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Vaccine potential of antigen cocktails composed of recombinant *Toxoplasma gondii* TgPI-1, ROP2 and GRA4 proteins against chronic toxoplasmosis in C3H mice

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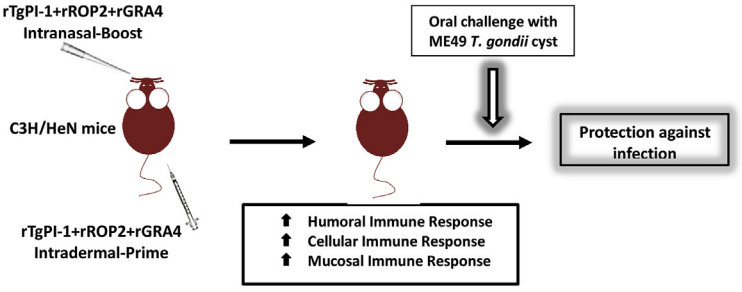
HIGHLIGHTS

- Combination of TgPI-1 with ROP2 and/or GRA4 *Toxoplasma gondii* recombinant proteins for immunization of C3H/HeN mice.
- Vaccine formulations including safe and effective adjuvants suitable to use in humans.
- Combination of intradermal and intranasal immunizations induces mucosal and systemic immune responses.
- The highest level of protection against chronic toxoplasmosis was achieved by the mixture of rTgPI-1 and rROP2.

GRAPHICAL ABSTRACT



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ABSTRACT

The development of an effective and safe vaccine to prevent *Toxoplasma gondii* infection is an important aim due to the great clinical and economic impact of this parasitosis. We have previously demonstrated that immunization with the serine protease inhibitor-1 (TgPI-1) confers partial protection to C3H/HeN and C57BL/6 mice. In order to improve the level of protection, in this work, we combined this novel antigen with ROP2 and/or GRA4 recombinant proteins (rTgPI-1+rROP2, rTgPI-1+rGRA4, rTgPI-1+rROP2+rGRA4) to explore the best combination against chronic toxoplasmosis in C3H/HeN mice. All tested vaccine formulations, administered following a homologous prime-boost protocol that combines intradermal and intranasal routes, conferred partial protection as measured by the reduction of brain cyst burden following oral challenge with tissue cysts of Me49 *T. gondii* strain. The highest level of protection was achieved by the mixture of rTgPI-1 and rROP2 proteins with an average parasite burden reduction of 50% compared to the unvaccinated control group. The vaccine-induced protective effect was related to the elicitation of systemic cellular and humoral immune responses that included antigen-specific spleen cell proliferation, the release of Th1/Th2 cytokines, and the generation of antigen-specific antibodies in serum. Additionally, mucosal immune responses were also induced,

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characterized by secretion of antigen-specific IgA antibodies in intestinal lavages and specific mesenteric lymph node cell proliferation. Our results demonstrate that rTgPI-1+rROP2 antigens seem a promising mixture to be combined with other immunogenic proteins in a multiantigenic vaccine formulation against toxoplasmosis.

1. Introduction

Toxoplasma gondii is an opportunistic pathogen with a worldwide prevalence in animals and humans (one third of the world's population) (Pappas et al., 2009). Infection can cause severe complications in congenitally infected and immuno-compromised people (Pittman and Knoll, 2015), and although in healthy individuals is usually asymptomatic, there has been an increase in the number of reports showing that *T. gondii* chronic infection is not safe (Sinai et al., 2016). The most common form of human infection is through ingestion of cysts present in meat products from infected livestock or vegetables contaminated with oocysts released in the feces of infected cats (Pittman and Knoll, 2015).

Currently available drugs are only effective during the acute phase of infection, but they are unable to eliminate tissue cysts in the chronically infected host. The only commercial vaccine is Toxovax® for veterinary use, which contains live attenuated tachyzoites of the S48 *T. gondii* strain and it is used in sheep and goats to prevent abortions induced by congenital toxoplasmosis. No other vaccines for use in cats, food-producing animals or humans are available so far (Garcia et al., 2014).

Recombinant subunit vaccines have a great potential for prevention of diseases caused by intracellular parasites, as they offer an efficient, safe and an alternative way to immunize humans. This kind of vaccines has been widely used to evaluate the immunogenicity of several antigens in combination with different adjuvant compounds that are not always suitable to use in humans (Zhang et al., 2015). In this context, our laboratory has been investigating the immunogenicity of subunit vaccines based on recombinant proteins in combination with safe and effective adjuvant compounds that could be used in any host (Cuppari et al., 2008; del L. Yácono et al., 2012; Martin et al., 2004; Sánchez et al., 2015, 2011). In the quest for promissory vaccine molecules exhibiting protective value, both GRA4 and ROP2 antigens are considered valuable candidates for prophylactic toxoplasmosis vaccines since they have both shown to induce immune responses that resulted in protection in various mouse strains (Dziadek et al., 2012, 2011; Martin et al., 2004; Sánchez et al., 2011). In the search of novel vaccine targets, we have previously introduced the serine protease inhibitor-1 of *T. gondii* (TgPI-1). We demonstrated that vaccination with recombinant TgPI-1 (rTgPI-1) resulted in partial protection to chronic infection in C3H/HeN and also in C57BL/6 mice (Cuppari et al., 2008; Sánchez et al., 2015).

Since the natural site of *T. gondii* infection is the intestinal mucosal surface, the development of a vaccination strategy that elicits effective immune responses both at systemic and mucosal levels is highly desirable (Garcia et al., 2014). In this sense, we have already shown that immunization with rTgPI-1 following a homologous prime-boost strategy, consisting on intradermal priming with rTgPI-1 plus alum and intranasal boost with rTgPI-1 plus CpG-ODN, induced systemic and mucosal immune responses that resulted in protection of highly susceptible C57BL/6 mice against chronic toxoplasmosis (Sánchez et al., 2015).

Multiantigenic vaccines are usually more powerful and more efficient than monovalent vaccines, due to the possibility to combine antigens expressed at different stages of the parasite, or with different functions in its life cycle. In an effort to find new antigen combinations which result in high levels of protection against infection, in the present work we combined the novel TgPI-1 antigen with ROP2 and GRA4 proteins, in order to explore the best immune-protective combination.

