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Toxoplasma gondii: Identification and immune response against a group of proteins involved in cellular invasion

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ABSTRACT

Toxoplasma gondii is an ubiquitous intracellular parasite, causative agent of toxoplasmosis, and a world-wide zoonosis for which an effective vaccine is needed. A group of proteins secreted by tachyzoites during host-cell invasion was isolated from the interaction medium. It induced the permeability of the cells as assessed by alpha-sarcin and consequently facilitated the entry of the parasite into the cells. SDS–PAGE of the purified proteins showed a pattern of four proteins of 67, 42, 32 and 27 kDa. MRC-5 cells incubated with the total protein and the different electroeluted bands endured a high cellular death in presence of alpha-sarcin. BALB/C mice immunized with the group of proteins had a mixed Th1/Th2 response and were protected upon challenge with the parasites.

1. Introduction

Toxoplasma gondii is an obligate Apicomplexan intracellular pathogen that enters both phagocytic and non-phagocytic cells by active penetration (Dobrowolski and Sibley, 1996; Morisaki et al., 1995). It has an extremely broad host range (Dubey and Beattie, 1988) and a unique capacity to invade all nucleated cell types from warm-blooded vertebrate hosts. Toxoplasmosis is one of the major causes of opportunistic infections in AIDS patients and can result in severe damage of newborns during congenital infection. Invasion is an essential step of the pathologies associated with these protozoan parasites that also include *Plasmodium* spp., the causative agents of malaria.

Despite the efficiency of invasion of its host cells by *Toxoplasma* tachyzoites, it is still unresolved whether it occurs by direct penetration or by induction of a host-mediated endocytic event. The chemotherapeutic agents presently used against toxoplasmosis are inadequate, expensive, often toxic and there is still no commercial vaccine for use in humans. A vaccine developed for veterinary use has shown some efficacy (Buxton, 1998). Therefore, the development of an effective vaccine against *T. gondii* would be extremely valuable for preventing both primary fetal infection and reactivation in immunocompromised individuals and might also reduce economic losses by preventing abortions in farm animals.

Although several types of vaccine against toxoplasmosis, which include attenuated and inactivated vaccines, genetically engineering vaccine and DNA vaccine, have been tested for their immunologic effects in animal models, few have been licensed for use, mainly due to poor efficiency or biosafety concerns (Innes and Vermeulen, 2006; Darcy et al., 1992). Inactivated vaccines can only elicit moderate levels of protection (Mishima et al., 2002; Wang et al., 2007). A live attenuated vaccine has been used in some countries, but it is inadequate and expensive, may cause side effects and has the possibility to revert to a pathogenic strain (Bhopale, 2003).

We describe here the isolation of a group of proteins secreted by *T. gondii* tachyzoites during cell invasion, with a capacity to cause the entry of the parasite inside the cells and highly immunogenic to mice.

2. Materials and methods

2.1. Cells and parasites

The RH strain of *T. gondii* was routinely maintained in the human myelomonocytic cell line THP-1 (ECACC 88081201, European Collection of Animal Cell Cultures, Sophia Antipolis, France). The medium used was RPMI 1640 (Sigma–Aldrich, France) supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin (Sigma–Aldrich, L'Isle d'Abeau, France), and 10% fetal calf serum (PAA, les Mureaux, France). The parasites were grown in 150-cm² plastic tissue culture flasks (Falcon, Becton Dickinson, Meylan,

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France). The adherent fibroblasts MRC-5 (CCL-171, ATCC, Manassas, USA) were used for permeability tests.

