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Humoral responses and immune protection in mice immunized with irradiated *T. gondii* tachyzoites and challenged with three genetically distinct strains of *T. gondii*

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ABSTRACT

Toxoplasma gondii is an obligate intracellular parasite that infects a variety of mammals and birds. *T. gondii* also causes human toxoplasmosis; although toxoplasmosis is generally a benign disease, ocular, congenital or reactivated disease is associated with high numbers of disabled people. Infection occurs orally through the ingestion of meat containing cysts or by the intake of food or water contaminated with oocysts. Although the immune system responds to acute infection and mediates the clearance of tachyzoites, parasite cysts persist for the lifetime of the host in tissues such as the eye, muscle, and CNS. However, *T. gondii* RH strain tachyzoites irradiated with 255 Gy do not cause residual infection and induce the same immunity as a natural infection. To assess the humoral response in BALB/c and C57BL/6J mice immunized with irradiated tachyzoites either by oral gavage (p.o.) or intraperitoneal (i.p.) injection, we analyzed total and high-affinity IgG and IgA antibodies in the serum. High levels of antigen-specific IgG were detected in the serum of parenterally immunized mice, with lower levels in mice immunized via the oral route. However, most serum antibodies exhibited low affinity for antigen in both mice strain. We also found antigen specific IgA antibodies in the stools of the mice, especially in orally immunized BALB/c mice. Examination of bone marrow and spleen cells demonstrated that both groups of immunized mice clearly produced specific IgG, at levels comparable to chronic infection, suggesting the generation of IgG specific memory. Next, we challenged i.p. or p.o. immunized mice with cysts from ME49, VEG or P strains of *T. gondii*. Oral immunization resulted in partial protection as compared to challenged naive mice; these findings were more evident in highly pathogenic ME49 strain challenge. Additionally, we found that while mucosal IgA was important for protection against infection, antigen-specific IgG antibodies were involved with protection against disease and disease pathogenesis. Most antigen responsive cells in culture produced specific high-affinity IgG after immunization, diverse of the findings in serum IgG or from cells after infection, which produced low proportion of high-avidity IgG.

1. Introduction

The causative agent of toxoplasmosis, *Toxoplasma gondii*, is an obligate intracellular protozoan parasite that is of significance as it infects a wide variety of animals in addition to humans. Although approximately 40% of adults worldwide exhibit evidence of *T. gondii* infection, toxoplasmosis is usually an asymptomatic disease in normal individuals. Disease occurs in two stages, the acute phase, characterized by rapid tachyzoite proliferation that can occasionally cause symptoms, and the chronic phase, where the parasites form cysts that can remain in tissues throughout the lifetime of the host [1]. In AIDS, transplant, oncologic or other similarly immunosuppressed patients, toxoplasmosis can be severe, and encephalitis is the leading cause of death in these individuals [2]. Congenital toxoplasmosis is also of great clinical importance. It occurs in acute maternal infection during pregnancy and affects the foetus, resulting in foetal malformations, central nervous system impairment, and blindness; further, vertical transmission of virulent tachyzoites through the placenta can lead to spontaneous abortion [3].

In livestock, infection with *T. gondii* has economic importance due to neonatal loss predominately in sheep and goats [4]. Furthermore, eating raw or undercooked meat from infected animals

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is the main source of parasite transmission to humans [5]. Another important route of infection is the ingestion of vegetables or water contaminated with oocysts eliminated by cats.

Chronic infection is similar in humans and animals and results in lifelong immune protection against reinfection [6]; specific immunity against *T. gondii* infection involves both cellular [7] and humoral [8] mechanisms. Studies evaluating immune responses to toxoplasmosis vaccines based on recombinant proteins or attenuated or mutant parasites have reported varying levels of vaccine efficacy [9].

A live vaccine based on attenuated parasites, TOXOVAX® (Schering-Plough, Walton, Greater, London, England) is currently being used in animals in New Zealand; however, the vaccine does not exhibit high levels of protection and the logistics of immunizing animals in countries far from the site of vaccine production are difficult [10]. Other attempts using intact altered parasites provided promising results, as thermosensitive mutants [11] or parasites defective in pyrimidine salvage pathways parasites [12].

Vaccines containing gamma-irradiated parasites have been used successfully for both helminths [13] and protozoa [14]. *T. gondii* tachyzoites that have been irradiated at a dose of 255 Gy with Cobalt-60 maintain most biological features, including the preservation of cellular structures, DNA and protein synthesis, and the ability to invade mammalian cells; however, irradiated parasites lose reproductive capacity and undergo mitotic death after host cell invasion. Mice immunized with irradiated parasites show high levels of protection against challenge with *T. gondii* ME49 strain cysts [15], although immunization with gamma irradiation *T. gondii* tachyzoites does not promote infection, it induces immunity similar to natural infection with no residual tissue cysts.

Even though all aspects of an immune response should be analyzed to evaluate a vaccine [9], most studies assess only one portion. For example, TOXOVAX® (Schering-Plough, Walton, Greater London, England) vaccination was evaluated by examining antigen specific IgG antibody responses by Western blot [16]. Further, while studies with recombinant products generally only assess antigen-specific antibody responses [17], few studies are devoted to cellular responses or the immune memory of vaccinated animals [18].

