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Homologous prime-boost strategy with TgPI-1 improves the immune response and protects highly susceptible mice against chronic *Toxoplasma gondii* infection

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ABSTRACT

Subunit-based vaccines are safer than live or attenuated pathogen vaccines, although they are generally weak immunogens. Thus, proper combination of immunization strategies and adjuvants are needed to increase their efficacy. We have previously protected C3H/HeN mice from *Toxoplasma gondii* infection by immunization with the serine protease inhibitor-1 (TgPI-1) in combination with alum. In this work, we explore an original vaccination protocol that combines administration of recombinant TgPI-1 by intradermal and intranasal routes in order to enhance protection in the highly susceptible C57BL/6 strain. Mice primed intradermally with rTgPI-1 plus alum and boosted intranasally with rTgPI-1 plus CpG-ODN elicited a strong specific Th1/Th2 humoral response, along with a mucosal immune response characterized by specific-IgA in intestinal lavages. A positive cellular response of mesentheric lymph node cells and Th1/Th2 cytokine secretion in the ileon were also detected. When immunized mice were challenged with the cystogenic Me49 *T. gondii* strain, they displayed up to 62% reduction in brain parasite burden. Moreover, adoptive transfer of mesenteric lymph node cells from vaccinated to naïve mice induced significant protection against infection. These results demonstrate that this strategy that combines the administration of TgPI-1 by two different routes, intradermal priming and intranasal boost, improves protective immunity against *T. gondii* chronic infection in highly susceptible mice.

1. Introduction

Toxoplasma gondii is an obligate intracellular parasite that infects the nucleated cells of warm blooded vertebrates, including humans (30–40% worldwide population) (Dubey and Su, 2009; Montoya and Liesenfeld, 2004). The main source of human infection is by ingestion of *T. gondii* tissue cysts in meat of infected livestock (Cook et al., 2000). At present, there are no drug treatments available to eliminate the parasite once chronic infection in human beings or animals is established (Coombs and Müller, 2002). The only vaccine commercially available, used in farm animals, is Toxovax® (Intervet Schering Plough, Boxmeer, The Netherlands), a live vaccine based on the attenuated S48 strain (Buxton and Innes, 1995). Since this is a live vaccine, it has a short shelf-life and, moreover, it is not safe to be used in humans. Therefore, much effort has been focused on the replacement of the attenuated vaccine with subunit vaccines based on parasite antigens (Jongert et al., 2009). In the search of novel vaccine targets, we have previously introduced the serine protease inhibitor-1 of *T. gondii* (TgPI-1) as a new antigen. This inhibitor is expressed during the three stages of the parasite and is secreted constitutively from dense granules to the excreted/secreted antigen fraction (ESA) (Morris et al., 2002) and also within the parasitophorous vacuole where *T. gondii* replicates in host cells. Moreover, since TgPI-1 is capable to inhibit a broad range of proteases including neutrophil elastase (Morris et al., 2002; Pszenny et al., 2002), it could be involved in parasite immune evasion. These properties let us hypothesize that this new antigen is an attractive vaccine candidate.

We have already shown that intramuscular vaccination with recombinant TgPI-1 (rTgPI-1) in combination with alum resulted in protection in C3H/HeN mice (Cuppari et al., 2008), with intermediate susceptibility to *T. gondii* infection. Since this strategy was not

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effective in the highly susceptible C57BL/6 mouse strain (unpublished data), the aim of the present study was to further investigate new TgPI-1-based vaccination strategies that could provide protective immunity in these highly susceptible mice. 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 (Buzoni-Gatel et al., 2006; Kasper et al., 2004). Several studies have been carried out using the intranasal route in mice as an alternative to obtain immunity against *T. gondii* infection (Hedhli et al., 2009; Lim and Othman, 2014; MA et al., 2014; Wang et al., 2014). Some of them have used cholera toxin, an adjuvant not recommended in humans due to its well-known pathogenicity (Debard et al., 1996; Igarashi et al., 2008; Velge-Roussel et al., 2000). Herein, we explored an original homologous prime-boost immunization protocol using rTgPI-1 administered by two different routes with adjuvant compounds approved for human vaccines. Mice intradermally primed with rTgPI-1 in combination with Alum and intranasally boosted with rTgPI-1 plus CpG-ODN, showed a significant reduction in brain parasite burden after a nonlethal challenge with *T. gondii* cysts.

