

## Studies on the protective efficacy and immunogenicity of Hsp70 and Hsp83 based vaccine formulations in *Leishmania donovani* infected BALB/c mice

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### ABSTRACT

Visceral leishmaniasis, a chronic systemic infection, is the major cause of morbidity and mortality in many parts of world. The current drugs for the treatment of leishmaniasis are toxic, expensive, difficult to administer and becoming ineffective due to the emergence of drug resistance. In the absence of effective treatment, vaccination remains the only hope for control of the disease. We have evaluated the protective efficacy of two heat shock proteins (Hsp70 and Hsp83) in combination with two different adjuvants (MPLA and ALD) in *Leishmania donovani* infected inbred BALB/c mice. The proteins were isolated by SDS-PAGE and the mice were immunized subcutaneously with Hsp70 + Hsp83, Hsp70 + Hsp83 + ALD and Hsp70 + Hsp83 + MPLA. These were challenged with  $10^7$  promastigotes of *L. donovani*. The animals were sacrificed on 30, 60 and 90 days post challenge for the assessment of parasite load and generation of cellular and humoral immune responses. The vaccines induced a strong protective response against experimental visceral leishmaniasis as shown by reduced parasite load in liver of all immunized groups as compared to the infected controls. The vaccines also led to the augmentation of DTH responses, increased levels of IgG2a, IFN- $\gamma$  and IL-2. Both the adjuvants raised significantly the level of protection imparted by the proteins but MPLA was more effective in comparison to ALD.

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### 1. Introduction

Leishmaniasis, a clinically heterogeneous group of diseases, caused by infection with protozoa of the genus *Leishmania*, is one of the world's most important infectious diseases. Infection with *Leishmania* spp. results in a wide range of clinical symptoms that can be classified into three main forms: cutaneous leishmaniasis, muco-cutaneous leishmaniasis and visceral leishmaniasis. *Leishmania* are protozoan parasites shuttling between sand fly vector where they multiply as free promastigotes in the gut lumen, and mammalian host where they proliferate as obligatory intracellular amastigotes in the mononuclear phagocytes (Handman, 1999). When promastigotes are transmitted from the sand fly to a mammalian host, the parasites experience a rapid temperature elevation from 22–28 °C to 33–37 °C. Indeed, it has been shown that exposure of promastigotes to elevated temperatures results in increased synthesis of heat shock proteins (Hsps) (Van der Ploeg et al., 1985). These are highly conserved molecules and are present in sub-cellular compartments in eukaryotes and prokaryotes (Oladiran

and Belosevic, 2009). Under normal physiological conditions, heat shock proteins are expressed at low levels but during stress a vast array of physiological, environmental and pathological stimuli can induce significant up regulation of Hsp mRNAs (Lindquist and Craig, 1988). In contrast to eukaryotes where the regulation of the Hsp expression is given by transcriptional mechanisms, gene expression in trypanosomatids relies highly on parasite-specific mechanisms involving polycistronic transcription and transsplicing. Expression of the major heat shock proteins is constitutive, even if heat shock may induce a transient synthesis, which has been shown to be regulated exclusively at the posttranscriptional level (Morales et al., 2010). Both the promastigotes and amastigotes contain transcripts which are specific for Hsp's 70 and 83 (Van der Ploeg et al., 1985).

In an effort to find new vaccine candidates against leishmaniasis, we focused on two heat shock proteins (Hsp70 and Hsp83) which have previously been reported to induce a strong cell mediated and humoral immune response in various infectious diseases. The Hsp70 has been found to be most inducible by stress (Moore et al., 1989) and is a major polypeptide antigen in many autoimmune diseases (Engman et al., 1990). It is a potent activator of innate immunity and aberrant expression of it in certain organs promotes immunopathology. The functions of the Hsp70 have recently been extended to their participation in the immunological defense against infectious diseases and cancers (Srivastava et al., 1998) and it is suggested that it might have immunotherapeutic potential as

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Hsp70 purified from malignant and virally infected cells can transfer and deliver antigenic peptides to antigen-presenting cells to elicit peptide specific immunity. A Th1-type of response was potentiated with immunizations of Hsp70 of *Leishmania infantum* (Rico et al., 1999). Hsp70 consists of an NH<sub>2</sub>-terminal nucleotide (ATPase) domain of 44 kDa and a COOH-terminal 25-kDa domain that binds peptide or polypeptide substrate (Becker et al., 2002). In its ATP-bound state, Hsp70 binds and releases peptide rapidly, whereas after hydrolysis, in the ADP state, bound peptide is held tightly (Flynn et al., 1989). The B and T cell epitopes of Hsp70 and the Th1-like expression pattern elicited *in vitro* by this molecule in canine PBMC suggest that Hsp70 is immunogenic (Carrillo et al., 2008). The conservation of epitopes among *Leishmania* species and the capacity of Hsp70 to generate a specific response against *Leishmania* in distinct hosts (human patients and dogs) and different clinical manifestations (cutaneous, muco-cutaneous and visceral) show the potential of Hsp70 as a candidate for vaccine trials (Rafati et al., 2007). The immunization of mice with Hsp70 produced a Th1-type immune response (Rico et al., 1999; Morell et al., 2006) and recently, Hsp70 has been found to enhance the immunogenicity of gp63 based protein and DNA vaccines against *Leishmania donovani* in mice (Kaur et al., 2011; Sachdeva et al., 2009). The Hsp83 in trypanosomatid parasites (trypanosomes and *Leishmania*) (Dragon et al., 1987; Shapira and Pedraza, 1990) share high identity to mammalian Hsp90 (Moore et al., 1989). Hsp83 also has been shown to be an immunodominant antigen recognized by sera from diffuse cutaneous leishmaniasis patients (Skeiky et al., 1997). In an earlier study, Hsp83 of *L. infantum* has been found to be useful for serodiagnostic assays for canine leishmaniasis (Angel et al., 1996). Hsp83 of *L. infantum* when physically linked to the Maltose Binding Protein (MBP) causes an immunostimulatory effect (Rico et al., 1999). It elicits an increased secretion of IgG2a and IgG1 isotypes (Echeverria et al., 2001).

Therefore in the current study, the protective efficacy of two heat shock proteins, that is, Hsp70 and Hsp83 of *L. donovani* in combination with different adjuvants (MPLA and ALD) was evaluated in BALB/c mice.

## 2. Materials and methods

### 2.1. Parasite strain

*L. donovani* strain MHOM/IN/80/Dd8, originally obtained from London School of Tropical Medicine and Hygiene, London, was used for the present study and maintained *in vitro* at 22 ± 1 °C in RPMI-1640 with 10% Foetal Calf Serum.