2.2. Permeability tests

2.2.1. Permeability to alpha-sarcin of *Toxoplasma*-infected cells

To assess the potential capacity of tachyzoites to permeabilize the infected cells, MRC-5 cells were infected at a ratio of 10:1 (parasites/cell) in serum-free medium, and subsequently grown for 12 h. Alpha-sarcin, a 16,800 kDa protein able to block the cellular protein synthesis only if the cells are permeabilized, was then added at final concentrations of 5, 10, 20 and 30 µg/ml. After an additional 4 h-incubation, the cells were washed four times with serum-free RPMI and subsequently cultured in fresh medium with RPMI supplemented with 10% of fetal calf serum at 37 °C for 24 h. The cytopathic effect was assessed using a MTS/PMS solution (CellTiter 96®; Aqueous non-radioactive cell Proliferation Assay, Promega, Madison, WI, USA). Twenty microliters of the solution were added and after 2 h of incubation, the quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture.

2.2.2. Permeability of the cells in the presence of secreted proteins

The MRC-5 cells were cultured in presence of the purified proteins (0.0025; 0.005; 0.025; 0.05; 0.1 µg/ml) for 12 h. Then, the cells were incubated for 4 h with alpha-sarcin, the cells were washed four times with serum-free RPMI and subsequently incubated with RPMI supplemented with 10% (v/v) serum at 37 °C. The cytopathic effects of the toxin were evaluated after 24 h using a MTS/PMS solution as it was detailed above.

2.3. Protein isolation and electroelution of the different bands

The released proteins were purified from the medium as follows: the serum was removed from the cells and from the tachyzoites by at least five washes with serum-free medium. The cells were then infected with tachyzoites at a ratio of 1:10 for 16 h. After cell invasion, the infected cells were centrifuged at 1500g, the supernatant was filtered through a 0.2 µm membrane and incubated overnight at 4 °C with 70% ammonium sulfate to precipitate the proteins. After centrifugation at 22000g for 1 h, the protein pellet was resuspended in 0.1 M phosphate buffer, pH 7.1, dialyzed for 72 h and lyophilized. The protein concentration was assessed with a modified Bradford method (RotiNanoquant®, Roth, Brumath, France). The proteins were analyzed with 12% SDS–PAGE gels and visualized either after Coomassie Brilliant Blue or silver staining. The bands of interest were excised from the gel and electroelution was performed using a Hoefer-GE 200, six-Pac gel eluter (Hoefer Scientific Instruments, San Francisco, CA, USA).

2.4. Mice immunization

Eight weeks old BALB/c females mice were purchased from Charles River laboratories (St. Germain sur l'Arbresle, France) and were immunized intraperitoneally (i.p.) with 200 µl of 40 µg of the antigen diluted in NaCl 0.9% containing 2:3 of a commercial adjuvant aluminium gel suspension (13 mg/ml, Sigma–Aldrich). The mice were divided into five groups of ten animals: (1) control healthy mice that did not receive any injection, (2) control adjuvant, mice that received only NaCl 0.9% emulsified in the adjuvant, (3) immunized mice that received the proteins without challenge, (4) immunized infected mice that were infected at day 21 and (5) control infected, naive mice that were infected at day 21 with RH strain tachyzoites. The animals were immunized on day 0 and received a booster injection on days 7 and 14. They were bled on days 0, 7, 14 and 21, and the sera were tested by enzyme-

linked-immunosorbent assay (ELISA) for antibody responses. On day 21, the first group of immunized mice was anaesthetized and sacrificed, their spleen was removed, washed and minced in phosphate buffer saline (PBS). After centrifugation for 10 min at 1000g, the red blood cells were removed by hypotonic lysis for 30 s, the resulting cell suspension was washed in PBS several times and centrifuged. The T-cells were then analyzed by two-color flow cytometry as described below. The sera were stored at –80 °C for cytokine assay. The second group of immunised mice was challenged as described in the next paragraph. All the other animals groups were sacrificed at day 42.

2.5. Challenge infection

Twenty days after the first immunization procedure, mice were intraperitoneally infected with 3×10^6 RH strain tachyzoites. A group of 10 mice was infected at the same time and used as infected control mice.