2. Materials and methods

2.1. Animals

Six to eight-week old female C57BL/6 mice were obtained from the Biotechnology Research Institute (IIB), National University of General San Martin (UNSAM), Buenos Aires, Argentina. All procedures requiring animals were performed in agreement with institutional guidelines and were approved by the Independent Ethics Committee for the Care and Use of Experimental Animals of UNSAM (C.I.C.U.A.E., IIB-UNSAM).

2.2. Parasites and preparation of excreted-secreted antigens

T. gondii tissue cysts (ME49 strain) were used to challenge mice and maintained in C3H/HeN as already described (Cuppari et al., 2008; Sánchez et al., 2011). *T. gondii* tachyzoites (RH strain) were propagated *in vitro* in human foreskin fibroblast cells. Excreted-secreted antigens (ESA) were prepared according to the protocol already described and stored at −80 °C until used (Golkar et al., 2007). As previously shown by Morris et al. (2002), TgPI-1 is secreted in the ESA fraction of the parasite. The presence of this protein in our ESA preparation was confirmed by Western Blot analysis. ESA proteins (10X) and rTgPI-1 (∼41 kDa) were resolved in 12,5% SDS-PAGE gel (Bio-Rad) under reducing conditions, and proteins were transferred to nitrocellulose membranes using a Transblot SD semi-dry transfer cell (Bio-Rad). Western blot was probed with mouse polyclonal anti-rTgPI-1 and alkaline phosphatase conjugated goat anti-mouse IgG (Sigma). The reaction was developed by the addition of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate. Prestained Protein Ladder (Life Tecnologies™) was used as molecular marker (Supplementary Figure).

2.3. Expression and purification of the recombinant protein

Recombinant TgPI-1 protein (rTgPI-1) was expressed and purified as already described (Cuppari et al., 2008). Before mouse inoculation, rTgPI-1 was dialyzed against PBS and endotoxins were removed with Detoxi-Gel™ Endotoxin Removing Columns (Pierce protein research products), filtered throughout a 0.22 μm-pore membrane and stored at −80 °C.

Table 1
Immunization strategies and control groups.

Group	Formulation/route			
	Day 0	Day 15	Day 30	Day 45
Control	PBS + Alum/I.D.	PB + Alum/I.D.	PBS + CpG/I.N.	PBS + CpG/I.N.
I.D.	PI + Alum/I.D.	PI + Alum/*i* I.D.	PI + Alum/I.D.	PI + Alum/I.D.
I.N.	PI + CpG/I.N..	PI + CpG/I.N.	PI + CpG/I.N.	PI + CpG/I.N..
I.D. + I.N.	PI + Alum/I.D.	PI + Alum/I.D.	PI + CpG/I.N.	PI + CpG/I.N.
PI I.D. + PBS I.N.	PI + Alum/I.D.	PI + Alum/I.D.	PBS + CpG/I.N.	PBS + CpG/I.N.
PBS I.D. + PI I.N.	PBS + Alum/I.D.	PBS + Alum/I.D.	PI + CpG/I.N.	PI + CpG/I.N.

PI: rTgPI-1; I.D.: intradermal; I.N.: intranasal; CpG: CpG-ODN.

2.4. Immunization and challenge

Mice were immunized four times with a 2-week interval according to the protocols described in Table 1. rTgPI-1 (10 μg) or PBS, plus 0125 mg of Alum (Al_2O_3, alhydrogel; Superfos Biosector a/s) in a final volume of 40 μl per dose was administered intradermally in the mouse base tail while rTgPI-1 (10 μg) or PBS plus 10 μg of CpG-ODN 1826 (Sigma) in a final volume of 6 μl per dose was administered intranasally (3 μl in each nostril). Two weeks after the last boost, mice ($n = 8$ per group) were orally challenged with 20 ME49 strain tissue cysts (non-lethal dose). One month after the challenge mice were sacrificed, their brains were removed and homogenized in 2 ml of PBS with a dounce tissue grinder. The mean number of cysts per brain was determined by observation under an optical microscope, by counting four samples of 25-μl aliquots of each homogenized brain.