### 2.2. Animals

Inbred BALB/c mice of either sex, weighing 20–25 g, were obtained from the Central Animal House of Panjab University, Chandigarh. They were kept in germ free environment and fed with water and mouse feed *ad libitum*.

### 2.3. Ethical clearance

The ethical clearance for conducting the experiments was obtained from the Institutional Animal Ethics Committee, Panjab University, Chandigarh, India.

### 2.4. Identification and electroelution of proteins

The parasite proteins were run on SDS-PAGE (miniVE vertical electrophoresis system, Amersham, USA) by the method of Laemmli (1970). The proteins were also localized by 2D gel electrophoresis in a previous study (Kaur et al., 2011). After staining

and destaining, the gel, the bands of interest were taken in the electrophoresis buffer (0.025 M Tris, 0.192 M glycine, 1% SDS) in the electro-eluter and a constant voltage of 50 V for 5–6 min was applied through the gel (Fig. 1). After elution, the proteins were dialyzed and suspended in PBS (pH 7.2) and were quantified by Lowry's method (Lowry et al., 1951).

### 2.5. Preparation of various vaccine formulations

Based on Hsp70 and Hsp83, three types of vaccine formulations were prepared in the present study: Hsp70+Hsp83 (without any adjuvant), Hsp70+Hsp83+ALD and Hsp70+Hsp83+MPLA (Sigma-Aldrich, USA). The ALD antigen was prepared by autoclaving the parasite suspension (10<sup>8</sup> per ml) at 15 lbs for 30 min and the protein content was quantified (Lowry et al., 1951). The Hsp70+Hsp83+ALD vaccine was prepared by mixing 250 µg of eluted proteins with 2.5 mg of ALD antigen while Hsp70+Hsp83+MPLA vaccine was prepared by the addition of 100 µl solution of MPLA (conc. 10 mg/ml) to 250 µg of the eluted proteins.

### 2.6. Immunization and infection of mice

BALB/c mice were immunized by subcutaneous injections of 10 µg each of Hsp70 and Hsp83 antigen free in PBS and along with different adjuvants. Two injections were given at an interval of two weeks. Animals receiving only PBS served as controls. Two weeks after the booster, the immunized and control mice were challenged intracardially with 10<sup>7</sup> promastigotes.

### 2.7. Assessment of parasite load

Six animals from each group were sacrificed on 30, 60 and 90 days post infection/challenge. Liver of all animals were removed and weighed. Impression smears of liver were made and the parasite load was assessed in terms of Leishman Donovan Units (LDU) by the method of Bradley and Kirkley (1977).

### 2.8. DTH responses to leishmanin

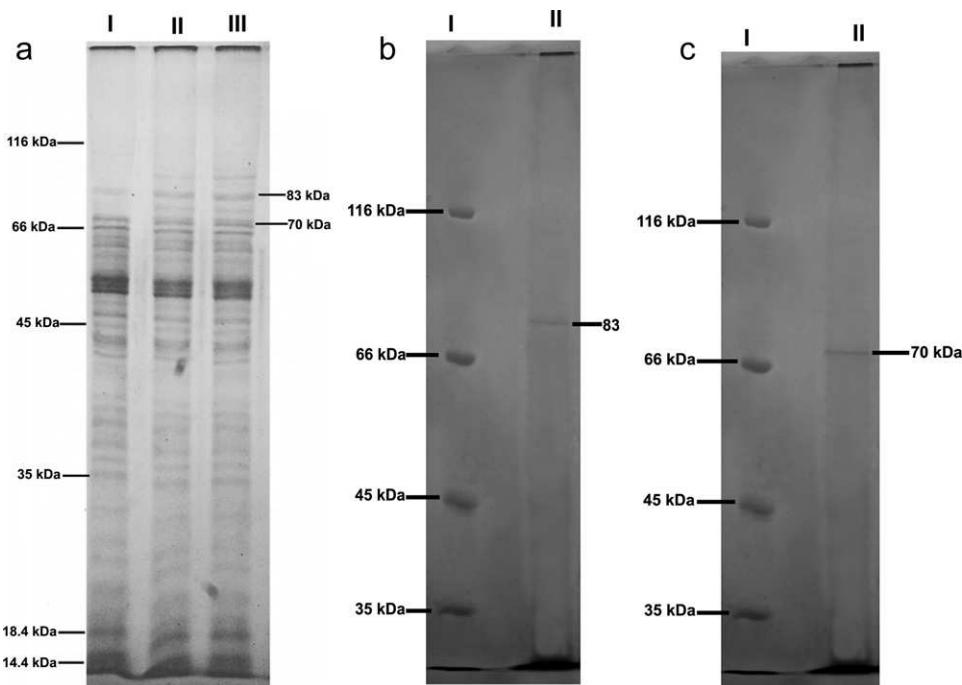
All groups of mice were challenged in right foot pad with a subcutaneous injection of leishmanin (Kaur et al., 2008). After 48 h, the thickness of right and left foot pad was measured using a pair of vernier callipers. Results were expressed as mean ± S.D. of percentage increase in the thickness of the right foot-pad as compared to the left footpad of mice.

### 2.9. ELISA for parasite-specific IgG1 and IgG2a isotypes

The specific serum immunoglobulin G (IgG) isotype antibody response was measured by conventional ELISA using commercially available kits (Bangalore Genei, India). 96-Well ELISA plates were coated with crude antigen. Serum samples were added at twofold serial dilutions, followed by addition of isotype specific HRP-conjugated secondary antibodies (rabbit anti-mouse IgG1 or IgG2a) after which the substrate and chromogen were added and absorbance was read on an ELISA plate reader at 450 nm.

### 2.10. Determination of vaccine-induced cytokine production

The lymphocytes from spleens of mice were cultured in 24 well plates in 1 ml of RPMI-1640 containing 20 mM NaHCO<sub>3</sub>, 10 mM HEPES, 10 U/ml of penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10% FCS. Cells were stimulated with 50 µg/ml of Hsp70+Hsp83. Cells were then incubated at 37 °C for 72 h and



**Fig. 1.** (a) SDS-PAGE profile of *L. donovani* promastigote extracts. (b) SDS-PAGE profile of electroeluted Hsp83 (lane II), the lane I displaying a protein marker. (c) SDS-PAGE profile of electroeluted Hsp70 (lane II), the lane I displaying a protein marker.

supernatant was collected and stored at  $-20^{\circ}\text{C}$ . This was then assayed for IL-2, IL-4, IL-10 and IFN- $\gamma$  by using ELISA kits (BenderMed Systems, Diaclone).