2.6. Antibody assay

The presence and levels of sera antibodies was analyzed by ELISA in 96-well microplates (Nunc-Maxisorb) coated with 1 µg/ml of the purified proteins in 30 mM carbonate–bicarbonate buffer, pH 9.6. After incubation overnight at 4 °C, the plates were washed with PBS containing 0.05% Tween 20 (PBS-Tween), and blocked with PBS-Tween containing 1% bovine serum albumin for 2 h at 37 °C. Pools of sera diluted at 1/100 in PBS-Tween were added in triplicate wells and the plates incubated at 37 °C for 2 h. After three washes with PBS-Tween, 100 µl/well of diluted specific antibody (1:1000) was added. Antibodies used were goat anti-mouse monoclonals IgG1, IgG2a, IgG2b, IgG3, IgA, IgM (Sigma) IgG (Southern Biotechnology). After incubation for 2 h at 37 °C, the plates were washed, and 100 µl/well of peroxidase conjugated rabbit anti-goat IgG (Sigma) diluted 1:5000 in PBS-Tween was added. After incubation (1 h at 37 °C), Reaction was developed using the substrate 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB, Sigma). Optical densities were read spectrophotometrically at 450 nm with an ELISA plate reader (Microplate Reader, Bio-Rad Laboratories). Sera from twenty 8-week-old normal female BALB/c mice were pooled and used as a negative control to measure the background activity in all experiments. Each assay was performed in triplicate.

2.7. Cytokine assay

The cytokine concentration was determined using commercial sandwich ELISA kits for IL-2, IL-4, IL-12, γ-IFN and IL-6 (Biosource, Nivelles, Belgium). Each assay was performed in triplicate and optical densities were read spectrophotometrically at 450 nm with a plate reader (Microplate Reader, Bio-Rad Laboratories). Sera from twenty 8-week-old normal female BALB/c mice were pooled and used as a negative control.

2.8. Flow cytometric analysis

For immunofluorescence staining with single labeling, 10^6 cells were incubated with FITC-conjugated rat monoclonal antibodies against CD3 (KT3), CD4 (YTS191.1) or with PE-conjugated rat monoclonal antibodies against CD8 (KT15) for 30 min at +4 °C, then washed twice and fixed with 1% paraformaldehyde. Flow cytometric analysis was performed with a Dako Galaxy flow cytometer (Dako, France) equipped with a 488 nm argon laser and detectors for forward scatter (FSC) and 90° light scatter (side scatter, SSC) and for FL1 (band pass filter wavelength 530 nm) and FL2 (585 nm) fluorescence emission in the green part and red/orange part, respectively of the spectrum. Splenocytes stained

with single colors, with FITC- or PE-labeled antibodies were detected by FL1 or FL2, respectively and were identified by their characteristic appearance on a dot plot of FSC versus SSC and electronically gated to exclude platelets, red cells, and dead-cell debris. T-cells were gated on the basis of CD3 staining. The results were reported as percentages of positive cells within this gate. Rat monoclonal antibodies IgG2a-FITC (LODNP16) (Immunotech) and IgG2b-FITC (A-1) (SouthernBiotech) and IgG2a-PE (LODNP16) (Immunotech) were used as negative controls for antibody CD3-FITC, CD4-FITC and CD8-PE, respectively.

2.9. Statistical analysis

Calculations of specific antibody production, spleen cell proliferation, and cytokine concentrations were done using Microsoft Excel (Office 2007) and the GraphPad PRISM 4.0™ (San Diego, CA, USA) software. Comparative analyses were done with the Student's *t* test using INSTANT™ software (GraphPad, San Diego, CA, USA). Differences were considered as significant for $P < 0.05$ unless otherwise stated.

3. Results

3.1. Cell permeation by tachyzoites

Fig. 1 shows the viability of infected cells incubated with increasing doses of alpha-sarcin. A significant decrease in the O.D. of the infected cells incubated with 30 µg/ml of alpha-sarcin as compared to the non-infected cells was noticed. The calculated death ratio was about 40%.