The induction of humoral immune memory is very important for the success of a vaccine; and memory B cells are strategically located in places where antigen drainage occurs, including the spleen, mucosal epithelium and bone marrow [19]. Secondary lymphoid tissues such as the spleen, lymph nodes, and lymphoid tissues associated with mucosal surfaces provide an important environment for the development of immune response. These sites are essential for interactions between antigen presenting cells, T cells, B cells and other accessory cells; cooperation between these cells results in an effective humoral immune response [20]. Although a primary lymphoid organ, the bone marrow may also serve as a secondary lymphoid organ [21], and also participate in the induction of humoral immune responses [22]. Circulation memory B cells and neutralizing antibodies provide the first line of defence against various pathogens [23]. Humoral immunity after vaccination or infection predominately results from two cell types: plasma cells and memory B cells. Plasma cells maintain a basal production of antibodies, while memory B cells can proliferate in response to antigenic stimulation [24]. Affinity maturation of antibodies occurs during the primary response, with initial production of low affinity antibodies being replaced over the course of the immune response by the production of high-affinity antigen specific antibodies [25]. All these steps can be identified by antigen proliferation assays, which assess antigen driven proliferation of memory cells (immune memory) and the consequent production of antibodies or cytokines from these cells [26], especially for humoral immune response, allowing quantification and avidity of those antibodies.

We studied the immune memory in infection and after immunization with irradiated tachyzoites in *Toxoplasma* mouse models. We evaluated antibody-producing cells in the spleen and bone marrow and the avidity of these antibodies, compared with the production and avidity of antibodies in the serum. Additionally, we evaluated the IgA levels in the stools of immunized animals, in order to evaluate the protection induced by immunization after challenge with different strains that have distinct genetic profiles. All those findings were related to protection from infection or disease in immunized mice challenged with ME49, VEG and P *T.gondii* strains.

2. Material and methods

2.1. Parasites and animals

Four cryopreserved strains of *T. gondii* RH (I), ME49 (II), VEG (III) and P (III) were maintained in liquid nitrogen and recovered by passage in mice (Protozoology Laboratory, IMTSP). Isogenic 20 g young BALB/c and C57BL/6J male mice were obtained from our colony (Bioterism Center of School of Medicine of University of São Paulo), and maintained in sterilized cages with commercial food (Nuvital® – Nutrients Nuvital S/A, Colombo, PR, Brazil) and water were provided *ad libitum*. Animal manipulation was conducted in accordance with the rules for the care of laboratory animals and with the "Principles of Ethics in Animal Experimentation" – Brazilian Society of Laboratory Animal Science SBCAL). All animal protocols were submitted and approved by the Animal Experimentation Ethic Council – Institute of Biomedical Sciences/University of São Paulo (ICB/USP).

2.2. Irradiation and immunization

Viable irradiated tachyzoites were produced as previously described [15]. Briefly, *T. gondii* RH strain tachyzoite were obtained from the peritoneal cavity of infected mice i.p. by phosphate buffered saline (PBS) washings. Parasite suspensions were filtered through a 5 μm polycarbonate filter and maintained in ice-cold baths until the moment of irradiation with 255 Gy from a uniform source of 60 Cobalt (-rays (Gammacell®, Atomic Energy of Canada, Pinawa, Canada). Sham non-irradiated parasites were also produced and both suspensions were cryopreserved in liquid nitrogen. Mice in groups of 5 were immunized with 3 biweekly doses of 10^7 irradiated tachyzoites; parasites were administered either by i.p. injection or by oral gavage of 10^7 irradiated tachyzoites suspended 1:1 (v/v) in 6% aluminium hydroxide.

2.3. Stools collection and processing

The stools of immunized animals were collected three times per week during the immunization period by removing mice to individual cages without absorbent bedding for one hour. Stool pellets were collected and homogenized in 10 volumes of PBS pH 7.2, containing 0.05% Tween 20 and 0.1 μM phenyl methyl sulfonyl fluoride (PBSTP) in an ice bath. Next, faecal extracts were centrifuged at 10,000 × *g* for 3 min at 4 °C; supernatants were collected and immunoglobulins were recovered by precipitation in neutral 50% ammonium sulphate for 1 h in an ice bath. Samples were centrifuged at 10,000 × *g* for 3 min to recover globulin pellets; pellets were then washed with 1 volume of ice-cold 50% ammonium sulphate in PBS and recentrifuged. Supernatants were drained and the pellets were resuspended in the original stool volume of PBSTP and stored at −20 °C prior to the specific immunesorbent assay.

2.4. T. gondii antigen preparation, ELISAs and antibody affinity determination

T. gondii RH strain tachyzoites were harvested from the peritoneal cavities of previously infected mice using PBS washes; suspensions were filtered through a 5 μm polycarbonate filter, parasites were recovered by centrifugation, counted and recentrifuged. Pellets were suspended in ice-cold water at a parasite density of 10^8 tachyzoites/mL and submitted to sonication until complete cell lysis. Next, 1 volume of 0.3 M NaCl was added to the lysed suspensions and suspensions were centrifuged at 10,000 × *g* for 3 min at 4 °C. Supernatant were harvested and used as *T. gondii* antigen after determination of protein concentration. ELISA plates were coated overnight at 4 °C with 1 μg protein/mL of *T. gondii* antigen in 0.05 M carbonate buffer, pH 9.0. Plates were washed with PBST (PBS containing 0.05% Tween-20) for 5 min and blocked with 0.3% milk in PBST for 1 h at 37 °C. After blocking, serum or stool globulins diluted in PBST were added. To test for IgG avidity, an additional step of 15 min incubation with a 6 M urea chaotrope solution was added for the removal of low avidity antibodies. Next, plates were washed and appropriately diluted anti-mouse IgG or IgA peroxidase-conjugated antibodies were added (Sigma® – Sigma–Aldrich Co., St. Louis, MO, USA). After further washes, bound IgG was revealed with by the *o*-phenylenediamine system for 1 h; reactions were stopped by adding 4 M hydrochloric acid (HCl). Absorbance at 492 nm was determined using an automatic microplate reader (Multiskan MS® Labsystems, Vienna, USA).