### 2.11. Statistical analysis

All the experiments were performed three times independently. The data comparisons were tested for significance using SPSS software. Two-way comparison between all the groups was performed using Student's *t*-test while the multiple comparisons among different groups were performed by analysis of variance (ANOVA). *P* values of less than 0.05 were considered significant.

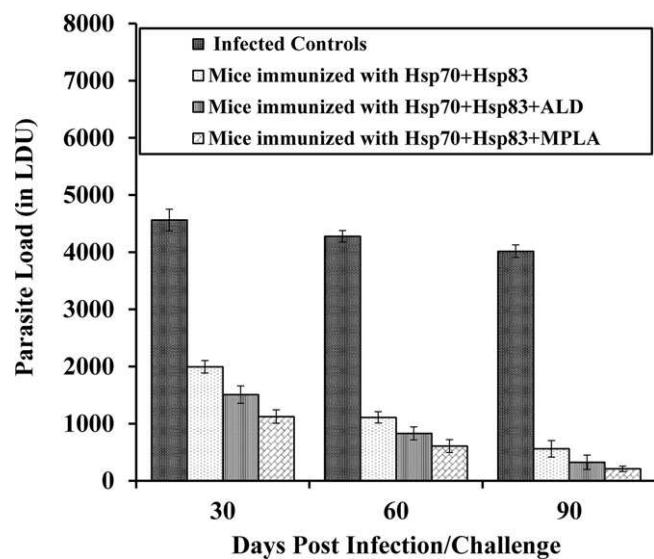
## 3. Results

### 3.1. Parasite load

The liver parasite load was measured in terms of LDU which increased significantly in infected BALB/c mice on different post infection days. Significant differences ( $p < 0.05$ ) in the hepatic parasite burden were detected between the infected controls and immunized animals and the maximum parasite load was observed in infected controls. Animals immunized with the combination of Hsp70 and Hsp83 showed a 56.27–86.06% decline in LDU as compared to the infected controls from 30 to 90 days post infection. However, the addition of adjuvants (MPLA and ALD) to the antigens increased their protective efficacy. Animals immunized with Hsp70 + Hsp83 + MPLA showed a reduction of 67–91.90% in the parasite load while least parasite load was observed in animals immunized with both the antigens in combination with MPLA with a reduction of 75.34–94.78% (Fig. 2).

### 3.2. Delayed type hypersensitivity (DTH) responses

The DTH responses in immunized animals were significantly ( $p < 0.05$ ) higher than the infected control group. Immunization with Hsp70 + Hsp83 along with MPLA antigen induced the

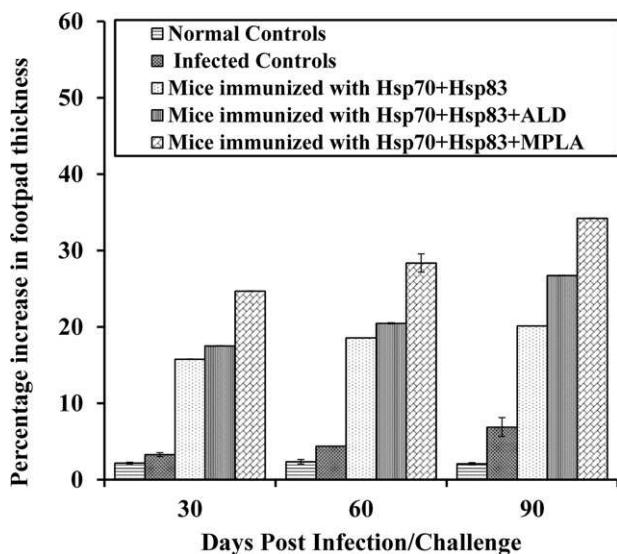


**Fig. 2.** Parasite load – assessed as LDU – in the liver on different days post challenge in BALB/c mice immunized with different vaccine formulations.

highest level (6–8 fold increase as compared to infected controls) of DTH response followed by those immunized with Hsp70 + Hsp83 adjuved with autoclaved antigen (4–6 fold increase) and Hsp70 + Hsp83 (3–5 fold increase), suggesting the generation of cell-mediated immune responses (Fig. 3).

### 3.3. Specific antibody responses

To monitor antigen specific immune responses primed by immunization with heat shock proteins adjuved with two different adjuvants, humoral immune responses were examined in immunized mice. IgG1 and IgG2a antibody responses were evaluated by ELISA using specific anti-mouse isotype antibodies. In the vaccinated group, where the mice received Hsp70 + Hsp83 + MPLA, the



**Fig. 3.** DTH responses on different days post challenge in BALB/c mice immunized with different vaccine formulations.

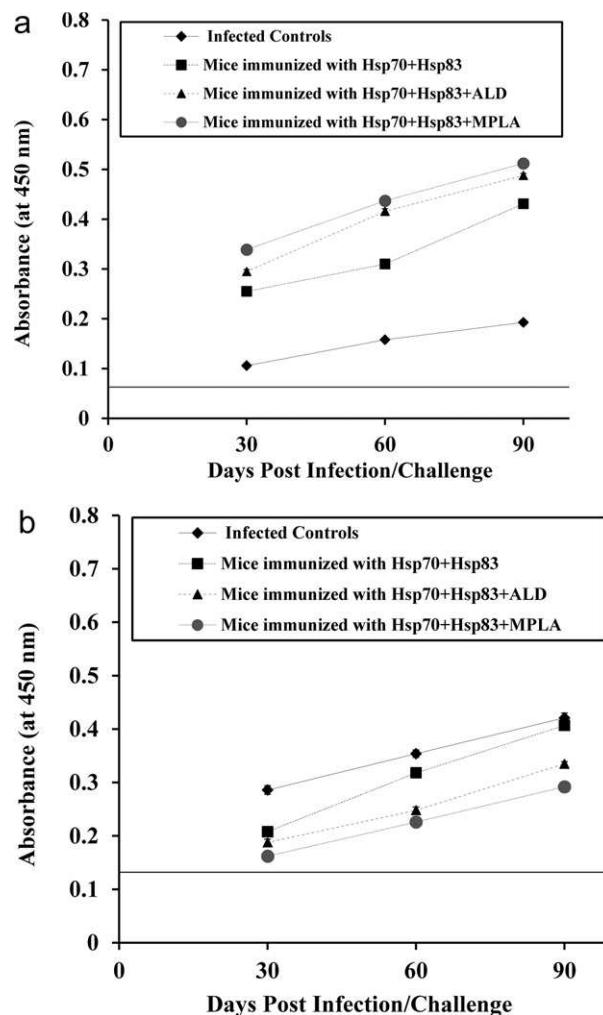
level of IgG2a was significantly higher ( $p < 0.05$ ) than the control group which suggested dominance of Th1 type of immune response. The Hsp70 + Hsp83 + ALD recipient mice also produced higher levels of IgG2a as compared to those mice who received only Hsp70 + Hsp83 (Fig. 4a).