3.2. Isolation of the proteins and permeability test

SDS-PAGE analysis showed the presence of four bands of 67, 42, 32 and 27 kDa (Fig. 2), the four bands were eluted and permeability was assessed with the total proteins at different concentrations (Fig. 3) and the four bands, separately (Fig. 4). A clear decrease in the viable cells was observed and the calculated death ratio reached 56% (Fig. 4). The eluted protein that caused the higher death ratio – 68% – was the 42 kDa band (Fig. 4).

3.3. Antibody response

In the mice immunized with the purified proteins, the IgG-specific response increased progressively from days 14 to 42 compared to controls (Fig. 5). There was no significant difference in the

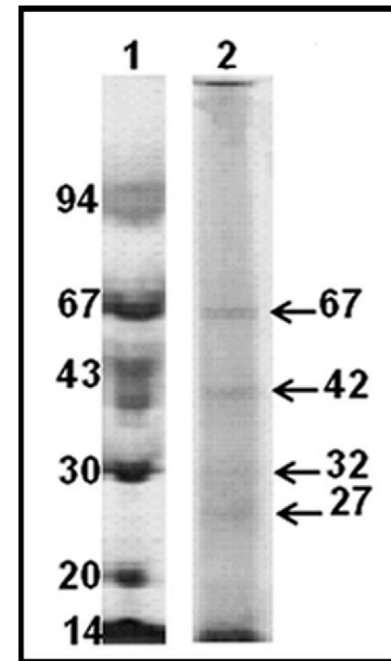


Fig. 2. SDS-PAGE of the purified proteins. (1) MW markers, (2) purified group of proteins (1 mg/ml). Four bands were observed.

humoral responses between the various groups. Upon infection, 8/10 of the mice died two weeks after infection, with low IgG levels. The different antibody subclasses produced after immunization with the group of proteins are shown in Fig. 6. IgG, IgM, and IgA were higher in immunized mice compared to controls, the highest being the IgM in all groups of mice. However, in comparison with the controls, immunization induced a light increase in IgG. The four isotypes were high in immunized mice, compared to the other groups, and the IgG1 were predominant. Except in immunized mice, IgG3 was quite high compared to the other isotypes. In immunized mice the ratios were >1 , whereas in all others groups it were generally <1 , with an IgG1/IgG3 highest ratio of 1.664 (Table 1).

3.4. Immunization by the released proteins reduces the mortality due to infection by *T. gondii*

All the immunized mice survived the acute phase of the infection, whereas eight mice from the infected control group died 11 days after the infection. The first signs of infection – paralysis

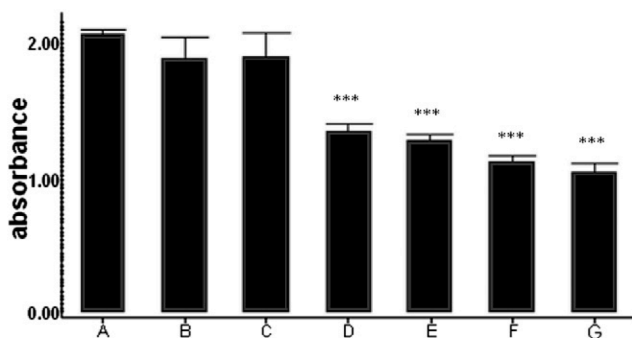


Fig. 1. Enhanced permeability to α -sarcin of MRC5 cells infected with *Toxoplasma gondii*. Absorbance is directly proportional to the number of living cells. (A) control; (B) infected cells; (C) uninfected cells +30 µg/ml of α -sarcin; infected cells with: (D) 5 µg/ml α -sarcin; (E) 10 µg/ml α -sarcin; (F) 20 µg/ml α -sarcin; and (G) 30 µg/ml α -sarcin. *** $P < 0.001$ (Student's *t* test). The values represent mean \pm SD. The experiment was done at least five times. Each assay was performed in triplicate.