2.5. Measurement of antibody responses

Two weeks after last immunization or one month after oral challenge, serum rTgPI-1-specific antibodies from 6 mice per group were analyzed by ELISA as previously described (Cuppari et al., 2008). Microtiter plates were coated with rTgPI-1 (5 μg/ml). Mouse sera were diluted 1:4,000 for IgG and 1:1,000 for subclass determination. HRP-conjugated goat anti-mouse IgG, IgG1 or IgG2b (Pharmingen) were used as a secondary antibody. Immune complexes were revealed with trimethylbenzidine substrate (TMB One-Step; Dako, Carpenteria, CA, USA). Plates were read in a plate reader (Sunrise RC, Tecan) at 450 nm with λ correction at 570 nm after the addition of stop solution (H_2SO_4). Intestinal lavages (LI) from 5 mice per group were obtained 2 weeks after last boost and stored at −80 °C (del L. Yácono et al., 2012). Total and rTgPI-1-specific secretory IgA was determined by ELISA as previously described (del L. Yácono et al., 2012). Results were normalized and expressed as percentage of rTgPI-1-specific IgA with respect to total amount of IgA.

2.6. Proliferation assays and flow cytometry

Mesenteric lymph nodes (MLN) were removed from 6 mice per group 2 weeks after last immunization. Cells (5×10^5) were *in vitro* stimulated with ESA (25 μl/well) (Golkar et al., 2007). After 72 h of culture, cells were stained with FITC-conjugated anti-mouse CD4 and PE-conjugated anti-mouse CD8 (BD, Biosciences) and acquired using a FACScan flow cytometer (BD, Mountain View, CA). Results were analyzed using WinMDI 2.9 software (De Novo Software, CA). MLN proliferative responses were assessed after stimulation with ESA for 5 days 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.

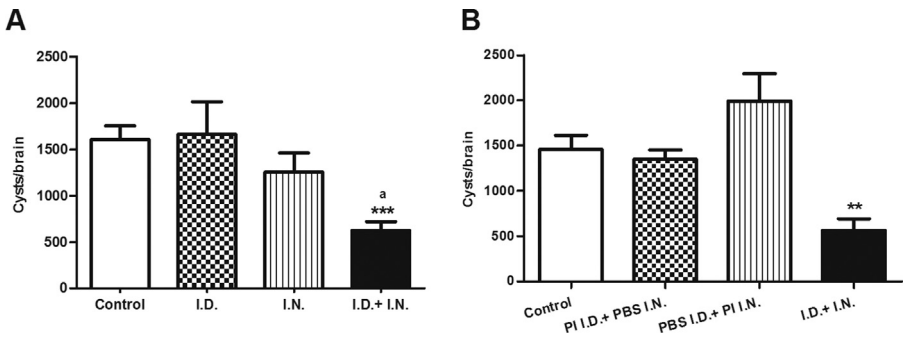

Fig. 1. Protection against chronic *Toxoplasma* infection in C57BL/6 mice vaccinated with rTgPI-1 by different immunization routes. Animals ($n = 8$ per group) were immunized with rTgPI-1 according to the protocols described in Table 1. Two weeks after the last vaccination dose, mice were orally challenged with 20 tissue cysts of ME49 *T. grondii* strain (non-lethal dose). One month later mice were sacrificed and their brains removed for cyst load determination. Each bar represents the group mean ± SEM. (A) Total brain cyst number in the following rTgPI-1-based vaccination groups: intradermal plus Alum (I.D.), intranasal plus CpG-ODN (I.N.) and a combination of 2 inoculations of rTgPI-1-Alum via intradermal route followed by 2 immunizations with rTgPI-1-CpG via intranasal route (I.D.+I.N.). (B) Control protection assay for the rTgPI-1-prime-boost strategy. Animals were immunized with rTgPI-1 according to the protocols described in Table 1: rTgPI-1 + Alum administered by intradermal route and boosted with PBS + CpG via intranasal route (PI ID + PBS IN); primed intradermally with Alum + PBS and boosted with rTgPI-1 + CpG intranasally (PBS ID + PI IN). Data obtained from one experiment. These experiments were performed three times, independently. ***$p < 0.005$ *vs.*Control and I.D. groups; [a] $p < 0.05$ *vs.*I.N. group; ** $p < 0.05$ *vs.* all groups.