2. Materials and methods

2.1. Animals

Six to eight-week old female C3H/HeN mice were obtained from the animal facilities of the School of Veterinary Sciences, University of Buenos Aires, Argentina, and maintained in our animal facilities for use throughout these experiments. Mice were used at the age of 6–8 weeks. All procedures requiring animals were approved by the Independent Ethics Committee for the Care and Use of Experimental Animals of National University of San Martin (CICUAE protocol ID # 005/16).

2.2. Parasites and preparation of excreted-secreted antigens (ESA) and soluble T. gondii antigens (sTAg)

T. gondii tachyzoites (RH strain) were propagated *in vitro* in human foreskin fibroblast cell line. Excreted-secreted antigens (ESA) were prepared according to the protocol already described and stored at −80 °C until used (Debard et al., 1996). To prepare the soluble *T. gondii* antigens (sTAg), the collected tachyzoites were filtered through a 5-μm polycarbonate filter for host cell exclusion, and the free parasites were recovered and washed by centrifugation. The pellets were suspended in ice-cold water at a parasite density of 10^8 tachyzoites/mL and submitted to sonication until the cells were completely lysed. One volume of 0.3 M NaCl was added to the lysed suspensions, and the suspensions were cleared by centrifugation at 10,000 × g for 3 min at 4 °C. The supernatants were harvested and used as the *T. gondii* antigen after determining the protein concentration.

2.3. Expression and purification of the recombinant proteins

TgPI-1 (rTgPI-1), ROP2 (rROP2) and GRA4 (rGRA4) recombinant proteins were expressed and purified as already described (Cuppari et al., 2008; Sánchez et al., 2015, 2011).

Before mouse inoculation, the recombinant proteins were dialyzed against PBS and endotoxins were removed with Detoxi-Gel endotoxin removal gel (Pierce, Rockford, IL), filtered throughout a 0.22 μm-pore membrane and stored at −80 °C.

2.4. Immunization and challenge

Mice ($n \geq 8$ per group) were immunized according to the protocol described in Table 1, four times with a 2-week interval. rTgPI-1, rROP2, rGRA4 (10 μg each) or PBS, were co-administered with 0,125 mg of aluminum ($Al(OH)_3$, Sigma) intradermally or co-administered with 10 μg of CpG-ODN 1826 (Sigma) intranasally.

Table 1
Immunization strategies.

Group	Formulation/route			
	Day 0	Day 15	Day 30	Day 45
Control	PBS+Alum/*i.d.*	PBS+Alum/*i.d.*	PBS+CpG/*i.n.*	PBS+CpG/*i.n.*
P	rTgPI-1+Alum/*i.d.*	rTgPI-1+Alum/*i.d.*	rTgPI-1+CpG/*i.n.*	rTgPI-1+CpG/*i.n.*
P+R	rTgPI-1+rROP2 +Alum/*i.d.*	rTgPI-1+rROP2 +Alum/*i.d.*	rTgPI-1+rROP2 +CpG/*i.n.*	rTgPI-1+rROP2 +CpG/*i.n.*
P+G	rTgPI-1+rGRA4 +Alum/ *i.d*	rTgPI-1+rGRA4 +Alum/*i.d*	rTgPI-1+rGRA4 +CpG/*i.n.*	rTgPI-1+rGRA4 +CpG/*i.n.*
P+R+G	rTgPI-1+rROP2+rGRA4 +Alum/*i.d.*	rTgPI-1+rROP2 rGRA4 +Alum/*i.d.*	rTgPI-1+rROP2 rGRA4 +CpG/*i.n.*	rTgPI-1+rROP2 rGRA4 +CpG/*i.n.*

i.d.: intradermal; *i.n.*: intranasal; CpG: CpG-ODN.

Two weeks after the last boost, mice were orally challenged with 20 ME49 strain tissue cysts (sublethal dose). One month later, mean number of cysts per brain was determined by observation under optical microscope.

2.5. Measurement of antibody responses

Two weeks after last immunization or one month after oral challenge, antigen-specific antibodies in sera were analyzed by ELISA as previously described (Sánchez et al., 2015, 2011). Microtiter plates were coated with rTgPI-1, rROP2 or rGRA4 (5 μg/ml) or with sTAg (10 μg/ml) in 0.05 M carbonate buffer, pH 9.6. Mouse sera were diluted 1:4000 for IgG and 1:1000 for subclass determination. HRP-conjugated goat anti-mouse IgG, IgG1 or IgG2a (Pharmingen) were used as a secondary antibody. Immune complexes were revealed with trimethylbenzidine substrate (TMB One-Step, Life Technologies). Plates were read in a plate reader (Sunrise RC, Tecan) at 450 nm with λ correction at 620 nm.

2.6. In vitro proliferation assays and cytokine production

Spleens and mesenteric lymph nodes (MLN) were removed from mice 2 weeks after last immunization. A single-cell suspension of cells was prepared by homogenization as it was described in our previous studies (Sánchez et al., 2015, 2011).

Cells (5×10^5) were seeded in triplicates in 96-wells plates in 100 μl of culture medium and stimulated with an equal volume of medium with ESA (25μl/well), rTgPI-1, rROP2 or rGRA4 in a final concentration of 10 μg/ml (Sánchez et al., 2015, 2011). After 5 days of culture, proliferative responses were assessed by addition of methyl-^{3}H-thymidine (1 μCi/well, PerkinElmer, Argentina) for the last 18 h. Incorporated radioactivity was measured in a liquid scintillation beta-counter (Beckman). From the row data obtained, an index was calculated as cpm incorporated by stimulated cells over those cultured with medium alone. Levels of lymphoproliferation are presented as mean normalized stimulation index (SI) values ± SD: individual SI value for each mouse after stimulation with the respective recombinant antigens or ESA/mean SI value of the control group. Cytokine production was measured in supernatants after 72 h of antigen stimulation, by capture ELISA commercial kits (BD Biosciences).