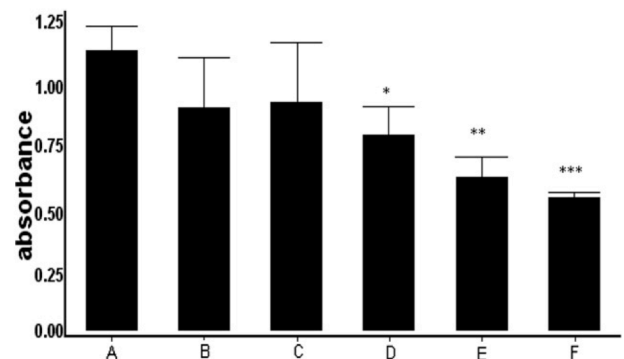


Fig. 3. Effect of α -sarcin on MRC5 cells incubated with various concentrations of purified group of proteins and 30 µg/ml α -sarcin. Absorbance is directly proportional to the number of living cells. (A) control; (B) 0.0025; (C) 0.005 µg/ml; (D) 0.025 µg/ml; (E) 0.05 µg/ml; and (F) 0.1 µg/ml. ** $P < 0.01$, *** $P < 0.001$ (Student's *t* test). The values represent mean \pm SD. The experiment was repeated at least five times. Each assay was performed in triplicate.

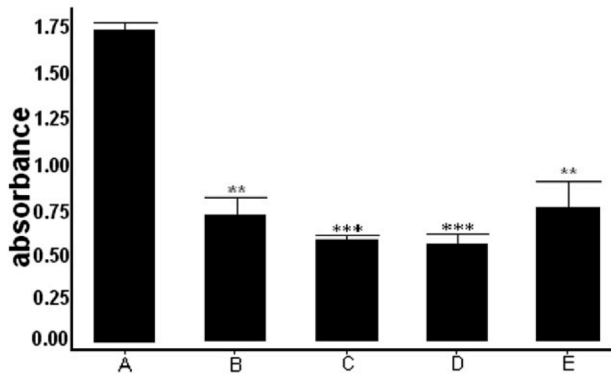


Fig. 4. Effect of the purified group of proteins and of the electroeluted bands on the permeability of MRC-5 cells to 30 µg/ml α-sarcin. Absorbance is directly proportional to the number of living cells. (A) control cells; (B) 67 kDa band (C) 42 kDa band; (D) 32 kDa band; and (E) 27 kDa band. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t test). The values represent mean \pm SD for a triplicate. The experiment was done three times. Each assay was performed in triplicate.

of the posterior limb – appeared a few days post-infection. In the immunized group, two deaths occurred later, at 20 and 25 days after infection, respectively. Consequently, compared with the control group, immunization with the group of proteins increased the survival rate (80% survival) up to 30 days after challenge.

3.5. T-cell analysis

The levels of CD4⁺/CD8⁺ are shown in Fig. 7. The CD4⁺ splenocyte population was higher than the CD8⁺ population in all groups of mice. In mice immunized with the purified proteins and challenged, the CD4⁺/CD8⁺ ratio was significantly higher than the controls ($P < 0.001$).

3.6. Cytokine response

The presence of IL-4 and IL-12 was detected in all groups of mice, but they were higher in immunized and in infected mice.

