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# Potent antigen-specific immunity to *Toxoplasma gondii* in adjuvant-free vaccination system using Rop2-*Leishmania infantum* Hsp83 fusion protein

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**Abstract**

The results of this study describe the immunostimulatory properties of *Leishmania infantum* Hsp83 (83) to elicit humoral and cellular response against the *Toxoplasma gondii* Rop2 protein in an adjuvant-free vaccination system. The analysis was performed by immunizing three different mice strains (BALB/c, C57BL/6 and C3H). Mice immunized with fusion Rop2-83 elicited a stronger humoral and cellular response in comparison to mice immunized with Rop2 alone, or a mix of LiHsp83 and Rop2. The fusion protein induced a Th1 type response, with predominance of specific IgG2a/IgG2c isotype and IFN-γ secretions, whereas Rop2 alone or mixed with LiHsp83 produced a Th1/Th2 mixed response. Vaccination with fusion protein conferred a remarkable resistance against oral infection with ME49 cysts in C57BL/6 and C3H mice, in comparison to mice immunized with Rop2 alone or the protein mixture. Following lethal challenge, a significant survival rate was observed in Rop2-83 immunized Balb/c and C57BL/6 mice in comparison to control groups.


*Keywords:* Hsp90; Immunity; *Toxoplasma gondii*; Vaccine

## 1. Introduction

Heat shock proteins (Hsps) are highly conserved molecules that play important roles in protein folding, assembly of protein complexes, and translocation of proteins across cellular compartments. Increasing evidence also favors an important role of Hsps in several immunological processes. On the one hand, Hsps are specialized carriers for representation of antigenic peptides [1]. On the other hand, Hsps induce release of cytokines by different immune cells [2,3]. In addition, *Leishmania infantum* and *Toxoplasma gondii* Hsp70 and/or Hsp90 (Hsp83), have been shown to be strong mitogens for mouse B cells [4,5]. These immunoproperties might be the reason why different Hsps showed a considerable efficacy to induce a strong humoral and cellular immunoresponse against antigens fused to them. Suzue and Young [6] have further shown the immunogenic potential of *Mycobacterium tuberculosis* Hsp70 by immunizing mice with recombinant human immunodeficiency virus type 1 (HIV-1) p24-Hsp70 fusion protein. Immunization of mice with a fusion of *M. tuberculosis* Hsp70-ovalbumin elicited a CD4-independent cytotoxic T lymphocyte (CTL) response [7]. Similar results were found for the fusion of *L. infantum* Hsp83 to maltose binding protein (MBP) [8,9], the *Trypanosoma cruzi* Kmp11 fused to *T. cruzi* Hsp70 [10] and with the *Plasmodium falciparum* EB200 fused to *P. falciparum* Hsp70 [11].

*T. gondii* is an obligate intracellular parasite, member of the phylum Apicomplexa which can infect mammals and birds. In humans, *T. gondii* is known to cause transplacental infection leading to abortion or severe neonatal malformations and eye disease. Recently, *T. gondii* has emerged as an opportunistic pathogen of major importance in immunocompromised individuals, frequently as cause of encephalitis

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[12]. The effective immune response is mediated by CD4+ and CD8+ T cells, and is associated to IFN-γ production [13–16]. Most of the immunization studies used DNA vaccination to induce immunity in animal models [17]. However, it was observed that DNA vaccines have a restricted value for their use in humans [18]. Recombinant antigens were further assayed in immunization experiments, but require the use of an appropriate adjuvant. Up to now, only for formulations that contain aluminum salts, evaluation of the adjuvant per se is not required [19]. Immunizations with rSAG1, rGRA4 and rRop2 adsorbed to alum conferred partial immunity against toxoplasmosis [20,21]. However, alum was shown to promote the production of Th2 cytokines with low level of CD8+ T cell activation, conversely to the Th1 response required to induce immunity against *T. gondii* in mice [22].

Taking into account the requirement of a Th1 response for an adequate immunity against the parasite, *T. gondii* arose as an interesting model to analyze the adjuvant and immunoprotective value of LiHsp83. In addition, there are different murine infection models with different strain-dependent susceptibility to *Toxoplasma*, available [23]. Balb/c mice ($H$-$2^d$) are resistant to infection, producing a very low level of cyst loading [24,25] but have been used as model for lethal infection by the high virulent *T. gondii* RH strain [26,27]. C3H ($H$-$2^k$) is a strain of intermediate susceptibility to infection [28] arising as an excellent model of chronical infection due to the number of cyst loading after oral infection with an avirulent *T. gondii* strain [21]. C57BL/6 ($H$-$2^b$) mice are highly susceptible to infection, presenting low rate of surviving at low dose of an avirulent *T. gondii* inoculation [29]. This susceptibility is produced by an exacerbated inflammatory response rather than by parasite growth [30,31]. Interestingly, Rop2 gave controversial immunoprophilactic results in different murine infection models, eliciting immunity in immunized C3H, but not in C57BL/6 mice [21,32]. In order to determine the value of *L. infantum* Hsp83 (LiHsp83) as a carrier of *T. gondii* antigens, we constructed a fusion protein containing rRop2 and LiHsp83 (Rop2-83). Different murine strains were immunized with the fusion protein, Rop2 alone and Rop2 mixed with LiHsp83, and the immune response was analyzed. Finally, different murine strains were used to perform lethal and non-lethal challenge tests.