In contrast to the IgG2a levels, the immunized animals revealed significantly ( $p < 0.05$ ) lesser IgG1 levels as compared to the infected controls. The animals immunized with Hsp70 + Hsp83 in combination with adjuvants (ALD or MPLA) revealed significantly ( $p < 0.05$ ) reduced IgG1 levels as compared to those immunized with Hsp70 + Hsp83 (Fig. 4b).

#### 3.4. Cytokine responses

Th1 specific cytokines, that is, IFN- $\gamma$  and IL-2 levels were significantly greater ( $p < 0.05$ ) in the immunized mice as compared to the infected controls. In the animals immunized with Hsp70 + Hsp83, the concentration of IFN- $\gamma$  increased 2.6–4.75 fold from 30 to 90 days post infection. In the animals immunized with Hsp70 + Hsp83 + ALD, the increase in the cytokine concentration was 2.67–5.27 fold. Maximum concentrations of the cytokine was found in animals immunized with the combination of Hsp70 and Hsp83 along with MPLA with a 3.42–6.31 fold increase in the concentration of IFN- $\gamma$  (Fig. 5a). Similarly, IL-2 levels showed an increase of 3.91–4.03, 4.05–4.82 and 6.72–8.81 fold in mice immunized with Hsp70 + Hsp83, Hsp70 + Hsp83 + ALD and Hsp70 + Hsp83 + MPLA, respectively (Fig. 5b). The difference within the groups was significant ( $p < 0.05$ ).

The levels of Th2 regulated cytokines IL-4 and IL-10 were significantly ( $p < 0.05$ ) lesser in immunized animals as compared to the infected controls. These were found to be minimum in the mice immunized with Hsp70 + Hsp83 + MPLA followed by those immunized with Hsp70 + Hsp83 + ALD and then the antigens alone (Fig. 5c and d). In the animals immunized with Hsp70 + Hsp83, the concentration of IL-4 decreased 1.13–1.49 fold from 30 to 90 days post infection. In the animals immunized with Hsp70 + Hsp83 + ALD, the decrease in the cytokine concentration was 1.15–1.32 fold. A minimum concentration of the cytokine was found in animals immunized with the combination of Hsp70 and Hsp83 along with MPLA with a 1.25–1.55 fold decrease in the concentration of IL-4. Similarly, IL-10 levels showed a decrease of 1.23–1.4, 0.75–1.8 and 1.56–2.97 fold in mice immunized with



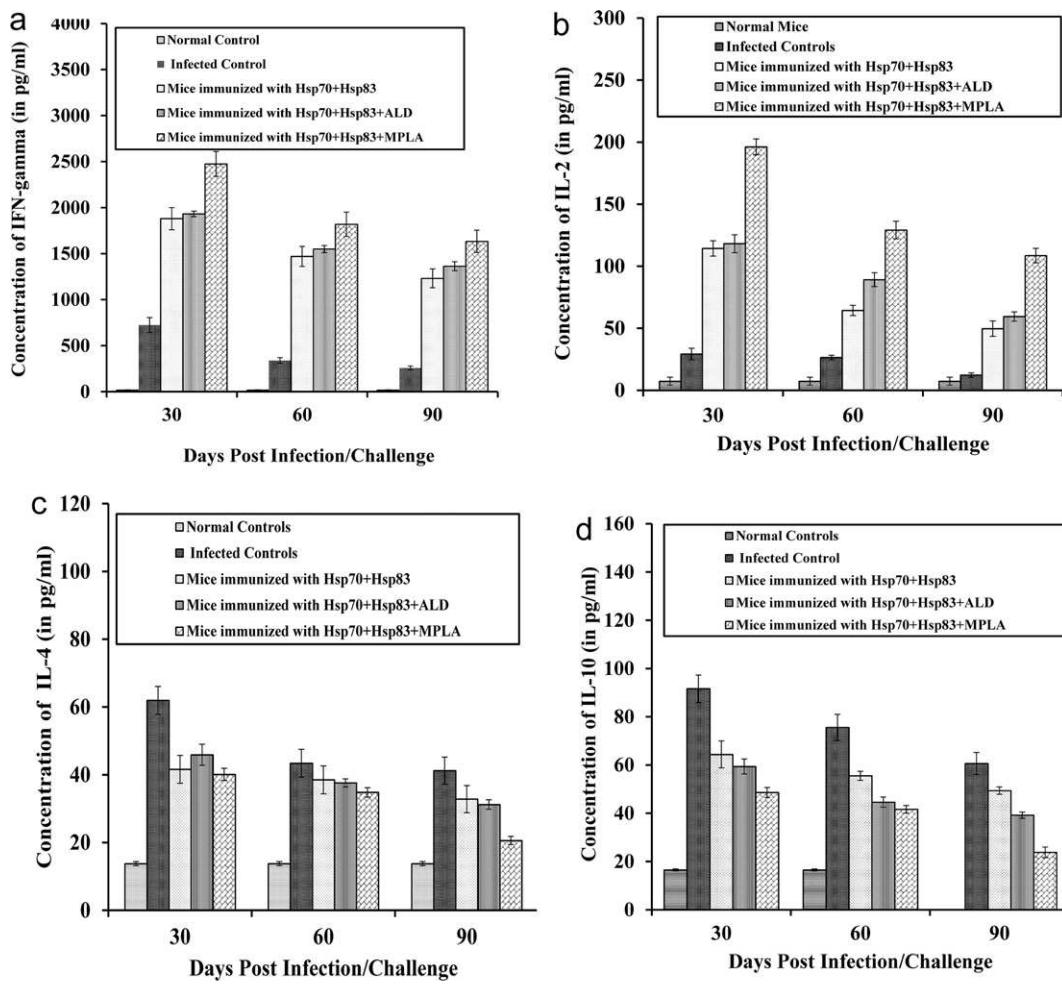
**Fig. 4.** Levels of *Leishmania*-specific antibodies ((a) IgG2a and (b) IgG1) in serum samples on different days post challenge in BALB/c mice immunized with different vaccine formulations.