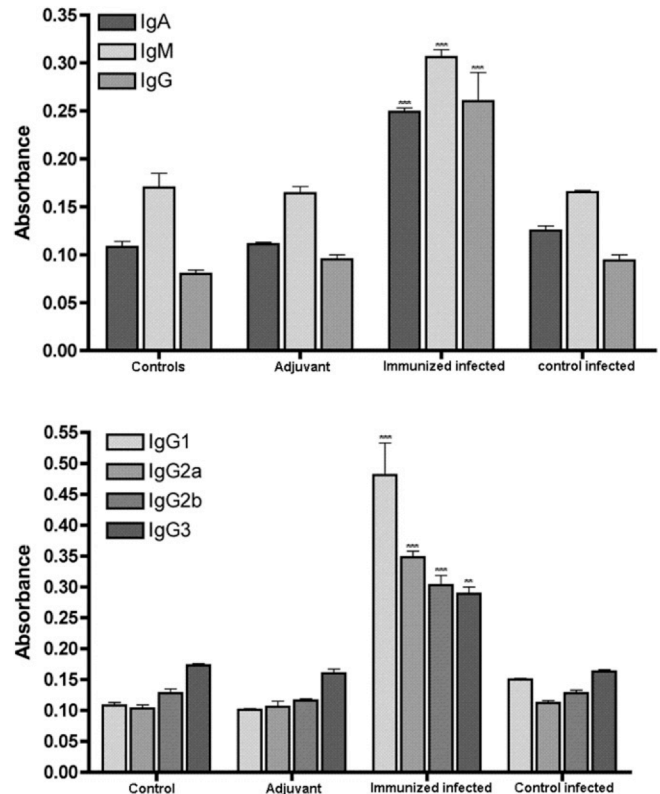


Fig. 6. Proportion of the different classes of antibodies in mice immunized with the purified group of proteins. The values represent averages \pm SD. ** $P < 0.01$, *** $P < 0.001$ (Student's t test). Each assay was performed in triplicate.

IL-12 was significantly increased in all mice except the controls. IL-2 and IL-6 were present only in immunized mice and as traces in infected mice. Although γ -IFN was present in immunized and in infected mice, it was higher in infected mice (Fig. 8).

4. Discussion

We had observed that infection of MRC-5 cells by *T. gondii* tachyzoites rendered them permeable to the toxin alpha-sarcin, a potent inhibitor of protein synthesis in cell-free systems, ineffective on intact cells since it is unable to cross the cell membrane under normal conditions (Fernandez-Puentes, 1983; Muñoz et al., 1985). Our experiments confirmed that the host cell plays a passive role in the parasite entry. Invasion by *Toxoplasma* occurs approximately 3–4 times more rapidly than phagocytic uptake and do not involve host cell membrane ruffling or pseudopod formation (Morisaki et al., 1995). Host cell invasion has been well described at the ultrastructural level, but the precise molecular interactions and the specific role of the released parasite proteins are still poorly understood. Proteins located on the surface of the parasite probably mediate the initial interaction with the cell.

A group of proteins released by *T. gondii* tachyzoites into the medium after interaction with MRC-5 cells was isolated, that made them permeable to alpha-sarcin. SDS-PAGE of the purified fraction exhibited four bands that were subsequently electroeluted and separately incubated with the cells and alpha-sarcin. The 42 kDa band appeared to be the more efficient. Mice were immunized with the group of proteins purified from the supernatant and challenged with tachyzoites of the RH strain.

Our purified group of proteins actively stimulated the mice immune response, the IgG levels increased significantly from day 0 to day 42, whereas they remained stable in uninfected and infected