2.7. Determination of secretory IgA in intestinal lavages

Two weeks after the last immunization, five mice per group were euthanized and intestinal lavages were obtained. The small intestine was flushed with several aliquots of PBS supplemented with 50 mM EDTA, 5% fetal bovine serum (FBS, GIBCO, Carlsbad, CA) and 10 mM PMSF in a final volume of 1 ml, centrifuged to remove debris (10 min at 3000×*g*), and supernatant fluids were stored at −80 °C. The amount of total and antigen-specific IgA present in the lavages was determined by ELISA as previously described (del L. Yácono et al., 2012; Sánchez et al., 2015). To establish the IgA standard curve, plates coated with goat anti-mouse IgA (Pharmingen) as capture antibody were further incubated with serial dilutions of purified mouse IgA (Pharmingen). As secondary antibody, biotinylated goat anti-mouse IgA (Pharmingen) was used and plates were revealed with trimethylbenzidine substrate according to the manufacturers' protocols (TMB One-Step; Dako, Carpenteria, CA, USA). The supernatants were added to plates coated with 10 μg/ml of the corresponding antigen to measure specific-sIgA levels. Specific sIgA amounts were calculated using the IgA standard curve described before. To compensate for variations in the efficiency of recovery of secretory antibodies between animals, the results were normalized and expressed as percentage of antigen-specific IgA with respect to the total amount of IgA present in the sample.

2.8. Statistical analysis

Representative results are presented as mean ± SEM. The mean of each variable (total IgG, IgG_1, IgG_{2a}, IFN-γ, IL-10, IL5, cell proliferation and cyst number) was compared between the different groups using one-way ANOVA with Bonferroni test “a posteriori”. Statistical analysis and graphics were carried out with Prism 5 Software (GraphPad, San Diego, CA). Statistical significance was accepted when $p < .05$.

3. Results

3.1. Humoral immune response

A prime boost protocol was developed to study whether GRA4 and ROP2 recombinant proteins could improve the immune protective response elicited with TgPI-1 immunization. C3H/HeN mice were intradermally primed and intranasally boosted with rTgPI-1+rROP2 (P+R), rTgPI-1+rGRA4 (P+G) and rTgPI+rROP2+rGRA4 (P+R+G) formulations, according to the protocols described in Table 1. In order to compare the type of immune response generated and extent of protection of these antigen combinations with rTgPI-1 single vaccination, a group of mice was immunized with rTgPI-1 alone (P).

To estimate the level of the humoral responses induced by vaccination, total soluble *T. gondii* RH strain tachyzoite antigens (sTAg) and rTgPI-1, rROP2 and rGRA4 proteins were probed by ELISA with sera collected two weeks after the immunization schedule was completed. As shown in Fig. 1A, rTgPI-1 induced the highest IgG production with similar levels in all experimental groups. Anti-rROP2 IgG antibodies were also detected, with significantly higher levels in the P+R+G group compared with P+R. While both P+G and P+R+G vaccinated groups generated anti-rGRA4 IgG antibodies, the level of the responses were very low compared to those generated by rTgPI-1 and rROP2 antigens. The distribution of IgG1 and IgG2a antibody subclasses are shown in Fig. 1B. rTgPI-1 induced significant production of both isotypes