Hsp70 + Hsp83, Hsp70 + Hsp83 + ALD and Hsp70 + Hsp83 + MPLA, respectively (Fig. 5d).

#### 4. Discussion

The heat shock proteins (also known as heat stress proteins) have mostly been regarded as intracellular molecules that mediate a range of essential housekeeping and cytoprotective functions. However, interest in their role as intracellular signaling molecules has been fuelled by the observations that these molecules can be released and are present in the extracellular environment under physiological conditions. They can elicit cytokine production by, and adhesion molecule expression of, a range of cell types, and they can deliver maturation signals and peptides to antigen presenting cells through receptor-mediated interactions. These functions suggest that heat shock proteins could be immunoregulatory agents with potent and widely applicable therapeutic uses (Pockley, 2003). These permit the utilization of heat shock proteins for the development of a new generation of prophylactic and therapeutic vaccines against infectious diseases (Srivastava and Amato, 2001). Therefore, we tried to test the prophylactic potential of two heat shock proteins (Hsp70 and Hsp83) against murine visceral leishmaniasis.

BALB/c strain of mice was chosen as animal model in the current study because it is the most widely studied model of VL. This is considered to be susceptible wherein the infection progresses



**Fig. 5.** Concentration of cytokines ((a) IFN- $\gamma$ , (b) IL-2, (c) IL-4 and (d) IL-10) in the supernatants of cultures of splenocytes incubated or not with Hsp70 and Hsp83 cocktail. The splenocytes were prepared from spleen collected on different days post challenge in BALB/c mice immunized with different vaccine formulations. These were cultured in presence or not of the Hsp70 and Hsp83 cocktail.

during the first two weeks, and it is then controlled by the host immune response (Murray et al., 1987). Thus, the short duration of the progressive illness and its self-controllable nature poses a limitation to extrapolate this model to generate data on clinical VL (Mukherjee et al., 2003). However, the mouse model is comparable to self-controlled oligosymptomatic cases and therefore is useful for the study of the protective immune response. On the other hand, the better model to study the progressive disease is hamsters infected with *L. donovani* or *Leishmania chagasi* that develop a disease similar to human progressive visceral leishmaniasis with hepatosplenomegaly, hypoalbuminaemia, hypergammaglobulinaemia, and pancytopenia (Rodrigues Junior et al., 1992). The main limitation in the use of this model has been the lack of available immunological reagents (Dea-Ayuela et al., 2007). Therefore, this model is mainly used to study the mechanisms of immunosuppression. Outbred mice are usually resistant to *L. donovani* infection. Most of the mice strains like C57BL/6, CBA/J, C3H and BIOD2 are uniformly resistant and BALB/c mice are consistently susceptible (Handman et al., 1979; Howard et al., 1980).

Thus, we tested the vaccine comprising of two antigens (Hsp70 and Hsp83) along with two different adjuvants, that is, ALD and MPLA. The efficacy of the three vaccine formulations was evaluated in terms of reduction in parasite load and the generation of humoral and cellular immune responses in BALB/c mouse model.

The hepatic parasite load in the immunized and control animals was calculated in terms of LDU (Bradley and Kirkley, 1977) after

1, 2 and 3 months post challenge. The cocktail vaccine comprising of Hsp70 and Hsp83 imparted significant protection against the *L. donovani* infection in BALB/c mice. In our previous study (Kaur et al., 2011), Hsp70 alone reduced the parasite burden to a significant level. Addition of Hsp83 to Hsp70 in the present study raised the level of protection which further increased to a significant extent when used with adjuvants. However, maximum protection was conferred by the vaccine formulation comprising of the two antigens in combination with MPLA. These results are in correspondence with our previous studies where addition of MPLA enhanced the level of protection of 78 kDa antigen in BALB/c mice by 56.5–92% in comparison to 51.3–71% imparted by ALD (Nagill and Kaur, 2010). Similarly, Leish-111f+ MPL-SE induced significant protection against *Leishmania* infection in mice and hamsters with a reduction of 99.6% in parasite load (Skeiky et al., 2002; Coler et al., 2002, 2007).

DTH is a typical *in vivo* manifestation of the cell-mediated immunity and the response can be measured easily and semi-quantitatively (Dannenberg, 1991). In our study, immunization of mice with the combination of heat shock proteins significantly raised the DTH responses in comparison to the infected controls. The addition of adjuvants further increased the DTH responses significantly. MPLA elicited strong and maximum DTH response followed by heat shock proteins plus ALD antigen. In a similar study in dogs from an endemic region of VL, an increase in DTH responses was observed when immunized with a recombinant protein, rLdc-

cys1 from *L. chagasi* (Pinheiro et al., 2009). In a previous study from our laboratory, in comparison to ALD, MPLA was found to be more immunogenic when given with 78 kDa in BALB/c mice as indicated by the enhanced DTH responses (Nagill and Kaur, 2010).

In leishmaniasis, resistance and resolution of the illness has been associated with IgG2a production (Mohammadi et al., 2006) and activation of Th1 cells with secretion of IL-2 and IFN- $\gamma$ . In mice, IL-4 secreted by Th2 cells induces an immunoglobulin isotype switch on B lymphocytes to IgG1 whereas IFN- $\gamma$  produced by Th1 clones increased the response of the IgG2a isotype (Stavnezer, 1996). To verify the cytokine responses and to understand the possible mechanism of protection, we measured serum levels of parasite-specific IgG1 and IgG2a isotypes. The low levels of IgG1 antibody detected in sera of the animals immunized with antigens in combination with different adjuvants suggests that the disease in immunized animals is being controlled, while in the control animals the infection progresses. Maximum level of IgG1 was seen in serum samples of mice immunized with Hsp70 + Hsp83 followed by the animals immunized with both antigens in combination with ALD and Hsp70 + Hsp83 along with MPLA. Moreover, higher levels of anti-*L. donovani* IgG2a were detected in serum samples of immunized animals again pointing towards the control of the infection in these animals. Maximum levels of IgG2a were seen in serum samples of mice immunized with MPLA vaccine followed by ALD vaccine and Hsp's alone. Indeed, IgG2a and IgG1 kinetics indirectly reflect the Th1/Th2 responses. The relative production of these isotypes can thus be used as a marker for the induction of Th1-like and Th2-like immune responses, respectively. Our studies are in accordance with Campbell et al. (2003) in which immunization with purified P4 nuclease protein and Hsp70 of *Leishmania amazonensis* resulted in significant increase in gamma interferon and reduction in parasite-specific IgG1, suggesting an enhancement in Th1 responses.