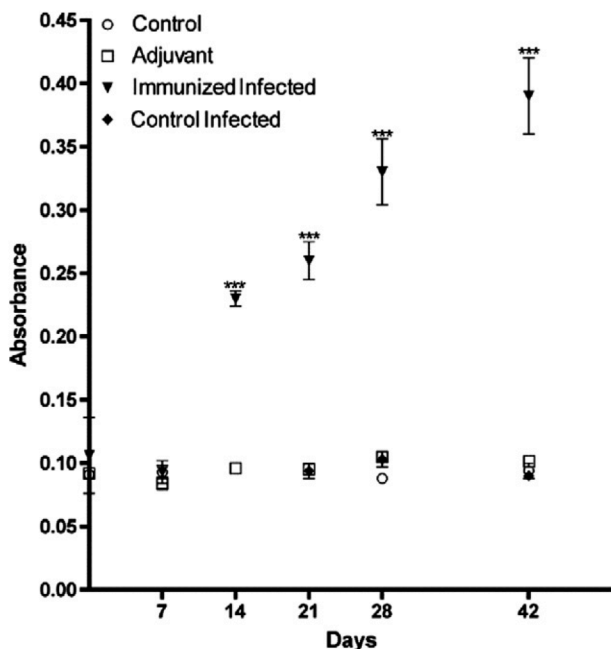


Fig. 5. IgG-specific antibody response in mice immunized with the purified group of proteins. *** $P < 0.001$ (Student's t test). The values represent mean \pm SD. Each assay was performed in triplicate.

Table 1

Ratios of IgG isotypes of antibodies against the group of proteins present in sera from immunized uninfected mice and infected mice with *T. gondii*.

	IgG1/ IgG2a	IgG1/ IgG2b	IgG1/ IgG3	IgG2a/ IgG3	IgG2b/ IgG3
Controls	1.048	0.843	0.624	0.595	0.73
Mice + adjuvant	0.952	0.870	0.63	0.66	0.725
Immunized infected mice	1.382	1.587	1.664	1.328	1.04
Control Infected	1.339	1.117	0.920	0.687	0.78

mice. IgM and IgA displayed comparable trends. Infection by *T. gondii* triggers a high level production of type 1 cytokines such as γ -IFN. Both IL-12 and γ -IFN are essential for resistance to this opportunistic pathogen (Denkers, 2003; Denkers and Gazzinelli, 1998; Gazzinelli et al., 1994; Lieberman and Hunter, 2002; Mun et al., 2003). *T. gondii* is also a potent stimulus for IL-12 production, which in turn is required for skewing the immune response toward Th1 (Scharton-Kersten et al., 1995; Sher and Reis e Sousa, 1998). In the present work, IL-12 increased significantly in both immunized and in infected mice, showing evidence that the group of proteins increased the resistance to the infection. Moreover, the measured γ -IFN was high in both the immunized and infected mice, albeit higher in infected mice.

IL-4 and IL-6 are synthesised by Th2 lymphocytes, which play a major role in the pathogenesis of parasitic diseases. Whereas IL-6 stimulates the production of antibodies and exerts a proinflammatory effect by stimulating the generation of acute phase proteins, the former inhibits cytokine synthesis and through the blocking of the IL-6 and TNF- α production, causes a shift in the immune response towards a Th2 involvement and B-cell activation. Our results show that IL-4 increased very significantly in both immunized and infected mice, the group of these proteins could thus improve the resistance to infection. IL-6 was present only in immunized mice and only as traces in infected mice.

The study of the four isotypes and the calculated ratios, confirmed the results obtained with the cytokine assay. The IgG1 subtype was present at very high levels and the IgG1/IgG3 ratio was the highest in immunized mice. Th2 patterns are characterized by a preferential production of complement-independent IgG1 antibodies, whereas Th1 responses are characterized by production of complement-dependent IgG2a antibodies. Consistent with these isotype profiles, antigen-specific CD4⁺ Th2-type T helper cells produce IL-4 that supports switching to IgG1, while Th1-type T helper cells are characterized by the generation of IFN- γ that supports

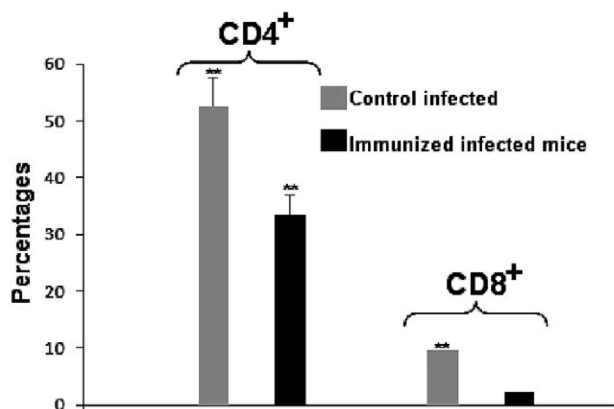


Fig. 7. Percentages of lymphocytes T, CD4⁺ and CD8⁺ in immunized infected mice. Two groups of animals were represented, the mice uninfected and infected by *T. gondii* tachyzoites. The values represent mean \pm SD. *** P < 0.001 (Student's t test). Each assay was performed in duplicate.