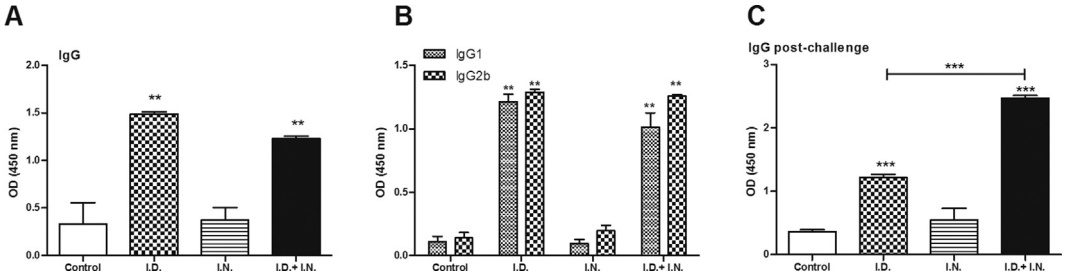

Fig. 2. Determination of humoral immune responses in rTgPI-1 vaccinated mice. Two weeks after the immunization schedules were completed, serum samples (6 mice per group) were obtained and IgG(A), IgG1 and IgG2b sub-classes (B) were determined by ELISA with rTgPI-1 as the bound target. ** $p < 0.01$ *vs.*Control and I.N. groups. (C) One month after infection, serum samples (6 mice per group) were obtained and rTgPI-1-specific IgG antibodies were determined by ELISA. *** $p < 0.005$ *vs.* all groups. Each bar represents the group mean ± SEM.

2.7. Determination of cytokine concentrations in ileum

Two weeks after last immunization, 5 mice per group were sacrificed and tissue samples from terminal ileum (~1 cm^2) were removed under sterile conditions and cultured over night, as previously described (Heimesaat et al., 2006). Supernatants were harvested and stored at −80 °C. Cytokine concentrations were determined by capture ELISA (OptEIATM Mouse IFN-γ, IL-10 and IL-5, BD Biosciences-Pharmingen) according to the manufacturer's instructions.

2.8. Adoptive transfer of MLN cells

Two weeks after last immunization, MLN were removed from vaccinated and control naive mice. Single-cell suspensions in RPMI—3% FBS were made using a cell strainer. 1.5×10^7 cells were injected i.v. in PBS in naive mice ($n = 6$ per group). For the protection assay, five days after the adoptive transfer, recipient mice were orally challenged with a non-lethal dose of 20 cysts of Me49 *T. gondii* strain. One month later, the mean number of cysts per brain was determined by observation under optical microscope, by counting four samples of 25-μl aliquots of each homogenized brain.

2.9. Statistical analysis

Each experiment was repeated at least 3 times. Representative results are presented as mean ± SEM. The mean of each variable (total IgG, IgG_1, IgG_{2b}, IFN-γ, IL-10, IL-5, cell proliferation, cytometric analysis 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 $p < 0.05$.

3. Results

3.1. rTgPI-1 prime-boost vaccination protects C57BL/6 mice against oral T. gondii infection

C57BL/6 mice were vaccinated according to single route protocols consisting of four intradermal doses of rTgPI-1 + Alum (I.D. group) or four intranasal doses of rTgPI-1 + CpG-ODN (I.N. group) or, alternatively, were submitted to a homologous prime-boost strategy with two intradermal doses of rTgPI-1 + Alum followed by two intranasal doses of rTgPI-1 + CpG-ODN (I.D. + I.N. group) (Table 1). 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. 1A, single route immunization strategies either I.D. or I.N. did not induce any protection against infection. On the other hand, mice vaccinated according to the homologous prime-boost regimen showed a significant decrease (62%) in brain parasite burden.

To test if adjuvant compounds induce nonspecific responses that could contribute to the protection observed, rTgPI-1 + adjuvant formulation was administered by one route and the corresponding adjuvant alone by the other route, as described in Table 1 . Only the prime-boost strategy including the administration of the recombinant protein by both routes of immunization resulted in significant protection (Fig. 1B).