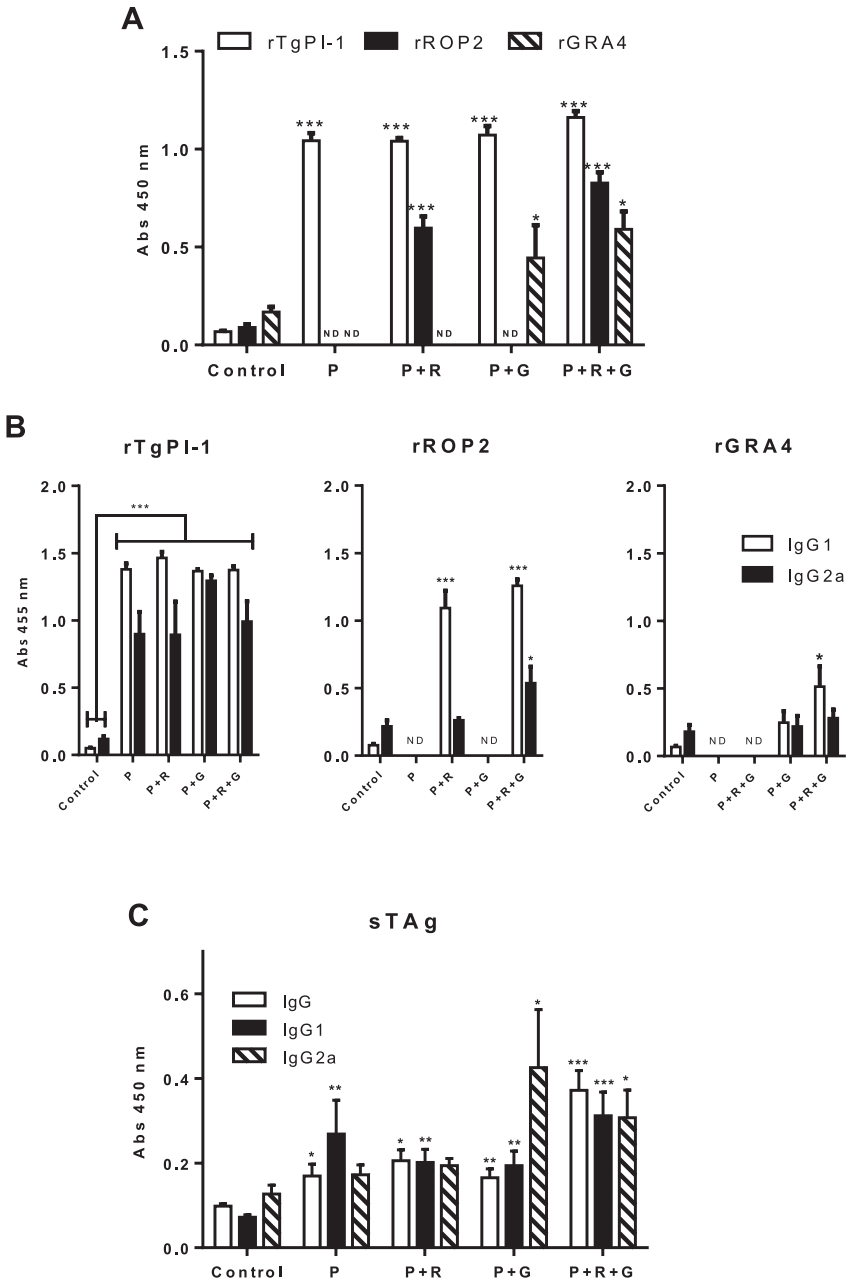

Fig. 1. Determination of humoral responses in vaccinated mice. Animals were immunized with rTgPI-1 (P), rTgPI-1+rROP2 (P+R), rTgPI-1+rGRA4 (P+G), or rTgPI-1+rROP2+rGRA4 (P+R+G) or PBS (Control) according to the protocol described in Table 1. Two weeks after the immunization schedules were completed, serum samples (6 mice per group) were obtained and IgG **(A)**, IgG1 and IgG2a sub-classes **(B)** were determined by ELISA with rTgPI-1, rROP2 or rGRA4 or **(C)** sTAg as the bound targets. Each bar represents the group mean ± SEM. $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .005$ *vs.* Control group. ND: not done.

in all experimental groups, with higher levels of IgG1 compared to IgG2a. On the other hand, rROP2 generated a predominant Th2 response both in the P+R and P+R+G vaccinated mice, while rGRA4 elicited only IgG1 antibodies in very low levels in the P+R+G group.

Additionally, we determined the immune response against natural proteins expressed by *T. gondii* tachyzoites (sTAg) (Fig. 1C). All vaccinated groups displayed significant levels of IgG antibodies, and the trivalent vaccine P+R+G showed the highest response. From the levels of IgG1 and IgG2a subclasses detected, it can be inferred that P and P+R groups generated responses with a Th2 profile, while both P+G and P+R+G groups showed a mixed Th1/Th2 immunity.

3.2. In vitro spleen cell proliferation and cytokine production

Two weeks after the last immunization, splenocyte proliferative responses to the antigens were assessed in the different groups of mice. As shown in Fig. 2, all vaccinated groups presented stimulation indexes (SI) significantly higher than that of the control group after stimulation with excreted-secreted antigens (ESA). Additionally, significant antigen-specific lymphoproliferation was induced by rTgPI-1 in all the groups, while only P+G vaccinated mice responded to rGRA4 stimulation. No positive responses could be detected after rROP2 stimulation.

The type of cellular immune response elicited by the different vaccine formulations was evaluated by the determination of

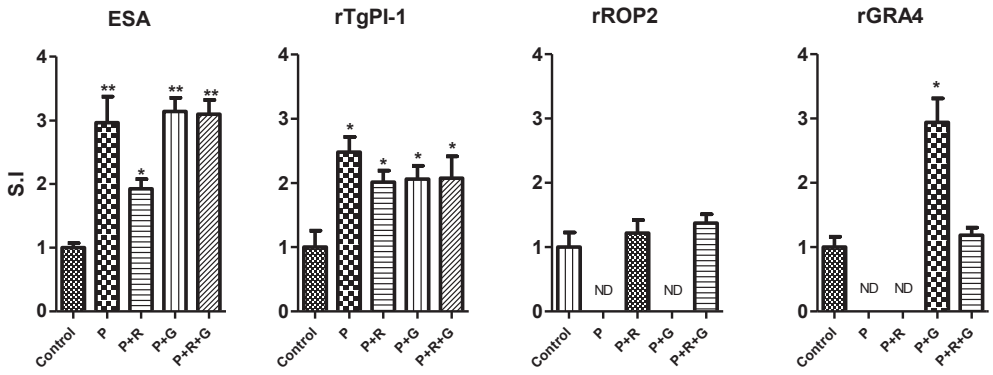

Fig. 2. *In vitro* proliferative responses of splenocytes from vaccinated and control mice. Two weeks after the immunization schedules were completed, splenocytes from vaccinated and control mice were cultured *ex vivo*, and proliferative responses were measured after ESA, rTgPI-1, rROP2 or rGRA4 stimulation. Levels of lymphoproliferation are presented as mean normalized stimulation index (SI) values ± SD detected with [^{3}H]-thymidine assay. SI values were calculated as: cpm of stimulated cells/cpm non-stimulated cells. $^*p < .05$; $^{**}p < .01$ *vs.* Control group. ND: not done.

cytokine release in the supernatant of antigen-stimulated spleen cell cultures. As shown in Fig. 3, the P+G vaccinated group released significant amounts of IFN-γ, IL-5 and IL-10 upon stimulation with ESA, rGRA4 or rTgPI-1. On the other hand, P+R+G splenocytes stimulated with rTgPI-1 or rGRA4 secreted both IFN-γ and IL-5, while IL-10 could be detected only after rTgPI-1 stimulation. Spleen cells from the P vaccinated group elicited IL-5 and IL-10 upon stimulation with rTgPI-1, while ESA-stimulated cells secreted only IL-5. Splenocytes from P+R immunized mice showed the weakest cellular response, with a trend towards a production of IFN-γ and IL-5 after ROP2 stimulation, and secretion of IL-5 and IL-10 in response to ESA and rROP2 stimulation respectively.

3.3. Mucosal immune response

Considering that the natural site of infection for *T. gondii* is the mucosal surface of the intestine, we investigated the mucosal immune responses generated by the different vaccine formulations. Intestinal lavages were collected 15 days after the immunization schedules were completed, and the presence of specific secretory immunoglobulin A (sIgA) anti-rTgPI-1, -rROP2 or -rGRA4 was evaluated by ELISA. Significant levels of anti-rTgPI-1 sIgA were observed in secretions from all vaccinated groups when compared to the control group (Fig. 4). Anti-rROP2 sIgA antibodies were detected in both P+R and P+R+G groups, while anti-rGRA4 antibodies were detected only in the P+R+G vaccinated group.