2.5. In vitro induced antibody production (IVIAP) by spleen and bone marrow cells

All steps were conducted in laminar flow hood with sterile handling. Sterile 96-well flat-bottom plates were coated overnight at 4 °C with sterile 10 μg/mL *T. gondii* antigen in 0.1 M carbonate buffer, pH 9.6. Plates were washed with PBST and blocked with sterile 2% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. After blocking, plates were washed with sterile saline. Serum, spleen and bone marrow cells were obtained from mice immunized with irradiated *T. gondii* tachyzoites, mice infected with ME49 strain and control mice that were not immunized. Harvested organs were dissociated with needles in RPMI 1640 culture medium with amphotericin B, 5 mg/mL; penicillin, 500 UI/mL; streptomycin, 200 mg/mL; and β-mercaptoethanol (complete culture medium). Suspensions were filtered through gauze for removal of debris and isolated cells were recovered by centrifugation at 1000 × *g* for 15 min. Pelleted erythrocytes were lysed by suspension in volume of 0.15 M ammonium chloride, sodium bicarbonate and 0.5 M EDTA pH 7.4. After centrifugation at 1000 × *g* for 15 min, supernatants were discarded and cells were resuspended in 1 mL of complete culture medium; cells were counted in a Neubauer chamber. Cell concentrations were adjusted to 10^7 cells/mL in complete culture medium and plated into pre-coated plates at a density of 10^6 cells/well. Next, complete culture medium with 10% foetal bovine serum and 10 μg/mL of the *T. gondii* antigen was added. Cells were incubated in 5% CO_2 at 37 °C for periods of 3 h, 4 days or 6 days. For avidity IVIAP, at this step an additional washing was performed with 6 M urea chaotrope solution. Plates were washed with PBST, and anti-mouse peroxidase-conjugated IgG was added in a volume of 100 μL/well; plates were then incubated for 1 h at 37 °C for 15 min. After further washings, tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) were added for 30 min. Reactions were stopped with 1 M sulphuric acid, and absorbance 450 nm was determined using an automatic microplate reader (Multiskan MS® Labsystems, Vienna, USA).

2.6. Challenge of immunized mice

Infective *T. gondii* ME49, P and VEG cysts were obtained from the brains of chronically infected mice. Briefly, after a minimum of 30 days of infection, mice were sacrificed, and their brains were removed and homogenized in 10 mL of sterile saline. Cysts counts were performed using a phase contrast microscope. Immunized or control mice were challenged with 10 cysts of the respective strain administered by oral gavage 15 days after the last immunizing dose; mice were observed for daily survival determination. After 30 days, surviving animals were sacrificed and brain cysts were counted by microscopy. Infection was defined as cyst presence and disease was expressed as cysts/brain in each challenged animal. We also defined infection protection as the proportion of challenged non-infected animals and disease protection as the percent decrease of cyst counts in brains of the immunized animals after challenge.

2.7. Statistical analysis

Comparisons of quantitative values, such as the percentage of avidity, antibody concentration, and the number of antibody-producing cells in the different groups were performed using the ANOVA test, after checking the homogeneity of variances. In the absence of this homogeneity, we used the nonparametric Kruskal Wallis test. Comparisons between groups were performed by post-test Bonferroni and Dunn tests, respectively. Comparisons were considered significant when a probability of equality was less than 5% ($p < 0.05$). The relationships between the various quantitative values were made using Pearson correlation with the same significance. All statistical estimates were made using the statistical package GraphPad Prism5.0® (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Humoral immune responses in the serum of BALB/c and C57BL/6J mice immunized by i.p. injection or oral gavage of irradiated T. gondii tachyzoites

We analyzed humoral immune responses induced by immunization with irradiated *T. gondii* tachyzoites by measuring the levels and avidity of IgG antibodies in the serum. We immunized groups of 5 BALB/c or C57Bl/6J mice according to the protocol described in Section 2.1; stools were collected throughout the experiment, and serum was collected 15 days after the last immunization. Mice infected with 10 *T. gondii* ME49 cysts by oral gavage were sacrificed 45 days after infection and used as positive controls. Serum and globulin stool fractions were tested in the anti-*T. gondii* IgA and IgG ELISA (Fig. 1).

3.1.1. Antigen-specific IgG in the serum

The production of antigen specific IgG antibodies in the serum of the immunized mice is shown in Fig. 1A; data represents one of four experiments. BALB/c mice immunized i.p. had higher serum levels of specific IgG antibody compared to mice immunized by oral gavage. Serum antigen-specific IgG levels in BALB/c mice immunized i.p. were similar to levels in control mice infected with strain ME49. Although animals immunized by oral gavage presented low levels of serum IgG, IgG levels were clearly higher than uninfected controls. C57BL/6J mice immunized i.p. showed serum levels of IgG intermediate between naive and ME49 infected mice. Overall, animals immunized by oral gavage failed to show significant serum levels of specific IgG compared to ME49 infected controls. Additionally, these data show significant production of IgG in mice