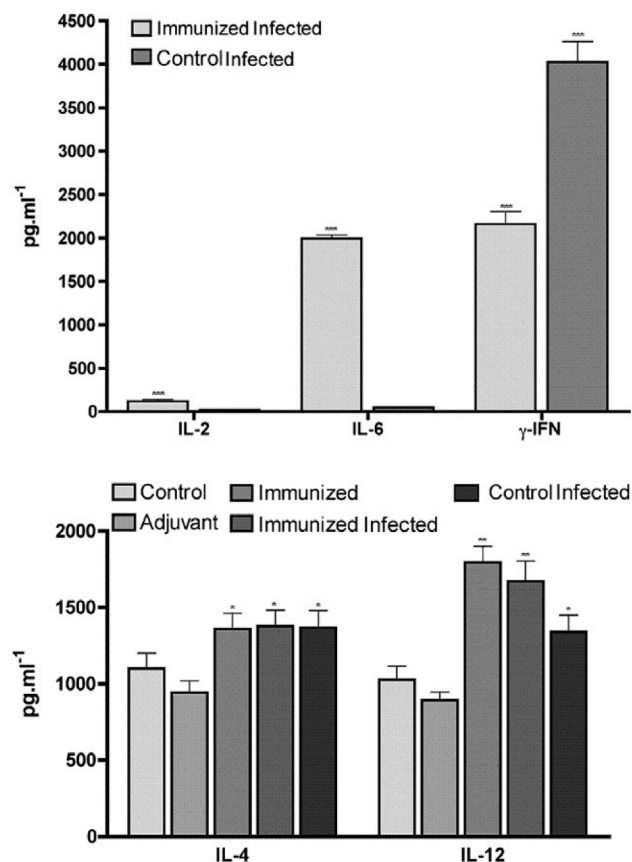


Fig. 8. IL-2, IL-4, IL-6, γ -IFN and IL-12 induction by the Balb/c mice immunized with the purified proteins. The specific differences between the stimulated immunized mice and controls stimulated are indicated; * P < 0.05, ** P < 0.01, *** P < 0.001 (Student's t test). The values represent mean \pm SD. Each assay was performed in triplicate.

switching to IgG2a (Mosmann et al., 1995, 1997). The cytokine assay gave a mixed cytokine profile of the Th1/Th2 types in infected as well as in uninfected immunized mice and the IgG subtype profiles evidenced a predominance of the Th2 response.

We then investigated the relative contribution of the CD4⁺ or CD8⁺ T lymphocytes subpopulations to the immune response generated by the purified proteins. CD4⁺ lymphocytes were very high in immunized mice, while CD8⁺ cells were low; infection by *T. gondii* induced a decrease of CD4⁺ and CD8⁺ lymphocytes. Infection by *T. gondii* produces predominantly CD4⁺ class II-restricted CTL (Canessa et al., 1988; Curiel et al., 1993) and CD8⁺ CTL are not generated (Yang et al., 1995). These CD4⁺ T cells subsequently synthesize large amounts of IFN- γ and IL-2, which partially correlates with our results, where only an increase in IFN- γ is observed (Fig. 8). Compared to the control group, immunization increased the survival rate (80% survival) up to 20 days after challenge, an indication that the mixed Th1/Th2 immune response induced by the purified group of proteins reduced the mortality in the immunized mice.

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