In order to study activation of the mucosal compartments, T cell-mediated immune responses were investigated in mucosal draining distal mesenteric lymph nodes (MLN). As shown in Fig. 5, the groups of mice vaccinated with the mix P+G and P+R+G exhibited a significant higher lymphoproliferative response of MLN cells compared to the control group after stimulation with ESA. In contrast, the P+R group showed a trend towards a positive response, while mice vaccinated only with rTgPI-1 resulted similar to the control group. Additionally, in the P+R and P+R+G groups, antigen-specific responses were induced by stimulation with all the correspondent recombinant antigens. In contrast, in the P+G group only rTgPI-1 induced significant cell proliferation, while no antigen-specific responses could be detected after stimulation with rGRA4.

3.4. Protection against infection

To evaluate the protective effect of the multiantigenic vaccines against chronic toxoplasmosis, two weeks after the last immunization mice were orally challenged with a non-lethal dose of *T. gondii* Me49 tissue cysts and one month later brain cysts were enumerated. As shown in Fig. 6, vaccination with the different proteins partially protected mice against chronic infection. The brain tissue cyst loads in vaccinated mice with the different protein formulations varied from 26% to 50% and were significantly lower compared to non-vaccinated mice ($p < .05$). Combination of rTgPI-1 with rROP2 provided more effective protection to the mice (50%) than the obtained in the other vaccinated groups of mice. However, the differences were not statistically significant ($p > .05$).

3.5. Humoral response after infection

In addition, we characterized the humoral response elicited in each vaccinated group of mice one month after parasite challenge. As shown in Fig. 7A, high levels of IgG anti-rTgPI-1 were observed in all experimental groups which resulted significantly increased

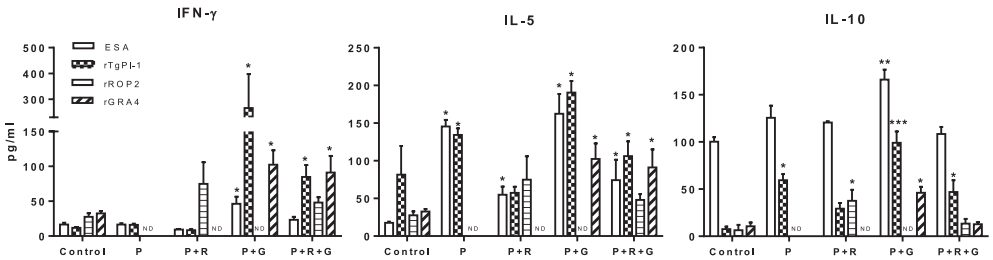

Fig. 3. Cytokine production elicited by spleen cells after vaccination. Two weeks after the immunization schedules were completed, splenocytes from vaccinated and control mice were cultured *ex vivo*, and cytokine production after ESA, rTgPI-1, rROP2 or rGRA4 stimulation was measured in cell supernatants by ELISA. Each bar represents the group mean ± SEM. $^*p < .05$, $^{**}p < .01$ and $^{***}p < .005$ *vs.* Control group. ND: not done.

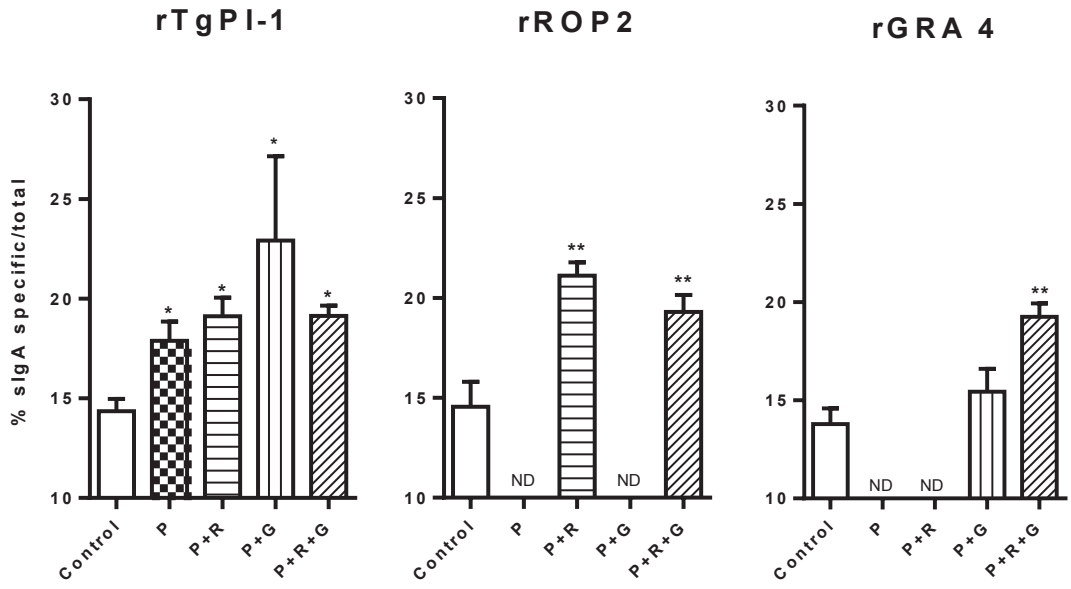

Fig. 4. Mucosal humoral immune response. Determination of specific anti-rTgPI-1, anti-rROP2 and anti-rGRA4 IgA in intestinal lavages (IL). Two weeks after the last immunization, IL samples from vaccinated or Control mice were obtained, and antibody concentration was determined by ELISA with rTgPI-1, rROP2 or rGRA4 as the bound targets. Results are expressed as the percentage of antigen-specific IgA with respect to the total IgA present in the sample ±SEM. $^{*}p < .05$ and $^{**}p < .01$ *vs.* Control group. ND: not done.