## 2. Materials and methods

### 2.1. Parasite

*T. gondii* tissue cysts of the Me49 strain were obtained from brains of C57BL/6 or C3H mice 1 month after intraperitoneally infection with 20 cysts. Cysts were purified by isopicnic dextran gradient and counted under optical microscope visualization. *T. gondii* of the RH strain was grown in human foreskin fibroblast monolayer with Eagle's Minimum Essential Media (Gibco) containing 10% Fetal Calf Serum (Gibco). Parasites were purified from infected monolayer by filtration through 3 μm pore size polycarbonate filters (Nucleoprotein) and counted in a Neubauer chamber.

### 2.2. Plasmids and recombinant proteins

All recombinant proteins are fused downstream and in frame with a sequence that encodes six histidine residues for purification with nickel resin. *T. gondii* $rRop2_{196–561}$ (Rop2) was induced and purified under non-denaturing conditions as described [33]. Lihsp83 was induced as described [34] and purified under non-denaturing conditions similarly as described for Rop2 but without glycerol. The region encoding Rop2 (residues 196–561) was amplified from pQE-$rRop2_{196–561}$ plasmid [35] by polymerase chain reaction (PCR). An upstream sense primer (Rop2-F 5′-ggatcccctggagacgtcgtcatt-3′) with the recognition sequence for the endonuclease *Bam* HI, and a downstream antisense primer lacking the stop codon (Rop2-R 5′-gcgcggtacctgccggttctccatcag-3′) with the recognition sequence for the endonuclease *Kpn* I were synthesized. The PCR product was cloned into pGEM T easy vector (Promega) and sequenced. The recombinant plasmid was digested with *Bam* HI and *Kpn* I restriction enzymes, the insert purified from agarose gel (Qiaex II, Qiagen), cloned into the corresponding sites of pQE-LiHsp83 vector in frame, generating the pQE-Rop2 ∼ LiHsp83 plasmid. The fusion protein was purified under non-denaturing conditions.

### 2.3. Immunization and challenge

The immunization doses and boosters of each antigen were the following: Rop2 (44 kDa, 2 μg), LiHsp83 (86 kDa, 6 μg), Rop2-LiHsp83 (∼120 kDa, 9 μg) and the mixture of Rop2 and LiHsp83 (2 + 6 μg, respectively). Balb/c, C57BL/6 and C3H mice were immunized by footpad injections on days 0, 21, 35 and 45 with PBS (control) or recombinant proteins. Each group was composed of eight animals. To analyze protection against virulent *T. gondii* strain (lethal challenge) immunized mice were challenged intraperitoneally with $10^5$ *T. gondii* RH tachyzoites 2 weeks after the last immunization. To analyze protection against non-lethal challenge, immunized mice were orally infected with 20 cysts of the ME49 *T. gondii* strain 2 weeks after the last booster. The brain parasite load was evaluated 1 month after infection. After removing brains, all of them were individually homogenized in PBS at equal volume. The mean number of cysts per brain was determined by observation under an optical microscope, counting four samples of 25-μl aliquots for each brain. Values are means plus standard deviation (S.D.).

### 2.4. IgG and subclasses determination

Immunized animals were bled at days 0, 21, 35 and 60 after first immunization and on day 30 after challenge.

Antigen-specific antibodies were analyzed by enzyme-linked immunosorbent assay (ELISA) as described previously [35]. Briefly, each well of the microtiter plate (Immuno Plate Maxisorp; Nunc) was coated overnight at 4 °C with 100 μl of 5 μg/ml recombinant proteins diluted in 0.05 M carbonate buffer (pH 9.6). Immune complexes were revealed with orthophenylenediamine (OPD, Sigma) as the chromogen and 0.15% $H_2O_2$ as the substrate for a horseradish peroxidase-conjugated goat anti-mouse IgG antibody [36] diluted 1:4000. Absorbance at 450 nm (A450) was measured with an automatic ELISA reader (Dynatech MR4000). Results were determined in duplicate for each serum. At least two independent ELISAs were performed for each serum.

Isotype-specific analysis was performed by ELISA using the horseradish peroxidase-conjugated goat anti-mouse IgG1 (rat anti-mouse IgG1 heavy chain, LO-MG1-2, Serotec) diluted 1:2000 and IgG2a (rat anti-mouse IgG2a heavy chain, LO-MG2a-9, Serotec) diluted 1:4000. Results were expressed as absorbance value at 450 nm (A450). C57BL/6 mice express the *Igh1-b* gene, which encodes the IgG2c isotype rather than IgG2a [37]. However, here we used an anti-IgG2a isotype which cross-reacts with IgG2c as observed in other cases [37]. This analysis was performed for three independent experiments.