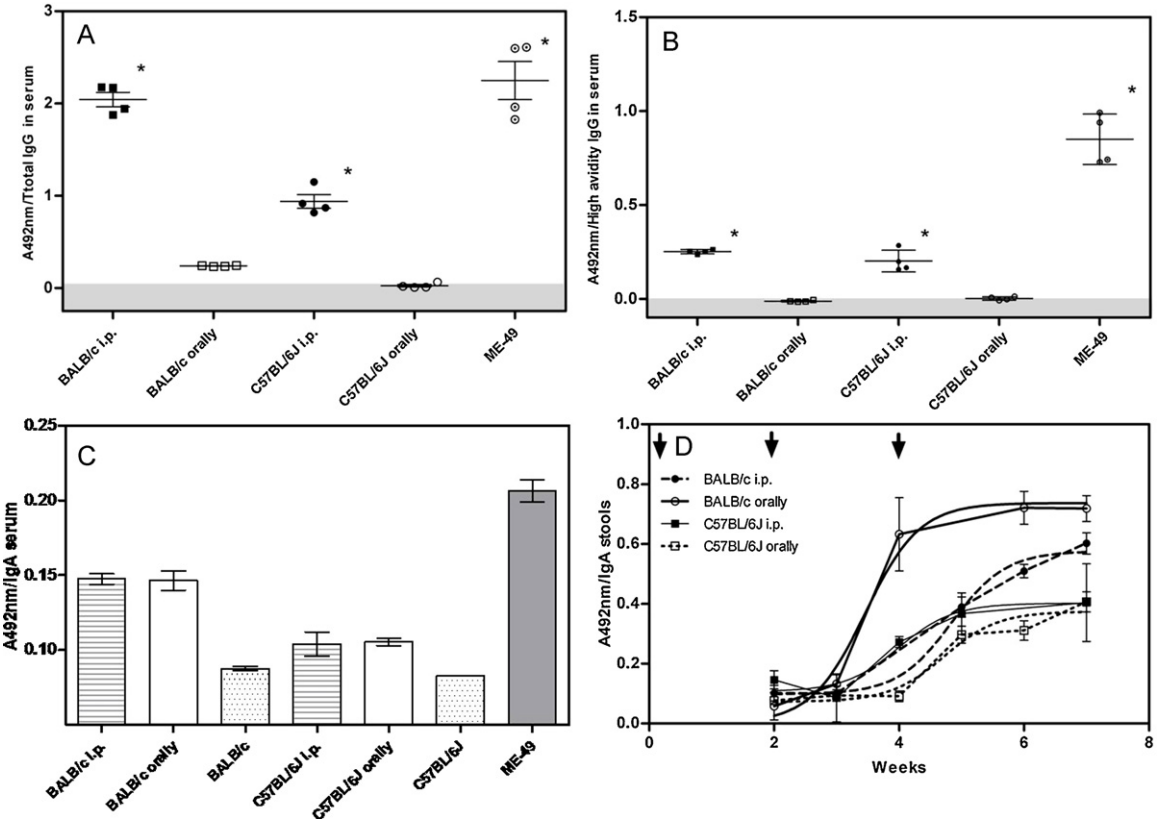

Fig. 1. Detection of total IgG antibodies (A), high-avidity IgG antibodies (B), IgA antibodies (C) in the serum and S-IgA antibodies in stools (D) of immunized mice by i.p. injection or by oral gavage with 10^7 *T. gondii* RH strain tachyzoites irradiated with 255 Gy of source 60 Cobalt. Bars represent the mean and standard deviation, and the presence of an asterisk indicates a significant difference ($^*p < 0.05$) between infection without immunization.

immunized i.p.; however, variations exist depending on mouse strain.

3.1.2. High avidity IgG in the serum

In addition to testing for total IgG, the same serum samples were tested for the detection of high-avidity antigen-specific IgG (Fig. 1B). BALB/c and C57BL/6J mice immunized by i.p. injection showed significant serum levels of high-avidity antibodies when compared to naive controls. However, both mice strains immunized by oral gavage failed to exhibit significant levels of high-avidity IgG in the serum. Mice infected with *T. gondii* strain ME49 had higher levels of high-avidity IgG in serum. Thus, our data show that immunization by oral gavage is not effective in inducing the production of high-affinity antibodies.

3.1.3. Antigen specific IgA in the serum

The presence of specific IgA was also studied in same serum samples (Fig. 1C). Immunized BALB/c mice had higher levels of antigen specific IgA in the serum as compared to immunized C57BL/6J mice. Interestingly, both strains of mice exhibited similar levels of antibodies for both i.p. and oral gavage immunization routes, which is different from the IgG results. Animals infected with *T. gondii* strain ME49 had the highest levels of specific IgA in the serum. Thus, immunization induces increased IgA levels in serum independently of the route of inoculation.

3.1.4. Anti-T. gondii specific IgA humoral responses in the stool

To evaluate intestinal mucosal immune responses, we examined antigen specific IgA in the stools of immunized mice. Stool IgA was measured throughout the immunization process and the results are shown in Fig. 1D. Quantitative data were compared at the end of the immunization schedule but also during the immunization to search for a plateau response. BALB/c mice immunized by oral gavage showed higher IgA levels in the stools as compared to BALB/c mice immunized i.p.; stool IgA levels in mice immunized by oral gavage rapidly established a plateau in production. Regardless of the route of immunization, C57BL/6J mice had lower Secretory immunoglobulin A (S-IgA) secretion and were delayed in reaching a level of stabilization compared to BALB/c mice. Additionally, we observed that the measurement of S-IgA in stools was highly variable due to issues related to measurement of faecal volume, faecal composition, and aspects related to excretion; further, IgA could be subjected to degradation by proteases from either the host or luminal intestinal bacteria. Despite these limitations, it is clear that BALB/c mice exhibit strong IgA independent of the route of immunization.