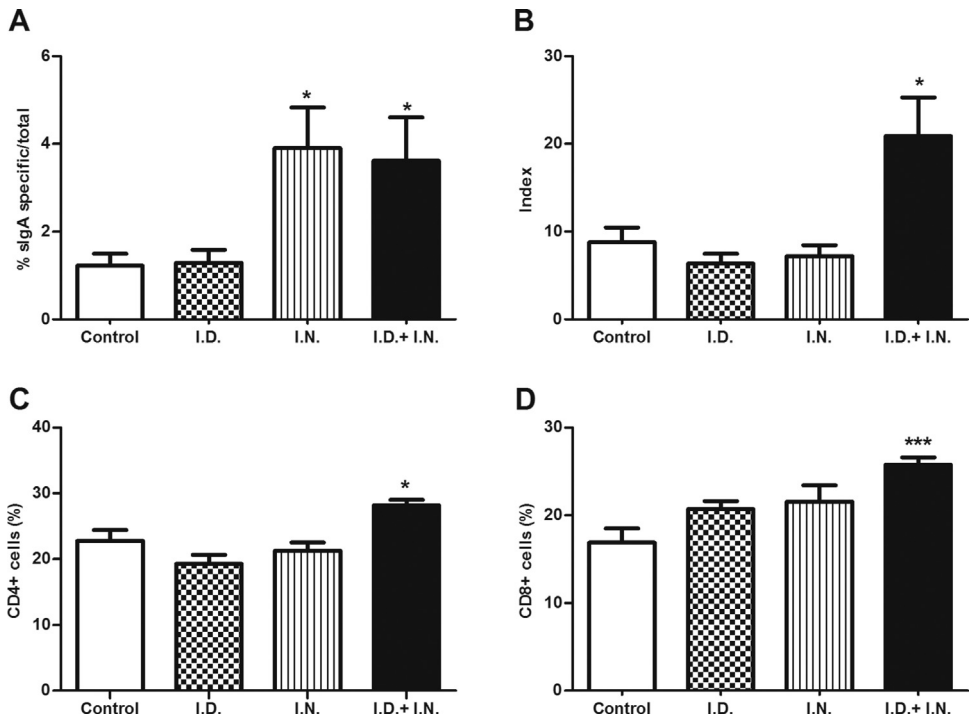

Fig. 3. Mucosal immune responses. (A) Determination of specific anti-rTgPI-1 IgA in intestinal lavages (IL). Two weeks after the last immunization, IL samples from vaccinated or Control mice ($n = 5$ mice per group) were obtained, and antibody concentration was determined by ELISA with rTgPI-1 as the bound target. Results are expressed as the percentage of TgPI-1-specific IgA with respect to the total IgA present in the sample ± SEM and are representative of two experiments. * $p < 0.05$ *vs.* Control and I.D. groups. (B) Proliferative responses of mesenteric lymph node (MLN) cells from vaccinated mice (6 mice per group) were determined 2 weeks after the last immunization by ^{3}H-thymidine incorporation after stimulation with ESA. Results are expressed as an Index (incorporation by cells stimulated over those cultured with medium alone) ± SEM. * $p < 0.05$ *vs.* all groups. Flow cytometric analysis of CD4$^+$ (C) and CD8$^+$ T cells (D) from MLN cells of vaccinated and control groups cultured with ESA (6 mice per group). Each bar represents the mean ± SEM of the animals tested individually. * $p < 0.05$ *vs.*all groups; *** $p < 0.05$ *vs.* control group. The results presented are representative of three independent experiments.

3.2. Antibody response

Two weeks after the immunization schedules were completed, the rTgPI-1 specific IgG antibody production was evaluated by ELISA assays. As shown in Fig. 2A, the highest levels of IgG antibodies were observed in I.D. and I.D. + I.N. groups. Conversely, mice from the I.N. group did not display significant IgG production. Besides, similar titers of IgG specific antibodies were observed in I.D. and I.D. + I.N. vaccinated mice, with positive values detected at a dilution as high as 1:20,000,000 (data not shown). In order to characterize whether a Th1 and/or Th2 response was elicited, the distribution of IgG subtypes was analyzed (Fig. 2B). Similar levels of IgG1 and IgG2b were observed in sera from I.D. and I.D. + I.N. groups, whereas no significant levels of either isotype were detected in sera from I.N. vaccinated mice.

We have also assessed the rTgPI-1 specific antibody response of vaccinated mice one month after the oral challenge, when brain parasite burden was measured. As shown in Fig. 2C, specific IgG antibodies were detected only in the I.D. and I.D. + I.N. immunized groups, which had elicited this immunoglobulin in response to vaccination, whereas surprisingly, no response was observed in I.N. and non-vaccinated control mice. On the other hand, the I.D. + I.N. vaccinated group produced significantly higher levels of anti-rTgPI-1 IgG antibodies compared to the I.D. group.