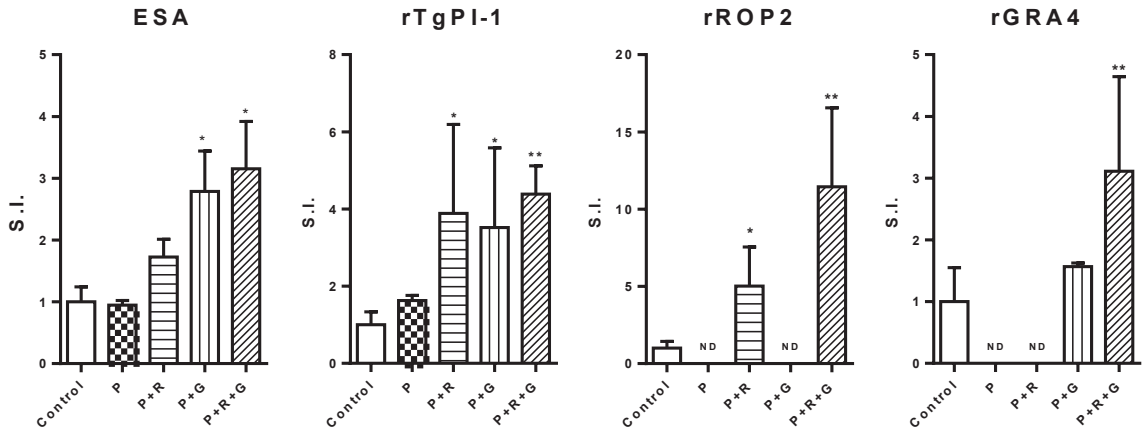

Fig. 5. Antigen specific *in vitro* proliferative response of mesenteric lymph node cells from vaccinated and control mice. Proliferative responses of mesenteric lymph node (MLN) cells from vaccinated and control mice were determined 2 weeks after the last immunization by [^{3}H]-thymidine incorporation after stimulation with ESA, rTgPI-1, rROP2 or rGRA4. Levels of lymphoproliferation are presented as mean normalized stimulation index (SI) values ± SD detected with [3H]-thymidine assay. SI values were calculated as: cpm of stimulated cells/cpm non-stimulated cells. ± SEM. (n = 6 animals per group). $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .005$ *vs.* Control group. ND: not done.

compared to non-immunized infected mice (control group). On the other hand, all immunized mice showed similar levels of anti-rROP2 and anti-rGRA4 IgG antibodies which resulted also similar to the control group. The levels of specific antibodies observed in the non immunized infected mice show that *T. gondii* infection generates strong humoral responses against ROP2 and GRA4 antigens, while against TgPI-1 this response was weak. As shown in Fig. 7B, similar levels of anti-rTgPI-1 IgG1 and IgG2a antibody subclasses were detected in all vaccinated mice which resulted both significantly higher compared to the control group. Similarly, anti-rROP2 response showed the same profile. Regarding the response generated against rGRA4, a predominant Th1 profile was detected not only in control but also in immunized mice. However, while no IgG1 was produced in control mice, both P+G and P+R+G vaccinated groups showed increased levels of this subclass. Moreover, the highest levels were obtained in the P+R+G group. Only specific-IgG2a antibodies against the 3 antigens were observed in the infected control group, corresponding to the Th1 profile that is expected after *T. gondii* infection.

4. Discussion

Recombinant subunit vaccines based on DNA or proteins have the potential to induce long lasting immune responses, as well as being safer and present lower overall costs (Lim and Othman, 2014). Accumulating evidence so far indicates that for *T. gondii*, the most effective strategy using recombinant proteins should consider the use of antigens expressed at the different stages in a multiantigenic combining vaccine. In the present study, we focused on the immunogenic and protective value of the novel TgPI-1 antigen when combined with two well-known antigen candidates, ROP2 and GRA4 proteins, in order to explore the best immune-protective combination in a murine model of chronic infection.

Both ROP2 and GRA4 antigens have shown protective efficacy

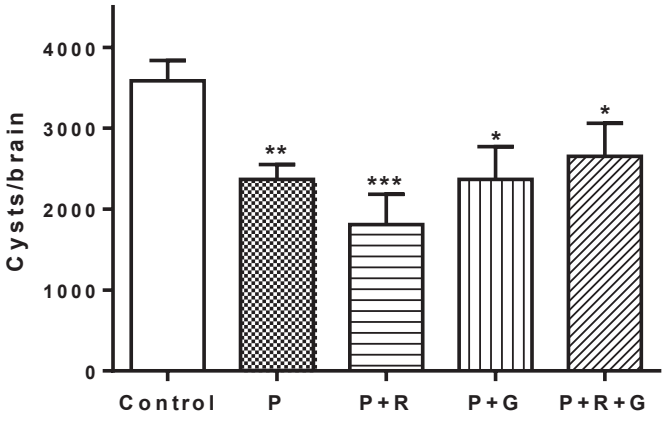

Fig. 6. Protection against chronic *Toxoplasma* infection in vaccinated C3H/HeN. Animals were immunized with rTgPI-1 (P group), rTgPI-1+rROP2 (P+R group), rTgPI-1+rGRA4 (P+G group), or rTgPI-1+rROP2+rGRA4 (P+R+G group) or PBS (Control group) according to the protocol described in Table 1. Two weeks after the last vaccination dose, mice were orally challenged with 20 tissue cysts of ME49 *T. gondii* strain (sublethal dose). One month later mice were sacrificed and their brains removed for cyst load determination. Each bar represents the mean total brain cyst number ± SEM. $^{*}p < .05$, $^{**}p < .001$, $^{***}p < .005$ *vs.* Control group.