Sera were used at 1:125 to 1:2000 dilutions. To graph the IgG profiles, serum samples were used at 1:500 because the low level of reactivity of those obtained after day 21. In order to attain a comparative analysis of isotype profiles serum samples obtained after immunization plan were used at dilution 1:2000, since they showed to be in lineal phase.

### 2.5. Analysis of Rop2-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 from mice 2 weeks after the last booster. Four phosphate-buffered saline (PBS)-injected mice were added as control group. Viable spleen cells were plated in triplicates in 200 μl of medium at $3 \times 10^5$ cells per well in 96-well flat-bottom microculture plates (Costar, Cambridge, MA). Cells were stimulated with optimal concentration of 10 μg of Rop2. Positive controls were assayed with concanavalin A in all experiments. Culture medium alone was used for negative controls. A 1 μCi/well of $^3$H-thymidine (specific activity 5 Ci/mol, Amersham Corp.) was added for 24 h. $^3$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 rRop2-stimulated cells divided by the mean of cpm from non-stimulated cells.

### 2.6. Cytokine analysis

Spleen cell cultures ($5 \times 10^6$ cells in 1 ml of complete medium in duplicate in 24-well flat-bottom microcultures plates) from mice 2 weeks after the last booster were stimulated with 10 μg of Rop2. Supernatants were harvested at 48 h to analyze interleukin-4 (IL-4) or 72 h to analyze gamma interferon (IFN-γ) and stored at −70 °C until samples were measured by ELISA. Briefly, 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 monoclonal antibodies (Pharmigen) diluted in 0.1 M $Na_2HPO_4$ pH 9. The wells were washed thoroughly with 0.05% Tween 20 in PBS. Empty binding sites were blocked by 2 h incubation at 37 °C with 1% bovine serum albumin in PBS. The supernatants from the cell cultures were tested in triplicates at 100 μl per well, and serial dilutions of recombinant murine IFN-γ and IL-4 (Pharmigen) proteins were used at 20–4000 pg/ml for the standard curves. After incubation for 1 h at 37 °C, the plates were washed four times and 1 μg/ml of biotinylated rat anti-mouse IFN-γ and IL-4 monoclonal antibodies (Pharmigen) were added for 1 h at 37 °C. Streptavidin-peroxidase conjugate (Sigma) diluted 1:1000 was added to the washed wells and allowed to react for 30 min at 37 °C. Bound complexes were detected by a solution of 0.15% $H_2O_2$–0.15% OPD (Sigma) in citrate (0.1 M)–phosphate (0.1M) buffer, pH 4.5. Absorbance was read at 450 nm in an automatic ELISA reader (Dynatech MR4000). At least two independent ELISAs were performed for each supernatant and the analysis was performed over three independent experiments.

### 2.7. Statistical analysis

Levels of significance between groups of mice for brain cyst loads were determined by a Student's *t*-test. Surviving curve was analyzed by product limit method of Kaplan and Meier, using the log-rank test for comparisons (Prism, version 3.0 GraphPad Software, San Diego, CA, USA).

## 3. Results

### 3.1. Expression and purification of Rop2-83 recombinant protein

Fig. 1 shows the recombinant proteins used in this work. Rop2, from residues 196 to 561, and LiHsp83 have been described previously [34,35]. Rop2-83 expression vector was generated by the fusion of a 6-histidine linked tag at the N-terminal part of a *Rop2* gene lacking the stop codon fused to the *LiHsp83* gene (Fig. 1A). Fusion protein migrated with an apparent molecular weight of ∼120 kDa (lane 3, Fig. 1B). In addition, a second product of ∼80 kDa was observed, which might be a degradation

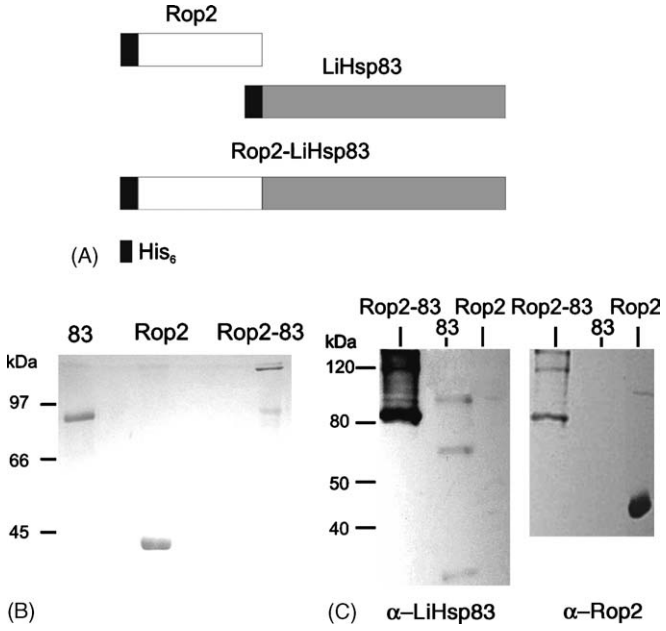


Fig. 1. Recombinant proteins. (A) Scheme of recombinant proteins utilized in this study. (B) Purified recombinant *T. gondii* antigens resolved by 10% SDS-PAGE and stained with Coomassie blue. Lanes: 83, LiHsp83; Rop2, $Rop2_{196–561}$; and Rop2-83, fusion of $Rop2_{196–561}$ to LiHsp83. (C) Same proteins were analyzed by Western blot using anti-LiHsp83 or -Rop2 serum samples. Molecular masses (*M*) are given on the left.

product. Both bands, 120 and 80 kDa, were recognized by anti-Rop2 and -LiHsp83 serum samples (Fig. 1C). Similar patterns were observed with whole bacteria extract (data not shown).