3.3. Mucosal immune responses

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 vaccination protocols. Intestinal lavages were collected 15 days after the immunization schedules were completed, and the presence of TgPI-1-specific secretory immunoglobulin A (sIgA) was evaluated by ELISA. Significantly higher levels of TgPI-1-specific sIgA was observed in secretions from I.N. and I.D. + I.N. groups compared to the control group (Fig. 3A).

In order to study activation of the mucosal compartments, T cell-mediated immune responses were investigated in mucosal draining distal mesenteric lymph nodes. Only prime-boost vaccinated mice exhibited a significant lymphoproliferative response of MLN cells after *ex vivo* stimulation with ESA (Fig. 3B). To further characterize the T cell responses elicited by the vaccination strategies, CD4$^+$ and CD8$^+$ T cell populations were examined. As shown in Fig. 3C and D, respectively, both I.D. and I.N. groups of mice presented similar percentages of CD4$^+$ and CD8$^+$ T lymphocytes which, in turn, were also similar to the control group. However, prime-boost rTgPI-1 vaccinated mice presented an expansion of both CD4$^+$ and CD8$^+$ cell populations after ESA stimulation as compared to the I.D., I.N. and unvaccinated groups.

After oral infection, the parasite infects the gut mucosa by direct invasion of epithelial cells in the ileum and also penetrates the epithelial barrier to enter the submucosal tissue in which it encounters a variety of resident leukocytes (Barragan and David Sibley, 2003; Courret et al., 2006; Ju et al., 2009). Hence, to further characterize the protective immunity stimulated by our prime-boost immunization strategy with rTgPI-1, we evaluated the cytokine profile elicited in the small intestine. For this, two weeks after the last boost, terminal ileum from each mouse was collected, cultured in complete media, and after 24 h, cytokines released were measured in supernatants. Only the I.D. + I.N. vaccinated mice showed significantly increased levels of IL-5 (Fig. 4A), IFN-γ (Fig. 4B) and IL-10 (Fig. 4C).

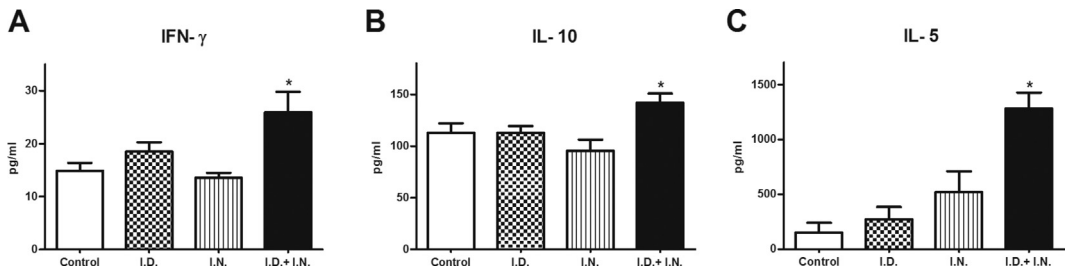

Fig. 4. Cytokines in Ileum from vaccinated mice. Two weeks after the last immunization, cytokines in supernatants of ileum explants from 6 mice per group were measured by ELISA after 24 h of culture in complete RPMI medium: IFN-γ(A), IL-10(B), IL-5(C). Results are shown as the group mean ± SEM. * $p < 0.05$ *vs.* Control group.

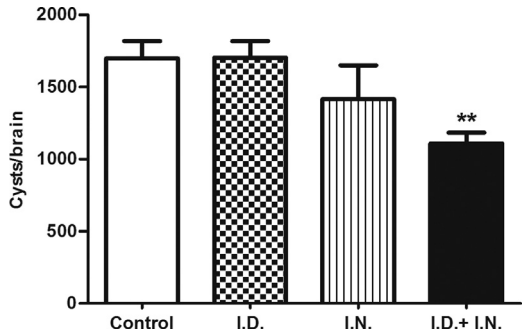

Fig. 5. Protection against chronic Toxoplasma infection in naive mice after adoptive transfer of MLN cells from rTgPI-1 vaccinated mice. C57/BL6 naive mice (6 per group) received $1{,}5 \times 10^7$ MLN cells intravenously from rTgPI-1-vaccinated according to the different protocols or from Control mice. Five days later recipient mice were orally challenged with 20 tissue cysts of ME49 *T. gondii* strain and one month later parasite cyst load was determined. Results are expressed as mean ± SEM. ** $p < 0.01$ *vs.* Control and I.D. groups.