against *T. gondii* infection in various mouse models. Previous results from our group showed that the bivalent recombinant subunit vaccine composed of these proteins, partially protected C3H/HeN (Martin et al., 2004) and C57BL/6 (Sánchez et al., 2011) mice from chronic toxoplasmosis. On the other hand, regarding TgPI-1 antigen, we have previously shown that intramuscular vaccination with this protein in combination with alum conferred partial protection in C3H/HeN mice (Cuppari et al., 2008), while in C57BL/6 mice a significant brain cyst load reduction was achieved following a homologous prime-boost immunization strategy (Sánchez et al., 2015). Herein, to study whether GRA4 and ROP2 recombinant proteins could improve the immune protective response elicited with TgPI-1 immunization, we compared the protective effects of rTgPI-1 (P), rTgPI-1+rGRA4 (P+G), rTgPI-1+rROP2 (P+R) and rTgPI-1+rGRA4+rROP2 (P+R+G) formulations in combination with alum and CpG-ODN in C3H/HeN mice, against an oral challenge with a non-lethal dose of ME49 *T. gondii* cysts. All formulations induced significant decreases in the number of cysts per brain compared to the control group. Many studies have demonstrated the advantages of multivalent over bivalent and monovalent vaccines for toxoplasmosis (Lim and Othman, 2014). Among them, a very high reduction in brain tissue cyst loads were obtained through immunization of mice with ROP2 or GRA4 antigens in combination with others like SAG1, ROP4 or GRA7 in multiantigenic vaccines (Dziadek et al., 2012, 2011; El Bissati et al., 2014). Herein, addition of the mixture of rROP2 and rGRA4 to rTgPI-1 failed to improve the degree of protection compared to vaccination with TgPI-1 alone. So in our hands, combination of the three antigens did not result in a synergistic protective effect. However, the addition of rROP2 (rTgPI-1+rROP2) increased the protection by 16%, appearing as the most successful vaccination procedure since P+G vaccinated mice exhibited the lowest parasite burden in the brain (50% of reduction).

Several studies have shown the involvement of the humoral response in providing protective immunity. Specific antibodies can limit the propagation of *T. gondii* by inhibiting the attachment of tachyzoites to host cell receptors and promoting parasite killing either by activation of the complement system or by opsonization of the parasites for phagocytosis and macrophage killing (Dupont et al., 2012). Consistent with previous reports (Cuppari et al., 2008; Sánchez et al., 2015, 2011), our results showed that all vaccinated groups of mice generated high specific IgG responses. While rTgPI-1 elicited a mixed Th1/Th2 type response with the highest levels of IgG antibodies in all groups of mice, rROP2 generated a Th2 profile. Surprisingly, the levels of anti-rGRA4 antibodies were remarkably low. These results indicate that rTgPI-1 antigen possesses a predominant humoral immunogenic value in this particular mix of antigens. Contrary to our results, other studies showed that recombinant GRA4 administered individually or as a component of a multiantigenic vaccine in combination with other antigens and with different adjuvants, induced high levels of specific antibodies (Dziadek et al., 2012, 2011; Martin et al., 2004; Sánchez et al., 2011). The absence of a specific anti-rGRA4 humoral response could be related to the use of this particular vaccination protocol, or that combination with rTgPI-1 antigen decreased its immunogenicity. The failure in the improvement of the level of protection in rGRA4 containing formulations could be related to the absence of specific antibodies generated by vaccination, since this protein plays an important role during cell penetration and intracellular survival of the parasite in the infected host (Nam, 2009).

When analyzing the antigen-specific splenocyte proliferation, we found that rTgPI-1 evoked a significant response in all immunized mice, rGRA4 only in rTgPI-1+rGRA4 vaccinated mice and no response could be detected upon rROP2 stimulation. On the other hand, splenocytes from all vaccinated groups proliferated after stimulation with ESA, suggesting their capability of responding to native forms of the proteins tested as potential vaccine components. Although mice immunized with rTgPI-1+rGRA4 showed better proliferative responses - *i.e.*, against ESA, rTgPI-1 and rGRA4, no improvement in protection could be elicited by this formulation compared to rTgPI-1 vaccine.

Immunization of mice with rTgPI-1 alone resulted in a cellular response with a Th2 profile. In contrast to these results, we have previously observed that intramuscular immunization of C3H/HeN mice with rTgPI-1 in combination with alum induced a systemic cellular Th1 response (Cuppari et al., 2008), while in C57BL/6 mice, using the same homologous prime-boost immunization protocol used in the present work, no systemic response could be detected (Sánchez et al., 2015). As we previously mentioned, ROP2 and GRA4 antigens were used in several vaccine formulations. Mice vaccinated with rROP2+rGRA4 in combination with CpG showed a Th1 cellular profile whereas in combination with alum a mixed Th1/Th2 response was generated (Martin et al., 2004; Sánchez et al., 2011). Other studies which used multiantigenic vaccines that include ROP2, GRA4, ROP4 and SAG1 antigens have shown little or no production of cytokines upon *in vitro* stimulation (Dziadek et al., 2012, 2011). Herein, vaccination with the mixture of rTgPI-1+rGRA4 or rTgPI-1+rROP2+rGRA4 induced a response with a mixed Th1/Th2 profile while splenocytes from mice immunized with rTgPI-1+rROP2 showed no cytokine secretion. All these results reinforce the concept that the outcome of the immune responses generated by a vaccine is influenced by many parameters such as the antigen composition, the adjuvants used, the administration strategy and also the mouse strain.

Considering that *T. gondii* gateway is the intestinal mucosa, efficient stimulation of a local response at this level seems to represent a priority that might be achieved by the administration of an oral or nasal vaccine. IgA secretion at mucosal surfaces is the main humoral mediator that can contribute to pathogen elimination, limiting the spread of *T. gondii* to other tissues such as muscle and brain (Ju et al., 2009). Herein, significant sIgA antibody responses against the three antigens were detected in intestinal washes from all the three groups of mice vaccinated with mixtures of antigens. However, the rTgPI-1+rGRA4 formulation didn't seem to induce sIgA against rGRA4.