### 3.2. Humoral response against Rop2 in immunized mice

The efficacy of vaccination in immunized mice was followed by serological analysis. Production of IgG antibodies against Rop2 was evaluated by ELISA on days 0, 21, 35 and 60. All immunized mice elicited a strong humoral response against Rop2, detected from day 35 (Fig. 2A).

Anti-Rop2 IgG1 and IgG2a (or IgG2c in case of C57BL/6 mice) antibodies were evaluated in serum samples obtained from immunized animals on day 60. All Rop2-83 immunized mice produced high levels of anti-Rop2 IgG2a/IgG2c isotype, whereas immunizations with Rop2 or Rop2 + 83 elicited a predominant specific IgG1 response (Fig. 2B). There were also significant differences in anti-Rop2 reactivity at inter-strain level, with C57BL/6 mice being the most responsive strain (Fig. 2B).

Mice immunized with PBS (Fig. 2) or LiHsp83 (data not shown) did not show any response against Rop2.

### 3.3. Cellular response against recombinant antigens

Individual mice spleen cell suspensions were obtained 14 days after the last booster. Stimulation with Rop2 was performed in order to analyze whether splenocytes from immunized mice were able to secrete IFN-γ or IL-4. Splenocytes from Rop2-83 immunized mice elicited significant higher production of IFN-γ than PBS, Rop2 and 83 + Rop2 groups (Fig. 3). In addition, it could be also observed a significant difference in IFN-γ production in immunized C57BL/6 and C3H mice with Rop2 or Rop2 mixed with LiHsp83 when

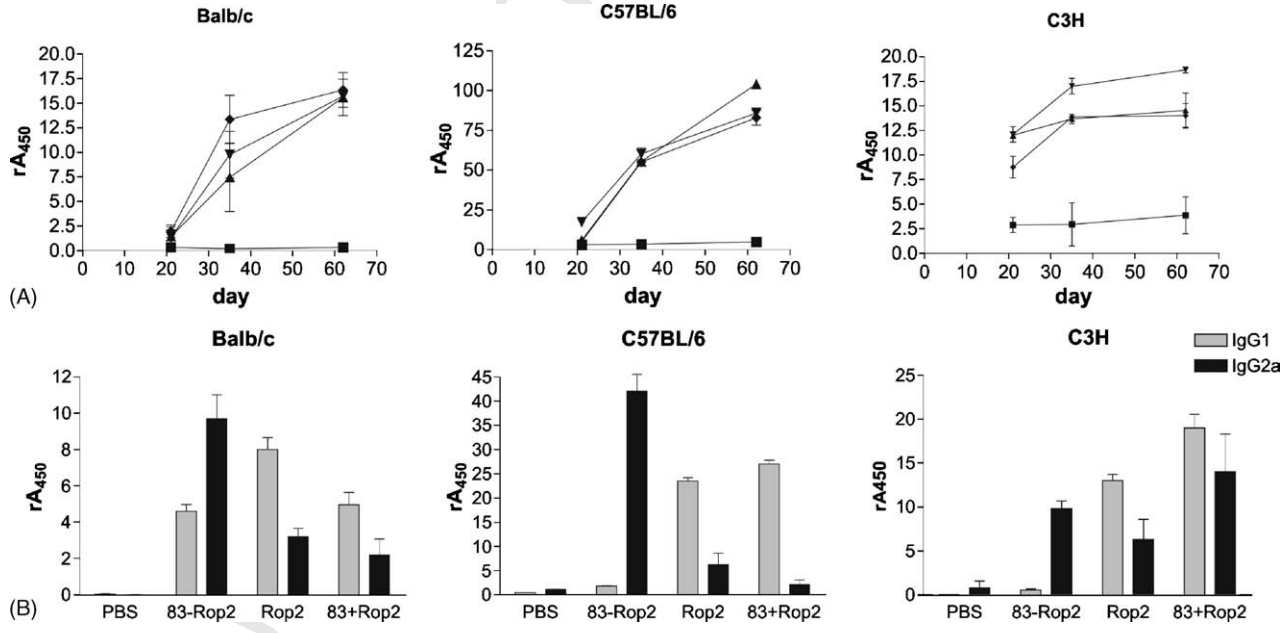


Fig. 2. Anti-Rop2 humoral response of immunized Balb/c, C57BL/6 and C3H mice analyzed by ELISA. Serum samples were used at dilution of 1:500. (A) Mice were immunized with PBS (■), Rop2-83 (▲), Rop2 (▼), 83 + Rop2 (♦). Production of IgG antibodies against Rop2 was evaluated by ELISA on days 0, 21, 35, and 60. (B) IgG isotype level generated against Rop2 protein. The antibody level was determined by ELISA. IgG1 and IgG2a antibodies produced against Rop2 were evaluated in serum samples obtained on day 60. Data are relative absorbance (rA, mean absorbance of each group vs. absorbance of pre-immune sera) values of pooled sera from eight mice per group. These data show representative results of at least three independent experiments.