In the current study, we observed a mixed Th1/Th2 type of immune response in the immunized animals. Immunization with heat shock proteins along with different adjuvants induced substantial production of both Th1 and Th2-specific cytokines. Though the concentrations of IFN- $\gamma$  and IL-2 were greater in the animals immunized with the two heat shock proteins in combination with adjuvants, the levels of IL-4 and IL-10 were also significant. Maximum levels of IFN- $\gamma$  and IL-2 were seen in culture of splenocytes from animals immunized with vaccine formulation with MPLA followed by those immunized with vaccine formulation with ALD. Minimum level was seen in cultures of splenocytes from animals immunized with Hsp's alone. In contrast, IL-4 and IL-10 levels were high in control animals but decreased significantly in immunized groups. The minimum levels of IL-4 and IL-10 were found in cultures of splenocytes obtained from mice immunized with MPLA vaccine followed by those immunized with ALD vaccine. These results are in agreement with other studies where anti-IL-4 and anti-IL-10 receptor antibody therapy led to a protective response against *Leishmania* infection in BALB/c mice (Belkaid et al., 2001; Murray et al., 2002).

**Therefore, we can conclude that addition of Hsp83 to a vaccine comprising of Hsp70 based vaccine increased the level of protection against *L. donovani* in BALB/c mice. This may be due to the cumulative response produced by both the antigens. These antigens have been reported to have similar immunostimulatory properties in case of *L. infantum* model (Rico et al., 1999, 2002).**

It is also clear from our study that MPLA is a better adjuvant than ALD when given along with Hsp70 and Hsp83 in *L. donovani* infected BALB/c mice. This is because of the ability of MPLA to activate monocytes and macrophages (Masihi et al., 1986). Presumably through the activation of these cells, vaccine antigens are more readily phagocytized, processed, and presented. In addition, MPLA either directly or indirectly stimulates the production of the

T helper cell type 1 (Th1) cytokines IL-2 and IFN- $\gamma$  (Odean et al., 1990; Gustafson and Rhodes, 1994). This capacity of MPLA to stimulate the cytokine cascades necessary for the induction of cellular immunity makes it an effective adjuvant by itself.

## 5. Conclusion

In conclusion, we have analyzed immune responses to two potent heat shock proteins of *L. donovani* in combination with two adjuvants MPLA and ALD. The differential cytokine elicitation patterns, DTH response, and antibody switching to IgG2a of Hsp's plus MPLA and Hsp's plus ALD suggest the potential of these Hsp's for designing vaccines with improved protective efficacy.

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# Analysis of the adjuvant effect of recombinant *Leishmania infantum* Hsp83 protein as a tool for vaccination

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## Abstract

The properties of *Leishmania infantum* hsp83 (LiHsp83) to elicit an immune response against a fused reporter antigen, maltose binding protein (MBP), was studied. CF1 mice were immunized with different purified recombinant proteins: MBP, LiHsp83 and MBP fused to LiHsp83 (MBP-LiHsp83). Serum samples were obtained at days 0, 21, 28, 60, 90, 120 and 150 post-immunization. MBP-LiHsp83 fusion protein elicited a strong humoral response against MBP, higher than that one obtained in mice immunized with MBP alone or MBP mixed with LiHsp83, showing the secretion of both anti-MBP IgG2a and IgG1 isotypes (IgG2a/IgG1 ratio: 2:1). This response was specific for recombinant proteins and was maintained for at least 150 days, whereas the reactivity in mice immunized with MBP alone disappeared at day 90. After in vitro stimulation with MBP, spleen cells from MBP-LiHsp83 immunized mice showed higher proliferation indices and produced higher secretion of IFN-γ than spleen cells from either control or MBP-immunized mice. In all groups of mice IL-4 was undetectable. Thus we consider that LiHsp83 may be a promising candidate to be used as carrier of fused antigens for adjuvant-free vaccination. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Heat shock protein; Hsp83; Carrier; Adjuvant; Vaccine

## 1. Introduction

Heat shock proteins (hsps) are a family of proteins that are constitutively expressed at high level, and which increase further when a cell is subjected to stress conditions. Hsps are also highly conserved from bacteria to mammals and are involved in many essential cell functions as cause of their molecular chaperonin features: protein translocation, folding and assembly. Compelling evidence exists demonstrating that hsps of bacterial and parasitic pathogens are strong immunogens [1]. In some cases adjuvant effects of hsps have been assessed. *Mycobacterium tuberculosis* hsp60 and hsp70 proteins have been used to conjugate synthetic *Plasmodium falciparum*-derived-peptides, eliciting a

long-lasting humoral response against the peptides [2]. Suzue and Young [3] have demonstrated that *M. tuberculosis* hsp70 enhance both humoral and cellular response against a recombinant HIV p-24 antigen when linked to the hsp carrier. Similar results were observed using a *Leishmania infantum* hsp70 fused to maltose binding protein (MBP) as reporter antigen [4]. In addition, it was shown that the ability to stimulate the humoral immune response lies in the amino terminal domain of the *L. infantum* hsp70, the most conserved region of this molecule [5].

Despite hsps gp96 and hsp90 associated with antigenic peptides triggers immune response against chaperoned-peptide [6,7], there is little evidence about the adjuvant effect of the hsp83-90 family. Preliminary results showed that *L. infantum* hsp83 (LiHsp83) also fires a strong humoral response to a fused protein, causing a mixed IgG1/IgG2a response [5]. The goal of the present study was to analyze the 'adjuvant' LiHsp83 as a tool for vaccination.

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## 2. Material and methods

### 2.1. Plasmids and recombinant proteins

Plasmids pMal-cRI, pM83 and pQE83 expressing the maltose binding protein (MBP), MBP fused to LiHsp83 (MBP-LiHsp83) and a 6-histidine tag fused to LiHsp83, respectively, have been described previously [4,8]. Recombinant proteins were expressed in M15 *Escherichia coli* strain. MBP and MBP-*Leishmania* proteins were purified by affinity chromatography on amylose resin (New England Biolabs) as described [10], and LiHsp83 was purified by non-denaturing conditions on nitrilotriacetic acid-Ni<sup>2+</sup> column (Qiagen) as described [9]. The purity of recombinant proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels in the Mini-Protean system (Bio-Rad).