3.2. In vitro IgG production (IVIAP) by spleen and bone marrow

3.2.1. Direct ex vivo in vitro IgG production

The direct *ex vivo* production of *T. gondii*-specific IgG from bone marrow and spleen cells in a 3 h culture was assessed as described in Section 2.5. Antigen-specific IgG production was evaluated in groups of 5 mice (BALB/c or C57Bl/6J) immunized by i.p. injection or by oral gavage. Additionally, 5 ME-59 infected C57Bl/6J mice were used as controls, similar to the previously described protocol for detecting serum IgG (Section 2.4). We show in Fig. 2 a compilation of data from three similar experiments. Spleen and bone marrow cells from BALB/c and C57BL/6J mice immunized either by i.p. injection or oral gavage exhibited a direct *ex vivo* production of *T. gondii*-specific antibodies regardless cell reproduction. In BALB/c mice immunized by i.p. injection, the direct *ex vivo* production of IgG antibodies by cells from bone marrow and spleen cells was higher compared to same mice immunized by oral gavage (Fig. 2A).

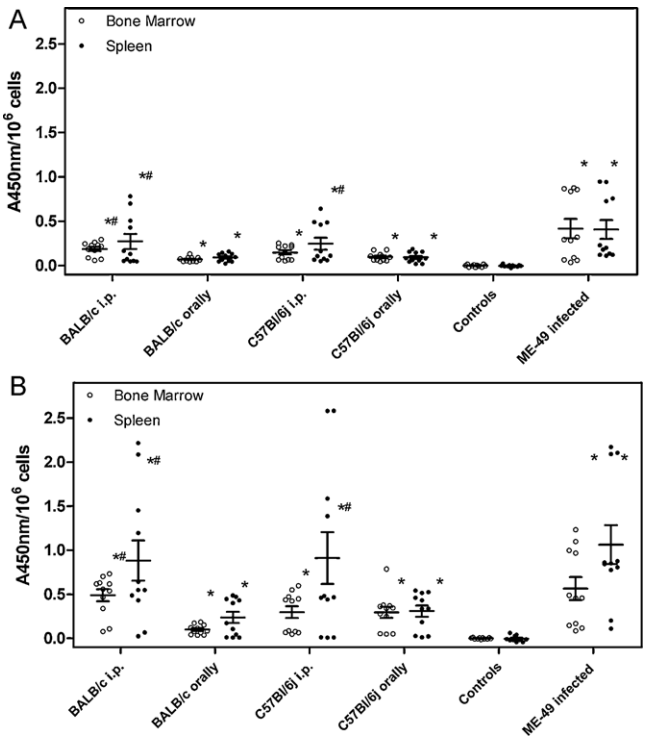

Fig. 2. Detection of IgG antibody production by bone marrow cells and spleen cells in BALB/c and C57BL/6J mice immunized either i.p. or orally with 10^7 *T. gondii* RH strain tachyzoites irradiated with 255 Gy from 60 Cobalt source. (A) *In vitro* direct *ex vivo* production after 3 h of culture. (B) Antigen-specific IgG production after 6 days of *in vitro* cell culture. The symbols represent single measurements of antibody production by cells from bone marrow (open symbols) and spleen (closed symbols). Bars represent the mean and standard deviation. (*) represents a significant difference compared to ME49 infected mice. (#) represents a significant difference between routes of inoculation in the same mice strain.

In C57BL/6J mice, we observed that both routes of immunization induced similarly low levels of antigen-specific IgG antibody production by bone marrow cells; however, spleen cell production of antigen-specific IgG was higher in mice immunized by i.p. injection compared to mice immunized by oral gavage (Fig. 2A). Compared to mice sacrificed 45 days after ME49 strain oral infection, i.p. immunized mice had smaller direct *ex vivo* production of IgG by bone marrow cells, however, this effect was not seen in spleen cells.

3.2.2. Late production of antigen-specific IgG from bone marrow and spleen cells

In order to examine the influence of continued cell–cell interactions and the possible selection of antigen responsive cells, spleen and bone marrow cells from the groups of immunized mice were also submitted to a longer culture period of 6 days. *In vitro* production of *T. gondii*-specific IgG increased in all groups, with greatest production of antibodies occurring after 6 days of culture (Fig. 2B). This result suggests a higher number of committed anti-*T. gondii* cells in these organs. BALB/c mice immunized by i.p. injection also showed greater production of antigen-specific IgG from both spleen and bone marrow cells after extended periods of culture compared to BALB/c mice immunized by oral gavage (Fig. 2B). In C57BL/6J mice, antigen-specific IgG production by bone marrow cells was similar and independent of immunization route; however, spleen cells from C57Bl/6J mice immunized by oral gavage produced more specific IgG than spleen cells from C57Bl/6J mice immunized by oral gavage. Compared to ME49 infected mice that were used as controls 45 days after infection, both i.p. immunized BALB/c or C57Bl/6J mice presented similar levels of late *in vitro* IgG production from spleen and bone marrow cells.

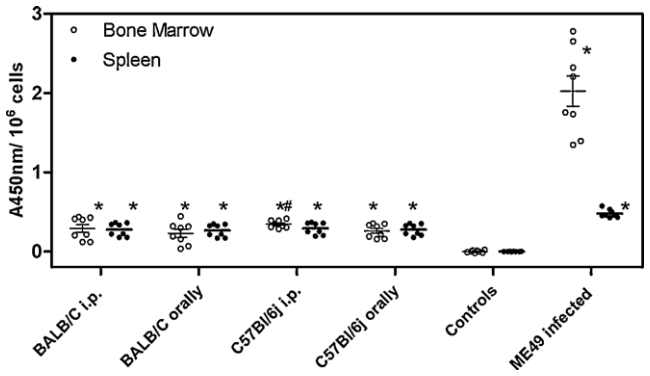

Fig. 3. Detection of high-avidity IgG antibodies after 24 h *in vitro* culture from bone marrow (open symbols) or spleen (closed symbols) cells from BALB/c or C57BL/6J mice immunized either i.p. or orally with 10^7 *T. gondii* RH strain tachyzoites irradiated with 255 Gy from a Cobalt 60 source. Additionally, mice were infected with ME49 strain of *T. gondii*. Bars represent the mean and standard deviation. (*) represents a significant difference compared to ME49 infected mice. (#) represents a significant difference between routes of inoculation in the same mice strain.