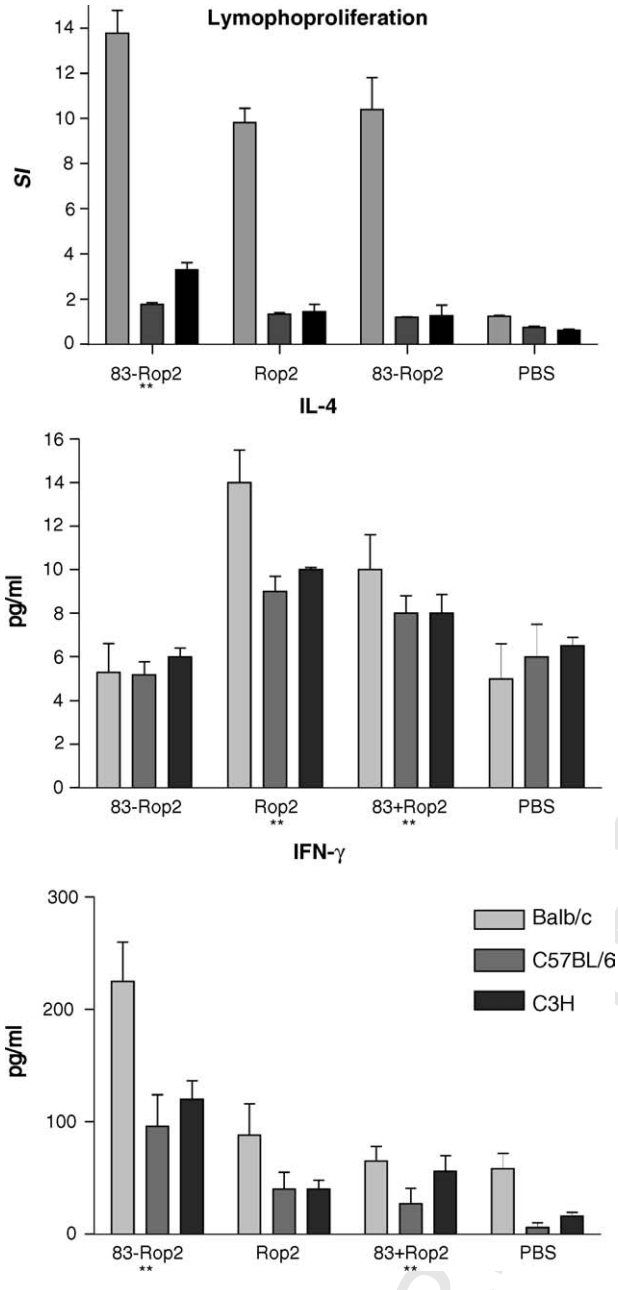


Fig. 3. Cellular response against Rop2 in immunized mice. Splenocytes from mice immunized with recombinant proteins or PBS (control) were stimulated in vitro 72 h (to analyze IFN-γ production and lymphoprolirferation) or 48 h (to analyze IL-4 production) with 10 μg of Rop2. Three (IFN-γ and IL-4) or 4 (lymphoproliferation) mice per group were analyzed. In the lymphoproliferation analysis results are represented as stimulation index (SI): the mean of counts per minute (cpm) of Rop2 stimulated cells divided by the mean of cpm from non-stimulated cells. SI of concavalin A stimulation ranged from 15.3 to 18.1. $^{**}P < 0.01$ (Student's *t*-test). In all cases, immunizations with 83-Rop2 showed a significant ($P < 0.01$) level of SI in comparison with PBS, Rop2 and 83 + Rop2 groups. In addition, groups Rop2 or 83 + Rop2 showed significant ($P < 0.01$ or <0.05) SI than PBS. To analyze cytokines, cell culture supernatants were analyzed by ELISA. Data are pg/ml (mean plus S.D.). IL-4: $^{**}P < 0.01$ vs. Rop2-83; IFN-γ: $^{**}P < 0.01$ vs. Rop2, 83 + Rop2 and PBS. IL-4 production of Rop2 and 83 + Rop2 immunized mice were also significant ($P < 0.05$) in comparison with PBS control.

compared with control. In contrast, only mice immunized with -Rop2 or 83 + Rop2 showed a significant IL-4 production in comparison with Rop2-83 or PBS (Fig. 3).

Splenocytes from Rop2-83 immunized mice showed a strong level of proliferation (stimulation index, SI) after stimulation with Rop2, significantly higher ($P < 0.01$) than those observed for Rop2, 83 + Rop2 and PBS groups in the three mice strains tested. In the Balb/c strain the stimulation level was the highest (Fig. 3). A significant level of lymphocyte proliferation was also observed when comparing 83 + rRop2 and Rop2 to PBS groups, especially in Balb/c mice.

