IgG1/IgG2a absorbance.
* Shows statistically significant difference comparing with PBS injected control group.

consideration. Induction of solid protection in BALB/c mice is difficult to achieve (Liew *et al.*, 1985). Parasite burden quantification is also a correlation of severity of leishmaniasis (Lima *et al.*, 1997) and therefore in this study is used to estimate the severity of the disease and the rate of possible protection. The results of parasite burden revealed a lower number of parasites in lymph nodes of 1 out of 3 mice in either groups of mice received different doses of Alum-ALM mixed with BCG and in 1 out of 3 mice in group of mice which received a low dose of Alum-ALM mixed with low dose of *M. vaccae*, but overall no significant difference was seen in the parasite burden of the related groups. In other studies comparing lesion size and parasite load, although no significant difference was observed in footpad swelling of different groups of mice but a significant difference was seen in parasite burden (Titus & Ribeiro, 1988; Chakkalath *et al.*, 1995).

In human, *in vivo* leishmanin skin test is a strong correlation of protection (Sharifi *et al.*, 1998; Momeni *et al.*, 1999; Mahmoodi *et al.*, 2003). A significantly ($P < 0.05$) stronger LST response was seen in groups of mice received different doses of Alum-ALM mixed with BCG in comparison with all other groups of mice, but this significant difference was not observed in groups of mice that received Alum-ALM mixed with *M. vaccae*.

IFN-γ is one of the key cytokines responsible for controlling intracellular parasites and role of IFN-γ in the resolution of *Leishmania* infection is described (Heinzel *et al.*, 1991; Scott, 1991). Induction of IFN-γ production in human after BCG inoculation is a subject of controversy (Mostafa *et al.*, 1986; Kemp *et al.*, 1997; Alimohammadian *et al.*, 2002; Mahmoodi *et al.*, 2003). Immunization of mice with *M. vaccae* expressing an antigen of *M. tuberculosis* induced a Th1 type of immune

response showed by IFN-γ production and a high titer of IgG2a (Abou-Zeid *et al.*, 1997). In the current study, at day 35 after the last booster a significantly ($P < 0.001$) higher IFN-γ production in lymph node culture of mice received different doses of Alum-ALM mixed with BCG or low dose of Alum-ALM mixed with *M. vaccae* was seen which is an indication of generation of Th1 type of immune response and might interpret the reason of lower parasite burden in a few mice. In addition a lower IL-4 production which was seen in group of mice immunized with Alum-ALM mixed with BCG or *M. vaccae* is an indication of suppression of Th2 type of response. It was shown by another study that *M. vaccae* significantly inhibited allergic inflammation by suppression of Th2 type of response showed by a lower IL-4 production (Adams *et al.*, 2004).

It is shown that Th1/Th2 related lymphokines control isotype of immunoglobulin which induced in response to microorganisms (Finkelman *et al.*, 1990). A Th1 type response which elicited in administration of a low dose of Alum-ALM mixed with BCG is further showed by a significantly higher ($P < 0.05$) IgG2a/IgG1 ratio.

A significantly higher level of IL-4 and higher titer of IgG1 antibody that was seen in group of mice received high dose of Alum-ALM mixed with high dose of *M. vaccae* might be due to a generation of Th2 type response which is associated with enhanced parasite burden and significantly increase in footpad thickness.

The results of this study showed that Alum-ALM alone or mixed with either BCG or *M. vaccae* is safe and tolerated in mouse model of leishmaniasis. A Th1 type of immune response in mice immunized with Alum-ALM mixed with BCG is induced which coincides with lower parasite burden and stronger LST response. Similarly, low dose of Alum-ALM plus low *M. vaccae* triggered a Th1 type response that caused lower parasite burden in some immunized mice. So, two groups of mice which received BCG mixed with high or low Alum-ALM and 1 group which received low *M. vaccae* mixed with low Alum-ALM is focused.

A significantly stronger LST response, higher production of IFN-γ and lower production of IL-4 was seen in BALB/c mice immunized with Alum-ALM mixed with BCG or low *M. vaccae*. Although this Th1 elicited immune response was not strong enough to completely protect animals against live challenge. BALB/c mouse is not an appropriate model to mimic human leishmaniasis, neither cutaneous nor visceral form but it is widely used due to lack of other options.

Based on this study, BCG mixed with first generation *Leishmania* vaccine induces stronger immune response than *M. vaccae*. The new formulation of Alum-ALM mixed with 1/10 of BCG, is tested with promising results in phase 1-2 clinical trials and clinical trials of immunotherapy in Sudan (Kamil *et al.*, 2003; Musa *et al.*, 2005; Khalil *et al.*, 2006; Musa *et al.*, 2008). Clinical trials of Alum-ALM mixed mixed with BCG followed by leishmanization are currently underway in Iran (Khamesipour *et al.*, unpublished data).

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