### 2.2. Immunizations

Two-month old female CF1 mice ( $n = 10$  per group) were immunized intraperitoneally (i.p.) with each antigen on days 0 and 21. The immunization doses and boosters of each antigen were the following: MBP (42 kDa, 1 µg), LiHsp83 (86-kDa, 2 µg) and MBP-LiHsp83 (128 kDa, 3 µg). Mice were periodically bled from the retro-orbital plexus.

### 2.3. Determination of IgG titers and isotypes

Enzyme linked immunosorbent assay (ELISA) was performed as described [10] using 500 ng/well of recombinant proteins to coat the plates. Immune complexes were developed with OPD (Sigma) as the chromogen and H<sub>2</sub>O<sub>2</sub> as the substrate of horseradish-peroxidase conjugated to anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) which was used as second antibody. Absorbance at 450 nm (A<sub>450</sub>) was measured with an automatic ELISA reader (Dynatech MR4000). Each serum was tested in duplicate. At least two independent ELISAs were performed for each serum. Iso-type-specific analysis were done by ELISA using the horseradish peroxidase conjugated anti-mouse IgG1 and IgG2a antibodies (Serotec), diluted 1:4000 and 1:2000, respectively.

### 2.4. Western-blot analysis

SDS-PAGE on 10% polyacrylamide gels was performed in the Mini-Protean system (Bio-Rad) using standard conditions. After electrophoresis, proteins were transferred to nitrocellulose membrane (Mini-Protean-Blot system, Bio-Rad), and the membrane was blocked with 5% non-fat dried milk powder in PBS-0.5% Tween 20 (blocking solution). The nitrocellulose

filters were probed with the first antiserum diluted in blocking solution. A peroxidase immunoconjugate (Jackson ImmunoResearch Laboratories) was used as the secondary antibody (1:5000), and specific binding was detected with H<sub>2</sub>O<sub>2</sub> as substrate and diaminobenzidine as the chromogen.

### 2.5. Analysis of MBP-dependent proliferation of spleen cells

In vitro proliferation assays were performed with RPMI culture medium supplemented with 10% fetal calf serum, 2-mercaptoethanol at a final concentration of  $5 \times 10^{-5}$  M, penicillin (100 U/ml) and streptomycin (100 µg/ml). Spleens from four immunized mice from each group were removed at day 120 after first immunization. Four phosphate buffer saline (PBS)-injected CF1 mice were added as control group. Viable spleen cells were plated by triplicate in 200 µl of medium at  $3 \times 10^5$  cells per well in 96-well flat-bottom microcultures plates (Costar, Cambridge, MA). Cells were stimulated with optimal concentration of 10 µg of MBP. Positive controls were assayed with concanavalin A in all experiments. Culture medium alone was used for negative controls. One µCi/well of <sup>3</sup>H-thymidine (specific activity 5 Ci/mmol, Amersham Corp.) was added for 24 h. <sup>3</sup>H-thymidine incorporation was measured at 72 h in a LKB (Gaithersburg, MD) liquid scintillation counter. Results were expressed as stimulation index (SI): the mean of counts per minute (cpm) of MBP-stimulated cells divided by the mean of cpm from non-stimulated cells.

### 2.6. Cytokines

To measure IFN-γ and IL-4, spleen cell cultures ( $5 \times 10^6$  cells in 200 µl of medium) from infected mice were stimulated as mentioned above. Supernatants were harvested at 48 and 72 h and stored at -20°C until IFN-γ and IL-4 contents were measured by ELISA. Microtiter plates (Immuno Plate Maxisorp; Nunc) were coated overnight at 4°C with 3 µg/ml of the capturing rat anti-mouse-IFN-γ and IL-4 (Pharmigen). Supernatants were tested by triplicate and serial dilutions of recombinant IFN-γ and IL-4 (Pharmigen) proteins were used at 20–4000 pg/ml and incubated for 1 h at 37°C. After washes, 1 µg/ml of biotinylated rat anti-mouse-IFN-γ (Pharmigen) and biotinylated rat anti-mouse-IL-4 (Pharmigen) were added for 1 h at 37°C. After washes, extravidin-peroxidase conjugate (Sigma) diluted 1:1000 was added. Bound complexes were detected by an OPD (Sigma) reaction. ELISA results were determined for each serum in duplicate at 450 nm. At least two independent ELISAs were performed for each supernatants. IFN-γ content was calculated as picograms per milliliter using as reference the recombinant IFN-γ curves.

### 3. Results and discussion

#### 3.1. Analysis of the humoral response of mice immunized with recombinant proteins

The presence of anti-MBP IgG and isotypes was evaluated over a period of 150 days in mice immunized with equimolar doses of MBP, MBP-LiHsp83, MBP mixed with LiHsp83 (MBP + LiHsp83) or LiHsp83. None of the immunizations included an adjuvant. Mice immunized with MBP-LiHsp83 elicited the highest anti-MBP IgG titer, averaging to 49 000 at day 28 (Fig. 1(A)). These titers descended to near 20 000 at day 60 and were maintained thereafter. Mice injected with MBP alone or MBP + LiHsp83 had also their highest anti-MBP IgG titers at day 28 averaging to 875 (Fig.

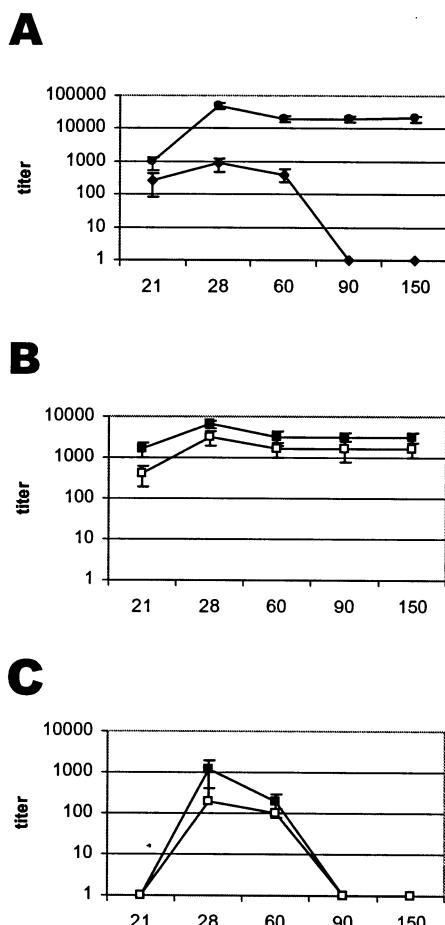


Fig. 1. Anti-MBP IgG and IgG isotypes profile of serum samples obtained from CF1 mice immunized with MBP and MBP fused to LiHsp83 proteins. Immunized mice were bled periodically. Data of serum samples obtained at days 21, 28, 60, 90 and 150 are represented. Titers were determined as the highest serum dilution giving absorbance value four times greater than the value obtained with preimmune sera. (A) Mean of anti-IgG titers of serum samples from mice immunized with MBP-LiHsp83 (circle), and MBP (square). (B–C) Anti-IgG1 (open square) and -IgG2a (filled square) titers from mice immunized with MBP-LiHsp83 (B) and MBP (C).