Immunization with LiHsp83 protein produced a similar level of cellular immune response in the PBS control group (data not shown).

### 3.4. Protection of immunized mice against challenge with T. gondii

To examine the immunoprotective value of Rop2-83 against the high virulent RH strain, groups of Balb/c and C57BL/6 mice were immunized with Rop2, Rop2 + 83 or Rop2-83 by footpad injections. Mice that received PBS were used as negative controls. The capacity of these immunizations to protect mice was evaluated by the surviving rate after intraperitoneal injection of $10^5$ RH tachyzoites. Immunization with Rop2-83 results in a significantly higher survival rate than the PBS control in Balb/c and C57BL/6 mice, whereas immunization with Rop2 alone showed a significant level of surviving only in Balb/c mice (Fig. 4A).

The immunoprotective value was also determined in a chronic model, using the non-virulent *T. gondii* strain Me49. Groups of C57BL/6 and C3H mice were immunized and 2 weeks after the last booster they were orally infected with 20 cysts of Me49. Cysts in brain were counted 1 month after challenge. In all cases, immunizations showed a significant ($P < 0.01$) level of protection in comparison to the PBS group (Fig. 4B). In addition, group Rop2-83 showed significant immunoprotection value in comparison to groups Rop2 and Rop2 + 83. The level of protection observed in Rop2 group did not show significant difference when compared with 83 + Rop2 immunized animals.

Immunization with LiHsp83 protein produced a similar level of cyst loading as in the PBS control group (data not shown).

### 3.5. Humoral response against Rop2 after challenge

Anti-Rop2 IgG1 and IgG2a/IgG2c levels in immunized C57BL/6 and C3H mices were individually tested by ELISA 1 month after challenge with 20 cysts of Me49 strain. Fig. 5 shows that in all cases, in both strains, a predominant anti-Rop2 IgG2a/IgG2c response was elicited. Mice from C57BL/6 and C3H strains immunized with Rop2-83

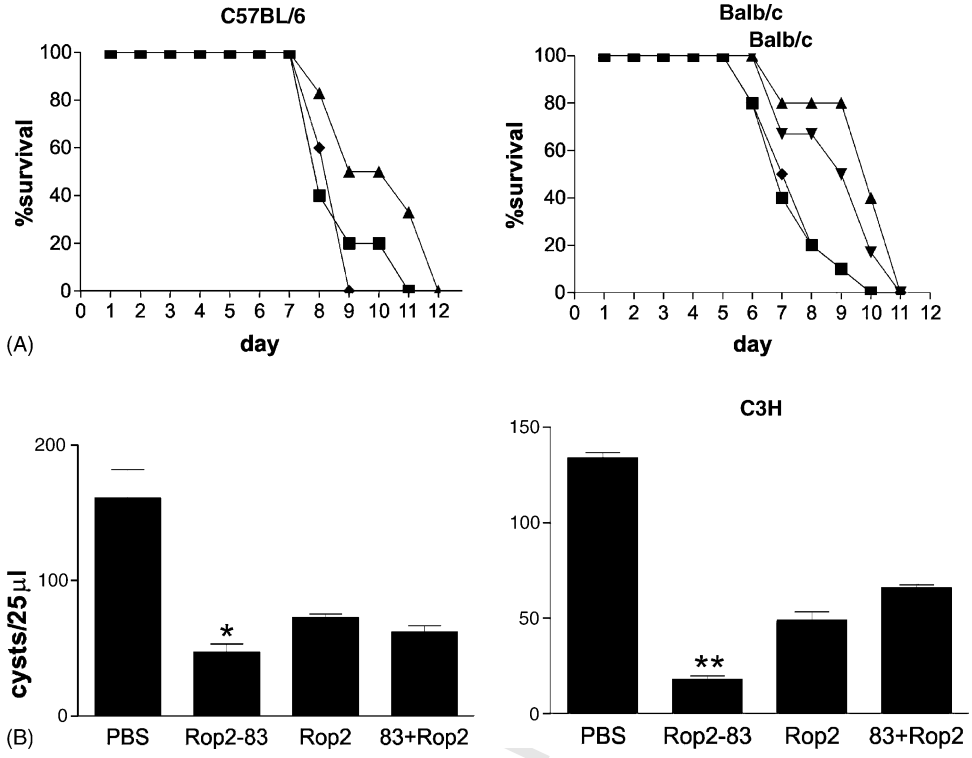


Fig. 4. Challenge test. (A) Survival curves of BALB/c and C57BL/6 mice immunized with the different recombinant proteins indicated, or PBS, and challenged intraperitoneally with $10^5$ tachyzoite of *T. gondii* RH strain 2 weeks after the last immunization. Mice were immunized with PBS (■), Rop2-83 (▲), Rop2 (▼), 83 + Rop2 (♦). Each group was composed of eight mice. Panel C57BL/6: PBS and Rop2 groups showed identical survival curves. $^{**}P < 0.01$; $^{*}P < 0.05$ (it was used the product limit method of Kaplan and Meier, to compare survival curves using the log-rank test for comparisons between immunized groups with PBS control group) (B). Assay for protection against oral challenge. Immunized C57BL/6 and C3H mice were orally infected with 20 cysts of the ME49 *T. gondii* strain 2 weeks after the last booster. The brain parasite load was evaluated 1 month after infection. The mean number of cysts per 25 μl of brain sample was determined by observation under an optical microscope. Values are means standard deviation (S.D.) of the results from experiments performed with groups of eight mice. $^{**}P < 0.01$; $^{*}P < 0.05$ (Student's *t*-test for comparisons between the Rop2-83 group and Rop2/Rop2 + 83 groups).