3.2.3. In vitro production of high-avidity antigen-specific IgG

Next, we wanted to assess the avidity of the antigen-specific IgG produced in *ex vivo* spleen and bone marrow cell cultures. Cells were cultured for 24 h *ex vivo* and high-avidity antibody production in the supernatants was assessed using an ELISA that was adapted to remove low avidity antibodies by adding a chaotrope wash step (Fig. 3). Bone marrow and spleen cells from mice immunized either by i.p. injection or by oral gavage produced significant levels of high-avidity IgG (Fig. 3). According to the route of immunization, bone marrow cells from i.p. immunized C57BL/6J mice produced higher levels of antigen-specific IgG when compared to C57BL/6J mice immunized by oral gavage, similar to our earlier findings (Section 3.2.1). Antigen-specific IgG production from spleen cells was equivalent in BALB/c and C57BL/6J mice, regardless of route of inoculation; however, antibody production was lower than production in spleen cells from infected mice with ME49 strain ($p < 0.001$). In these infected mice, antigen-specific IgG production by bone marrow cells was higher than by spleen cells ($p < 0.0001$). Our data support the concept that high-avidity memory immune cells are located mostly in bone marrow. Additionally, our findings suggest that while high-avidity memory immune cells are primarily generated during infection, low levels are also generated by immunization protocols.

3.3. Immune protection in immunized mice from challenge with genetically distinct strains of T. gondii

3.3.1. Quantitative protection

To assess the protective efficacy of the different immunization protocols we challenged groups of five immunized mice with three different and genetically diverse strains of *T. gondii*. In these experiments, protection was defined as the number of cysts present in the brain; results are depicted in Fig. 4. The numbers of brain cysts at 30 days after challenge or infection are shown in Fig. 4. Both BALB/c (Fig. 4A) and C57BL/6J (Fig. 4B) immunized mice exhibited a clear decrease in brain cyst numbers compared to mice infected without immunization. In ME49 strain infections, C57Bl/6J mice had higher cyst numbers compared to BALB/c mice. Interestingly, BALB/c mice exhibited similarly low numbers of brain cysts regardless of the *T. gondii* strain used in the challenge. Mice immunized by oral gavage had higher numbers of brain cysts as compared to their counterparts immunized by i.p. injection.