1(A)) and 1000 (data not shown), respectively, becoming negative at day 90. In conclusion, MBP linked to LiHsp83 elicited a humoral response greater than those observed in mice immunized with MBP alone or not linked to heat shock protein, similar as it was demonstrated to occur with hsp70s from *Mycobacterium* and *Leishmania* [3,4].

Fig. 1(B) showed that immunization of CF1 mice with fusion protein MBP-LiHsp83 elicited strong anti-MBP IgG2a and IgG1 antibody response, with a IgG2a/IgG1 ratio near 2 along the experiment. Immunization with MBP alone developed a predominant anti-MBP IgG2a isotype profile that decreased up to day 90 (Fig. 1(C)). Similar results were found for MBP + LiHsp83 (data not shown).

The anti-LiHsp83 reactivity of mice immunized with LiHsp83 and MBP-LiHsp83 recombinant proteins was determined at day 28. In all cases, serum samples assayed by IgG-ELISA showed titer values averaging  $10^5$ . Mice immunized with MBP-LiHsp83 or LiHsp83 resulted in the presence of both anti-LiHsp83 IgG1 and IgG2a isotypes (data not shown). Sera from mice immunized with MBP-LiHsp83 did not show cross reactivity with proteins from murine foreskin fibroblast cell line as determined by immunoblot (Fig. 2, lane 2), indicating that autoantibodies were not induced by immunization with LiHsp83.

#### 3.2. Cellular immune response induced by recombinant protein immunizations

MBP-dependent cellular immune response in recombinant protein-immunized mice was evaluated at day 90 post-immunization. Substantial specific lymphoproliferation was observed in spleen cell cultures obtained from MBP-LiHsp83-immunized mice after 72 h of culture (Fig. 3(A)). Proliferation of splenocytes from MBP-immunized mice was also high, but consistently lower than that obtained for splenocytes from MBP-LiHsp83 group.

Spleen cells from MBP-LiHsp83 immunized mice produced significant levels of IFN- $\gamma$  when stimulated with MBP, compared to controls (Fig. 3(B)). IL-4 levels were low for all groups ( $< 20$  pg/ml). These results are in agreement with those found for isotype analysis for CF1 mice immunized with MBP-LiHsp83, where anti-MBP IgG1 levels were lower than IgG2a.

Our results with LiHsp83 are promising and should be taken in consideration for developing of free-adjuvant vaccine systems. *L. infantum* hsp83 (LiHsp83) elicited a high and long lasting humoral and cellular immune response against the reporter antigen MBP, when co-administered as fusion protein. The association of IgG isotype and Th cell type is well defined in mice: an IgG1 antibody response is primarily driven by Th2, and IgG2a by Th1 T cells [11,12]. Mice immu-

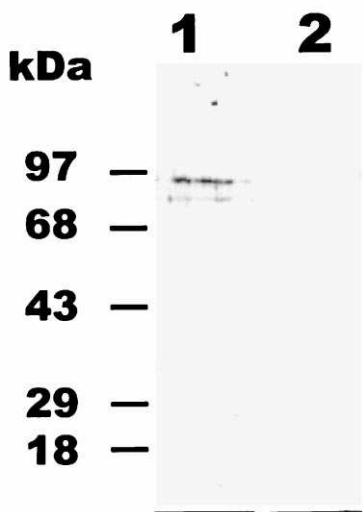


Fig. 2. Immunoblot of murine fibroblast foreskin cell proteins with rabbit anti-*Toxoplasma gondii* Hsp90 (lane 1) and MBP-LiHsp83 immunized mice (lane 2) sera. Sera were used at a dilution of 1:500 (lane 1) and 1:50 (lane 2). In lane 1, potential murine Hsp90 is detected. Molecular masses are given on left.

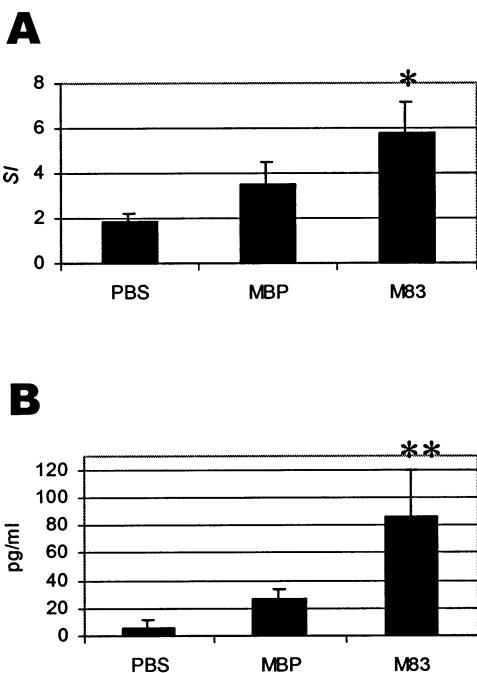


Fig. 3. Splenocytes from mice immunized with MBP, MBP-LiHsp83 and PBS (control) were stimulated in vitro with 10 µg of MBP. Four mice per group were analyzed. (A) lymphoproliferation analysis after 72 h of in vitro incubation represented as stimulation index (SI). SI of concavalin A stimulation ranged from 12.7 to 20.1. (B) IFN- $\gamma$  production after 48 h of in vitro incubation. The significance of data was determined by Student's *t*-test. \* and \*\*, significantly higher ( $P < 0.05$  and  $P < 0.01$ , respectively) than the MBP group.

nized with MBP-LiHsp83 elicited an anti-MBP IgG1/IgG2a response, inferring that both Th1 and Th2 responses were primed. In the present study, we demonstrated the production of specific IFN- $\gamma$  (Th1 cytokine). During *Toxoplasma gondii* infection IFN- $\gamma$  showed to be a major mediator against parasite infection [13]. At present, our interest is focused on the use of LiHsp83 as a carrier/immunostimulatory molecule for the Rop2 antigen [10], analyzing its potential capacity to stimulate an effector immune response against *T. gondii* in mice.

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