showed a significant fired up of IgG2a/IgG2c isotype in comparison with other groups, but mice immunized with Rop2 alone or Rop2 + 83 also showed a significant fired up of anti-Rop2 IgG2a/IgG2c isotype in comparison with the PBS group.

## 4. Discussion

In the present work, we have analyzed the value of *L. infantum* Hsp83 as carrier of the *Toxoplasma* antigen Rop2 in the murine model. Our study showed that the fusion of Rop2

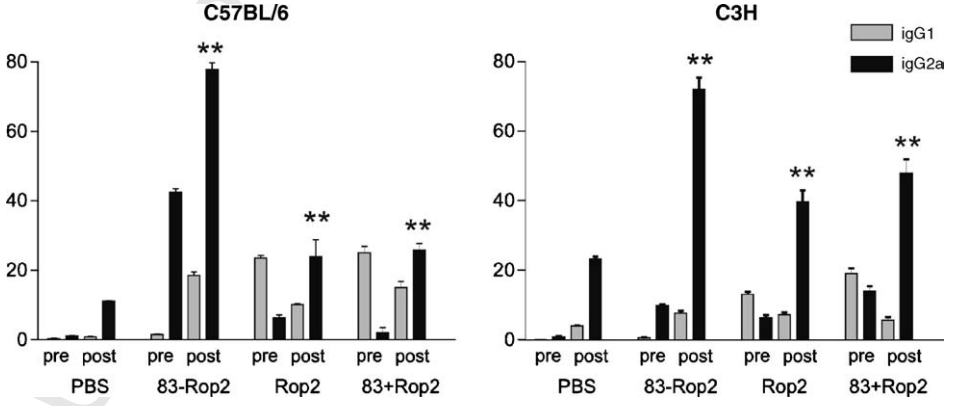


Fig. 5. Anti-Rop2 IgG1 and IgG2a (IgG2c in case of C57BL/6) antibodies in immunized C57BL/6 and C3H mice pre-challenge (day 60) and post-challenge (day 90) with *T. gondii* cysts. Anti-Rop2 IgG1 and IgG2a/IgG2c levels in all the mice were individually tested by ELISA. The bars represent the means of relative absorbance (rA, mean absorbance of each group vs. absorbance of pre-immune sera).

with LiHsp83 elicited a strong humoral and cellular response against Rop2, with high levels of specific IgG2a/IgG2c isotype, IFN-γ production and lymphoproliferation level. Moreover, immunization with the fusion protein elicited a predominant Th1 response in all mouse strains (Balb/c, C57BL/6 and C3H), whereas mice immunized with Rop2 alone or Rop2 + LiHsp83 mixture showed a mixed Th1/Th2 and weak cellular immune response.

Considering the response in the different strains, Balb/c mice immunized with Rop2-83 showed the highest level of IFN-γ production and lymphoproliferation. By contrast, it was observed that Rop2-83 immunized C57BL/6 and C3H mice showed a stronger IgG2a/IgG2c response than Balb/c. Since the non-immunized (PBS) Balb/c group also showed a high level of IFN-γ production when stimulated with Rop2 in comparison to those observed in C57BL/6 and C3H-PBS groups, it could be considered that the Th1 response would be more marked in the latter mouse strains. Further analysis should be done to shed light on this point.

Our study clearly shows that the antigen must be fused to LiHsp83 to generate the adequate Th1 immune response in all of the three mice strains. A strong humoral and cellular immune response against an antigen has been also observed when it was fused to Hsp70 molecules as carrier [6–8,10,38]. This suggests that Hsp83 and Hsp70 proteins present similar mechanism of immunoresponse stimulation. In this sense, LiHsp83 and *L. infantum* Hsp70 had shown similar immunostimulatory properties [4,9]. This leads to the assumption that the antigen is carried to the Hsp83 pathway of immune response stimulation. In fact, Hsps have been shown to be involved in several aspects of immune response modulation [39]. For LiHsp83, a T-cell independent B cell mitogenic activity has been determined [4]. It was found that gp96, Hsp90 and Hsp70 are capable to recognize the CD91 receptor in phagocytic cells, antigen presenting cells (APC) and dendritic cells, inducing their activation and maturation [40–43]. In addition, gp96, Hsp70 and Hsp90, among other Hsps, can bind peptides for immune recognition [44,45], suggesting that Hsps can activate innate and adaptative immune response [46]. On the other hand, it has been observed that bacterial Hsps directly induce cytokine secretion in macrophages and other cell types [47,48]. These findings have led to the postulation that extracellular Hsps, either from pathogens or from damaged cells, act as damage signals, whose abnormal presence would turn on the immune response.