3.4. MLN cells from TgPI-1 vaccinated mice transfer partial protective immunity

The results obtained in the mucosal compartment led us to investigate the contribution of MLN cells to the reduction of brain cyst development. MLN cells from TgPI-1 immunized mice were isolated one week after the last boost and intravenously transferred to naive syngeneic mice. Control mice received an equivalent number of cells from non-immunized donors. Four days later, all recipients were orally challenged with a non-lethal dose of Me49 cysts and one month later, they were assessed for brain tissue cyst enumeration. Significantly fewer cysts were observed only in mice that had received MLN cells from the I.D. + I.N. group (35% reduction) (Fig. 5).

4. Discussion

Recombinant vaccines have a great potential for prevention of diseases caused by intracellular parasites like *T. gondii*. Many factors are important in the development of recombinant vaccines. The selection of parasite antigens, adjuvants and vaccination protocols is crucial to ensure that the formulation is appropriately exposed to the host immune system. In this context, our laboratory has been working on the development of a subunit vaccine based on recombinant proteins using safe and effective adjuvant-compounds suitable to use in any host (Cuppari et al., 2008; del L. Yácono et al., 2012; Martin et al., 2004; Sánchez et al., 2011). In the present study, we evaluated the use of two adjuvants and inoculation routes of rTgPI-1 in order to improve the immune protective responses against toxoplasmosis.

It is now widely accepted, that vaccine strategies based only on systemic immunizations, although able to induce good systemic T-cell responses, rarely induce optimal sustained mucosal T- or B- cell immunity (Ranasinghe and Ramshaw, 2009). In a previous study from our group, intramuscular immunization with this novel antigen in combination with alum, resulted in partial protection against acute and chronic *T. gondii* infection in C3H/HeN mice (Cuppari et al., 2008). In order to extend the study of the protective efficacy of rTgPI-1, the same vaccination protocol was evaluated in the highly susceptible C57BL/6 mouse strain. Since no significant protection could be achieved against an oral challenge (unpublished data), in the present study we assayed different vaccination strategies with the purpose of improving the immunogenicity of this antigen. Intradermal immunization of C57BL/6 mice with rTgPI-1 in combination with alum, an adjuvant authorized for human vaccines (Baylor et al., 2002), did not result in a significant protection. Considering that the natural site of infection for this parasite is the mucosal surface of the intestine, the possibility of inducing mucosal immunity is of great interest. Few studies have been carried out using the intranasal route in mice as an alternative to obtain immunity against *T. gondii* infection (Debard et al., 1996; Igarashi et al., 2008; Velge-Roussel et al., 2000). Some of them have used cholera toxin as adjuvant, whose use is not recommended in humans due to its well-known pathogenicity. In our work, we used the intranasal route to immunize mice using TgPI-1+ODN-CpG, an adjuvant recently included in phase 1 and 2 trials (Carpentier et al., 2010; Ellis et al., 2010). Since this strategy did not induce significant protection, we devised a prime-boost immunization protocol that includes intradermal priming with rTgPI-1+Alum followed by intranasal boost with rTgPI-1+ODN-CpG, pointing to stimulate both mucosal and systemic immunity. Using this homologous prime-boost strategy, we achieved an increase in the immunity that resulted in protection against chronic infection (62 % brain cyst reduction). Concerning *T. gondii* vaccines, several studies have investigated the potential of different antigens like GRA4, SAG1, SAG2, GRA7 and AMA1 administered by heterologous prime-boost regimens combining different recombinant virus expressing *T. gondii* antigens, or recombinant virus with DNA, or DNA with protein, obtaining diverse levels of protection depending the animal model used (Meng et al., 2013; Mendes et al., 2013; Yu et al., 2012; Laguia-Becher et al., 2010; Machado et al., 2010; Shang et al., 2009; Zhang et al., 2007; Caetano et al., 2006). In our work, we have improved the immune-protective value of the recombinant TgPI-1 protein using a combination of two routes of administration of the same form of the antigen.