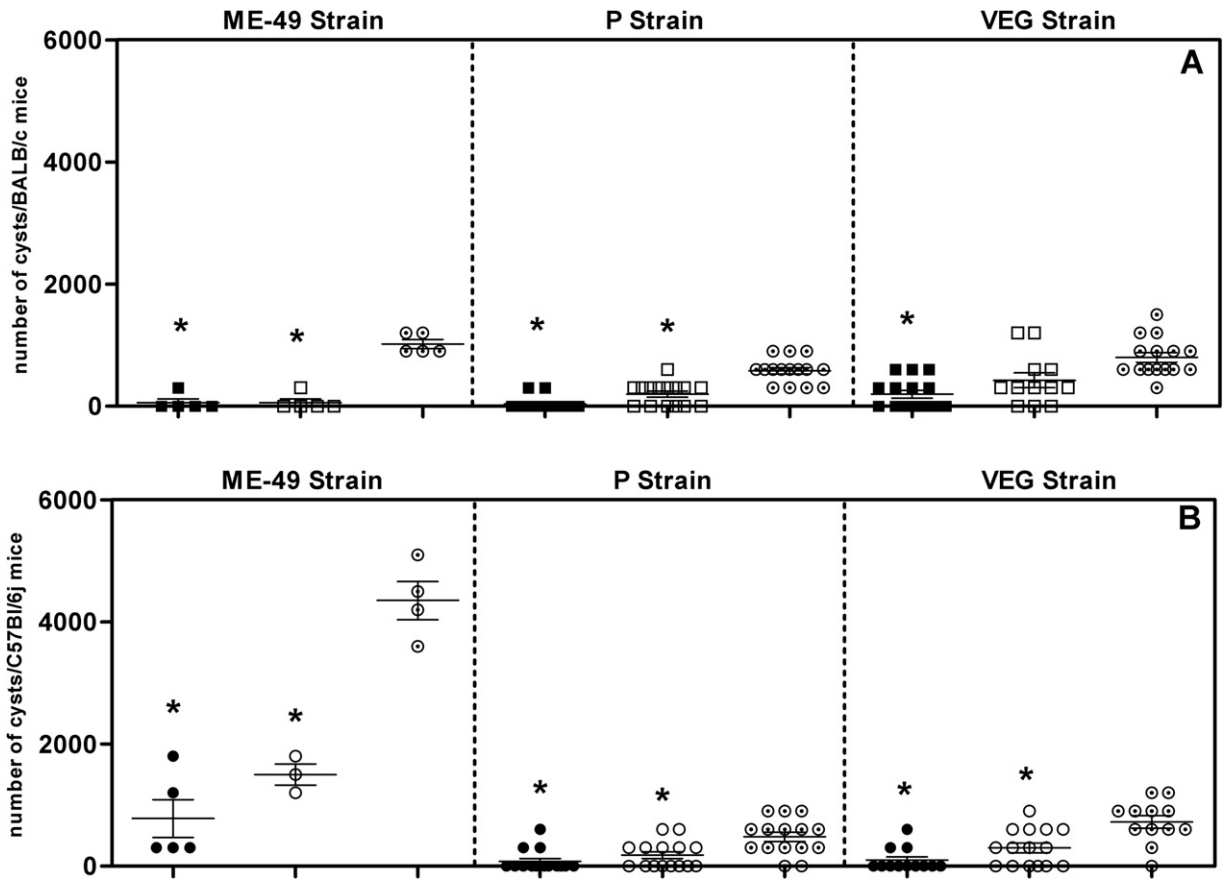

Fig. 4. Numbers of brain cysts of immunized BALB/c mice (A) and C57BL/6J mice (B) immunized either i.p. or orally with 10^7 *T. gondii* RH strain tachyzoites irradiated with 255 Gy from a Cobalt 60 source and challenged with 10 cysts of *T. gondii* ME49, P or VEG stain. Solid symbol: i.p. immunization; empty symbol: oral gavage immunization; symbol with point: ME49 infected mice. Bars represent the mean and standard deviation, and the presence of an asterisk indicates a statistically significant difference ($^*p < 0.05$) compared to infection.

In order to quantify the effect of immunization regardless of the challenging infection, we normalized the numbers of cysts observed in immunized mice to the mean of cysts observed in primary infection, thus determining the proportion of infection (Fig. 5). Using this proportion, the effect of immunization is more evident, and shows that immunization by oral gavage generally provided less protection than i.p. immunization for both BALB/c (Fig. 5A) and C57Bl/6J (Fig. 5B) mice.

3.3.2. Qualitative protection

Protection can also be measured in terms of the proportion of animals without cysts in the brain; the percentage of animals free of detectable brain cysts is presented in Table 1. BALB/c mice immunized i.p. and by oral gavage showed significant protection in this analysis. Protection was 80% when BALB/c mice were challenged with ME49 strain for both routes of immunization. When challenged with strain P, BALB/c mice immunized by oral gavage showed a 40% protection while mice immunized by i.p. injection exhibited 84% protection. However, when BALB/c mice were infected with the VEG strain, i.p. immunization showed only 53% protection and immunization by oral gavage showed 25% protection (Table 1). C57BL/6J mice showed susceptibility when challenged with ME49, as no animals were protected from *T. gondii* cysts in the brain. However, i.p. immunization of C57BL/6J mice induced significant protection as 80% of the animals were protected against P strain challenge and 75% of mice were protected against VEG strain. Overall, the protection by oral gavage immunization was not very effective; as only 53% of mice were protected against strain P and 40% protected against VEG strain challenge (not significant).

Table 1
Qualitative distribution of absolute protection (lack of infection) in mice immunized with irradiated *T. gondii* tachyzoites and challenged with 3 different strains of *T. gondii*.

Strain	Immunization	Protection (infected mice/total challenged)	
		BALB/c	C57BL/6J
ME49	None	0 (5/5)	0 (4/4)
	Per os	80% (1/5)*	0 (3/3)
	i.p.	80% (1/5)*	0 (5/5)
VEG	None	0 (15/15)	8% (11/12)
	Per os	25% (9/12)	40% (9/15)
	i.p.	53% (7/15)*	75% (3/12)*
P	None	0 (15/15)	13% (13/15)
	Per os	40% (9/15)*	53% (7/15)*
	i.p.	84% (2/12)*	80% (3/15)*

* $p < 0.05$ by Fisher's exact test, when compared to the non-immunized mice.

3.4. Correlation between immunologic events and protection against challenge in mice immunized with irradiated T. gondii tachyzoites

Immunological events were quantified and analyzed in all models, according to each challenging strain, immunization route and host mouse strain looking for mathematical Pearson correlation with protection both either as the observed brain cyst reduction, quantitative protection; or frequency of animals without brain cysts, qualitative protection (Table 2).

We used mean data for immunological events after 2 weeks of the end of immunization protocol for each group. Events