It is known that during lysis of bacterial cells for protein purification, endotoxin molecules are released from the outer membrane of *Escherichia coli* into the lysate and it is not surprising that traces of endotoxins like LPS are frequent contaminants of biochemical preparations. In general, the LPS levels of purified recombinant antigens are below the standards set for pharmaceutical use. In addition, recently it was shown that a fusion protein Hsp65-P1 can activate DC independently of LPS, in spite of the requirement of a Toll-like receptor 4 for an optimal CD8 T cell response [49]. Since the mix of LiHsp83 with Rop2 showed similar immune response and protective values as immunizations with Rop2 alone we considered that putative traces of LPS in our preparations are not interfering with the conferred immunity of immunized mice.

After purification, two forms of the recombinant Rop2-83 protein could be observed, which were recognized by anti-Rop2 and anti-LiHsp83 antibodies. Whereas the approximately 120 kDA band corresponds with the expected product of complete Rop2-83 fusion, the second band (80 kDa) seems to be a degradation product. Since the His tag is fused at the N-terminal portion of Rop2, it seems likely that the 80 kDa product has the complete Rop2 fragment and approximately the first 40 kDa of LiHsp83. Presence of 80 kDa product seems not to disturb with the elicited immune response of the full length. It will be interesting to examine if the N-terminal region of LiHsp83 conserves the immunostimulatory properties as observed for the N-terminal region of LiHsp70 [9].

According to the generation of a strong Th1-type immune response after immunization, fusion improved the immunity against *T. gondii* lethal and non-lethal infection in all mouse strains. In contrast, the Rop2 or 83 + Rop2 groups only presented a poor cellular immune response against rRop2. Since IFN-γ was produced in all cases, it can be assumed that protection could be directly related, at least in part, to the production of this cytokine. Non-lethal infections allow inferring what kind of immunological response was taking place in C57BL/6 and C3H mice groups after challenge. *T. gondii* showed to polarize a strong Th1 response after infection [50], as expected, we observed a marked fired up of IgG2a/IgG2c isotype antibody in all challenged mice. The highest level of specific IgG2a/IgG2c after infection was observed in Rop2-83 immunized mice. In addition, the isotype profiles after infection with *T. gondii* were inversed (IgG1 towards IgG2a/IgG2c) in both C57BL/6 and C3H mice, immunized with Rop2 and Rop2 + 83. On the one hand, these data indicate that LiHsp83, as adjuvant, does not interfere with the normal immune response against *T. gondii*. On the other hand, additional conclusions can be drawn. In all immunized groups the IgG2a/IgG2c isotype level was higher than the IgG2a isotype level of the PBS group. In addition, the number of cyst loading was inversely related to the specific IgG2a/IgG2c isotype levels observed after infection. So, these data indicate a correlation between the level of protection and the level of IgG2a/IgG2c and IFN-γ production not only before challenge but also during infection.

Here we observed a similar level of protection in both C57BL/6 and C3H mice immunized with rRop2-83. By contrast, immunization with recombinant Rop2 adsorbed with alum or *Rop2* gene conferred immunity in C3H mice but not in C57BL/6 [21,32]. In one case, the susceptibility or resistance was related to the generation of Th2 or Th1 response after immunization, respectively [21]. However, in the other case, a strong Th1 response was also obtained in immunized C57BL/6 mice [32], being suggested that Th1 response induced after vaccination with *Rop2* gene could be the cause of the high lethality observed in C57BL/6 mice [30,31]. In

spite of this, Desolme et al. [51] showed protection against parasite infection in C57BL/6 immunized with *Gra4* gene. In this case, vaccination elicited IFN-γ and IL-10, suggesting a modulation of the Th1 response. We measured high IFN-γ, but not IL-4 levels, after vaccination of C57BL/6 and C3H mice with Rop2-83. Probably, the Hsp-based vaccine can regulate the inflammation processes during *Toxoplasma* infection, besides providing a specific immune response against Rop2 antigen. Both features can be dependent of physical link between Hsp83 and Rop2 proteins. Also, it should be considered that Vercammen et al. [32] used other *T. gondii* strains, and observed high lethality in their model using little doses of parasites. In our case, we used the Me49 strain, which did not show this level of lethality at low dose.

In conclusion, here we demonstrate that a member of heat shock protein 90 family, LiHsp83, is a good candidate to carry antigens and develop an adjuvant-free vaccine. This carrier based vaccine system has the capability to produce an immunoresponse that activates antibody secretion, cytokine production and stimulates cellular immune response, all positive features to control parasite infection. It will be interesting to analyze the behavior of other *Toxoplasma* antigens, as Gra4 or SAG1, fused to LiHsp83, which have been shown to confer immunity in different animal models and immunization assays [17]. Furthermore, LiHsp83 could be a carrier for antigens from a variety of pathogens, which require the generation of a Th1 response to confer immunity.

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