Several studies have shown the involvement of antibodies in providing protective immunity to intracellular pathogens (Farris et al., 2010; Liu et al., 2012). No differences in the specific humoral immune response were obtained between rTgPI-1-intradermally and prime-boost immunized mice, and from the analysis of the subclass nature of the IgG response, a mixed Th1/Th2 type was generated (similar levels of IgG1 and IgG2b) in both groups. However and noteworthy, after the oral challenge, significant increased levels of specific-IgG were detected only in the prime-boost group (I.D. + I.N.). Particularly interesting is that this antigen seems not to be recognized during *T. gondii* infection since infected control

mice did not elicit any response against rTgPI-1. This suggests that this protein might be a very good target for vaccination representing an interesting and novel approach to reduce infection. Moreover, vaccination with rTgPI-1 would enable differentiation between vaccinated and infected hosts.

Induction of both systemic and mucosal responses is an important goal for any vaccine strategy targeting intracellular pathogens that invade through a mucosal route. Therefore, we investigated whether the rTgPI-1-based vaccination protocols could induce protective mucosal responses. A mucosal response in terms of specific-sIgA was detected both in the I.N. group and also in I.D.+I.N. vaccinated mice. In this last group, the high levels of IgA could be just the result of the two intranasal immunizations or in addition, the switch of intradermally-primed B cells to IgA producing cells after the intranasal boost with the protein. IgA secretion at mucosal surfaces contributes to pathogen elimination, limiting the spread of *T. gondii* to other tissues such as muscle and brain (Ju et al., 2009).

Lymphocytes that have been primed in the NALT compartment, not only return as effector cells to the organs in which they were activated, but they can also re-circulate as effector cells to other mucosal tissues such as the intestinal tract. Since MLN are the first lymphatic organization draining the gut, activation of lymphocytes at this site might account for protection. Remarkably, the rTgPI-1-prime-boost vaccination regimen was the only protocol capable of raising cell-mediated immunity at distal MLN characterized by a high proliferative response after ESA stimulation and an increase in both $CD4^+$ and $CD8^+$ T cell populations. To further study the immune response elicited in the GALT compartment, we analyzed the cytokine production in the small intestine. Increased IFN-γ, IL-10 and IL-5 levels in ileum section supernatants from I.D. + I.N. vaccinated mice were observed. These cytokines could be secreted by Th1 and Th2CD4+ effector T cells present in the lamina propria or by $CD8^+$ intraepithelial lymphocytes primed in the NALT with the intranasal boost after the intradermal priming. The decreased brain parasite burden detected in mice vaccinated according to the prime-boost regimen could be related to the IFN-γ-dependent parasite killing at the portal of entrance, the gut. Also, this local increased IL-5 could in turn enhance IgA production at mucosal level and collaborate with the immune response during *T. gondii* infection (Kuraoka et al., 2004; Yokota et al., 1987). IFN-γ and IL-5 production would be offset by a significant expression of the anti-inflammatory cytokine IL-10 (O'Garra and Vieira, 2007). All these changes at the intestinal mucosa could also modify the resident microbiota contributing with the protection observed in the I.D. + I.N. vaccinated mice (Heimesaat et al., 2006).

Finally and noteworthy, adoptive transfer of MLN cells from rTgPI-1-prime-boost immunized mice conferred partial protection against *T. gondii* challenge showing that lymphocytes from MLN stimulated by this immunization protocol participate, at least in part, in the host immunity against *T. gondii* oral infection.

Taken together, our results show that using a homologous prime-boost strategy that combines two different adjuvants and routes of immunization, we achieve protection against cyst formation during chronic toxoplasmosis in a highly susceptible mouse strain. Single route immunizations induced immune responses only in one compartment: intradermal immunization generated a strong humoral response while intranasal immunization generated a weak mucosal immune response, maybe due to the small amount of antigen used. None of these responses resulted in protection against infection, but using the same total amount of recombinant protein administered with this homologous prime-boost protocol, a protective immune response was generated. The protective effect obtained with this protocol seems to be the result of the addition of the individual single route responses and moreover, the improvement of the mucosal response. The design of this kind of strategies which induce intestinal immune responses would be useful in cats in order to diminish *T. gondii* oocyst shedding and thus reducing parasite transmission. Studies of new immunization approaches are essential to building up future vaccines which could be applied to prevent *T. gondii* infection in many host species.

5. Conclusion

TgPI-1 homologous prime-boost vaccination strategy improves systemic and mucosal immunity against *T. gondii* infection in C57BL/6 mice, highlighting the importance of testing different delivery systems in the case of a particular antigen.

Conflicts of interest

The authors have no potential conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2015.07.013

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