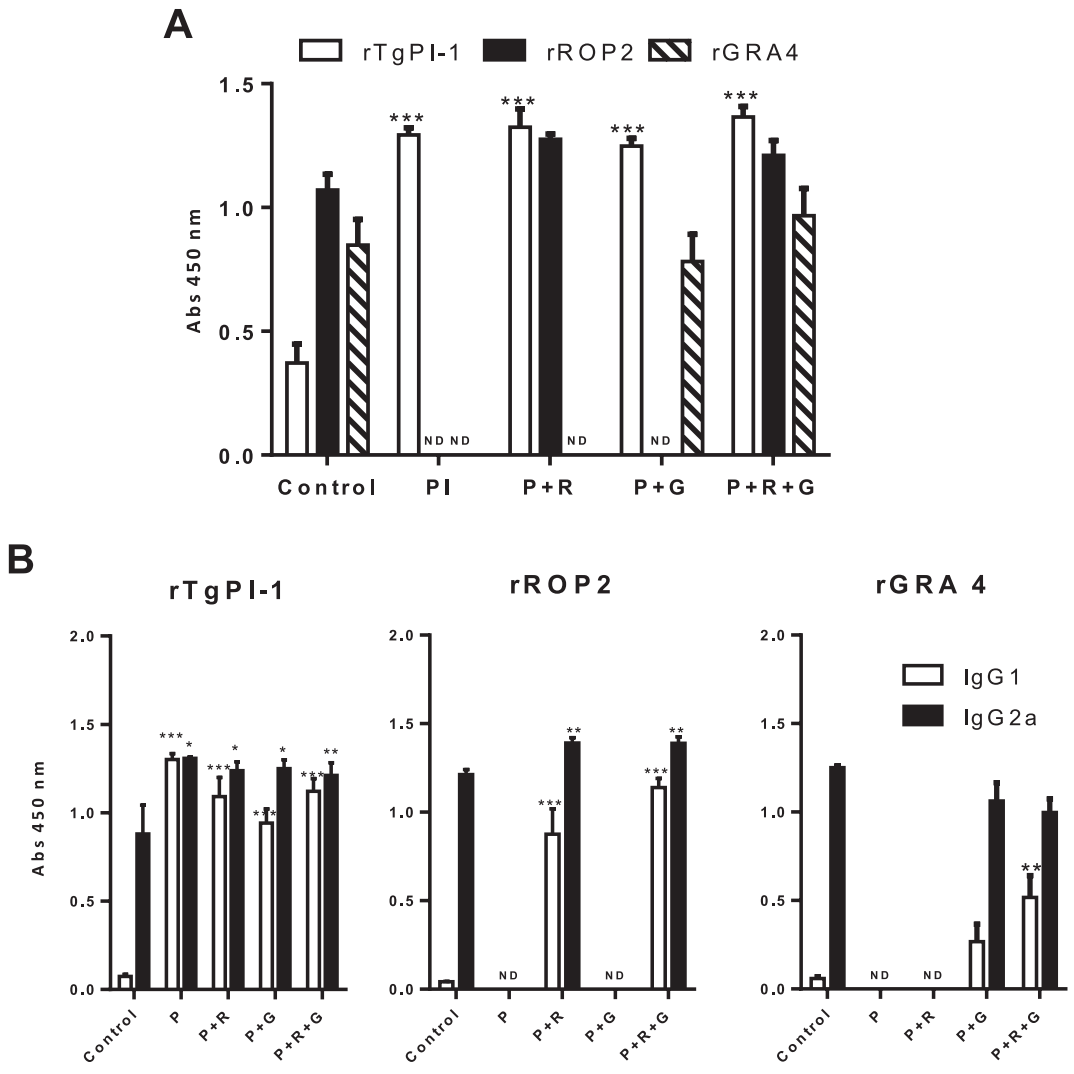

Fig. 7. Determination of humoral responses in vaccinated mice after the challenge. Two weeks after the immunization schedules were completed, mice were challenged with Me49 cysts. One month after infection serum samples (6 mice per group) were obtained and IgG **(A)**, IgG1 and IgG2a sub-classes **(B)** were determined by ELISA with rTgPI-1, rROP2 or rGRA4 as the bound targets. Each bar represents the group mean ± SEM. $^{*}p < .05$, $^{**}p < .001$, $^{***}p < .005$ *vs.* Control group.

Mucosal defenses are also mediated by a cellular immune response (Dimier-Poisson et al., 2003). After oral infection with *T. gondii*, the initial T cell activation likely occurs in Peyer's patches and mesenteric lymph nodes. Since this is the first lymphatic organization draining the gut, activation of lymphocytes at this site might account for protection. In our work, MLN cells from the P+G and P+R+G groups significantly proliferated after *in vitro* stimulation with ESA, whereas no response was observed in the single rTgPI-1 immunized group or the P+R group. Taken together, these results demonstrate that the rTgPI-1+rROP2+rGRA4 formulation resulted more effective in generating mucosal immune responses compared to single rTgPI-1 vaccination or the bivalent vaccines. However, even though the trivalent formulation induced the production of IgA and T cell proliferation against the three antigens, this vaccine didn't prove to be more effective than the other formulations.

Although the present results are promising in terms of inducing humoral and cellular immune responses at systemic and mucosal levels, protection was limited. A growing body of current research has focused on cocktail or multi-antigenic vaccines based on DNA or recombinant forms of proteins (Ching et al., 2016; Dziadek et al., 2012, 2011; Gedik et al., 2016; Pinzan et al., 2015; Zhang et al., 2016, 2015). Herein, we present the first report that evaluates the capacity of the rTgPI-1 protein in a multiantigenic vaccine formulation to increase the protection level against chronic infection. Unexpectedly, our results show that combination of this antigen with rROP2 or rGRA4 proteins may not have a synergistic effect to achieve this goal when compared to rTgPI-1 single vaccine efficacy. Future studies will focus on improving the multivalent vaccine immunogenicity by combining rTgPI-1 with other immunogenic proteins also expressed by all forms of the parasite.

Finally, our data also demonstrate that, when using C3H/HeN mice, the homologous prime-boost immunization protocol used in this work doesn't show to improve the protection compared to a single immunization route (Cuppari et al., 2008). Since this prime-boost protocol had enhanced the protective efficiency of rTgPI-1 in the highly susceptible C57BL/6 mice (Sánchez et al., 2015), our results emphasize the importance of testing experimental vaccines in mouse strains with different susceptibility to infection.

5. Conclusions

The present study demonstrate that immunization of C3H/HeN mice with rTgPI-1, rTgPI-1+rROP2, rTgPI-1+rGRA4 or rTgPI-

1+rROP2+rGRA4 formulations following a homologous prime-boost protocol that combines intradermal and intranasal routes, conferred partial protection against chronic toxoplasmosis. The vaccine-induced protective effect was related to the elicitation of systemic and mucosal immune responses. The highest level of protection was achieved by the mixture of rTgPI-1 and rROP2 proteins.

Conflict of interest

This work represents no conflict of interest for none of the authors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.exppara.2018.01.006.

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