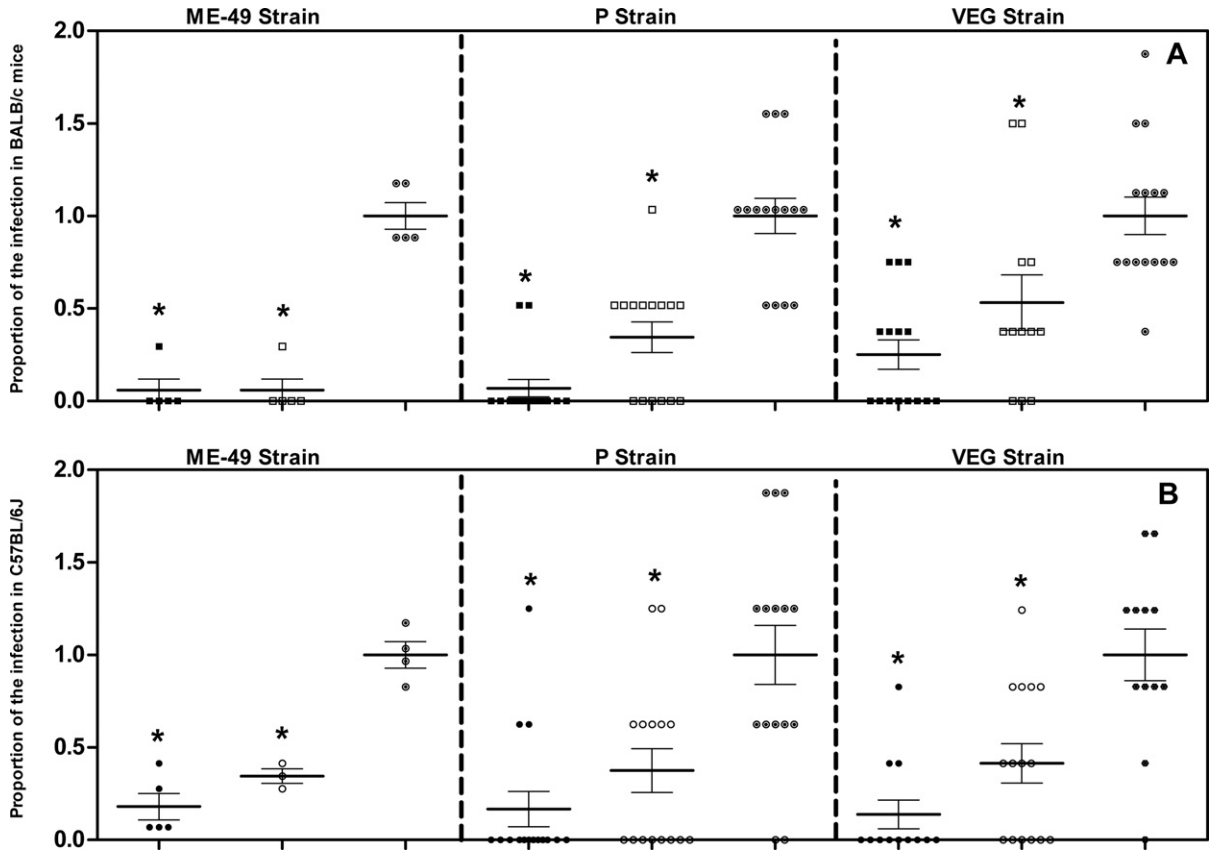

Fig. 5. Proportion of infection in immunized BALB/c mice (A) and C57BL/6J mice (B) immunized either i.p. or orally with 10^7 *T. gondii* RH strain tachyzoites irradiated with 255 Gy from a Cobalt 60 source and challenged with 10 cysts of ME49, P or VEG strain of *T. gondii*. Solid symbol: i.p. immunization; empty symbol: oral immunization; symbol with point: no immunization. Bars represent the mean and standard deviation, and the presence of an asterisk indicates a statistically significant difference (*$p < 0.05$) compared to infection without immunization.

were defined as serum levels of antigen specific IgG, high-avidity antigen specific IgG, antigen specific IgA, production of antigen specific IgG after 6 days of culture by bone marrow cells and spleen cells and the production of high-avidity IgG after 24 h of culture from bone marrow and spleen cells. These data were related to quantitative protection, as percent of cyst reduction in brain as compared to infection in each model or qualitative protection, as the proportion of challenged mice without brain

Table 2
Correlation between quantitative immunological events and the proportion of reduction of brains cysts or quantitative protection and percent of non infected animals or qualitative protection, analyzed in immunized mice challenged with different strains of *T. gondii* compared to mice infected without immunization. One-tailed Pearson correlation and 90% confidence interval are presented. Significant events are marked in bold, NS – not significant.

Event	ME49 + VEG + P (12) Protection		VEG + P (8) Protection		ME49 (4) Protection	
	Quantitative	Qualitative	Quantitative	Qualitative	Quantitative	Qualitative
IgG in serum (*r*, C.I. 90%, *p*)	**0.64**	0.47	**0.74**	0.62	0.57	0.42
	0.20–0.86	−0.14 to 0.82	**0.22–0.93**	−0.15 to 0.92	−0.76 to 0.98	−0.91 to 0.98
	0.012	NS	**0.017**	NS	NS	NS
IgG of high avidity in serum (*r*, C.I. 90%, *p*)	**0.68**	0.40	**0.88**	**0.81**	0.37	0.07
	0.27–0.88	−0.22 to 0.79	**0.56–0.97**	**0.25–0.96**	−0.85 to 0.97	−0.95 to 0.96
	0.007	NS	**0.002**	**0.007**	NS	NS
IgA in serum (*r*, C.I. 90%, *p*)	0.15	0.32	−0.10	−0.29	0.85	**0.99**
	−0.37 to 0.61	−0.31 to 0.76	−0.68 to 0.56	−0.82 to 0.52	−0.35 to 0.99	**0.94–1.00**
	NS	NS	NS	NS	NS	**0.001**
IVIAP in bone marrow (*r*, C.I. 90%, *p*)	**0.60**	0.25	**0.86**	**0.87**	0.12	−0.32
	0.14–0.84	−0.37 to 0.72	**0.50–0.96**	**0.42–0.97**	−0.91 to 0.94	−0.98 to 0.92
	0.020	NS	**0.003**	**0.002**	NS	NS
IVIAP high avidity bone marrow (*r*, C.I. 90%, *p*)	**0.52**	0.16	**0.81**	**0.85**	−0.06	−0.49
	0.02–0.81	−0.45 to 0.67	**0.36–0.95**	**0.37–0.97**	−0.93 to 0.92	−0.98 to 0.89
	0.042	NS	**0.008**	**0.003**	NS	NS
IVIAP in spleen (*r*, C.I. 90%, *p*)	**0.56**	0.21	**0.81**	**0.84**	0.10	−0.37
	0.09–0.83	−0.41 to 0.70	**0.38–0.95**	**0.32–0.97**	−0.913 to 0.94	−0.98 to 0.92
	0.028	NS	**0.007**	**0.005**	NS	NS
IVIAP high avidity in spleen (*r*, C.I. 90%, *p*)	0.38	0.04	**0.70**	**0.79**	−0.29	−0.69
	−0.14 to 0.74	−0.54 to 0.60	**0.12–0.92**	**0.19–0.96**	−0.96 to 0.87	−0.99 to 0.80
	NS	NS	**0.027**	**0.01**	NS	NS

cysts using unicaudal Pearson correlation *r* and its confidence interval.

The relationship of immunological events and the quantitative protection using all models show significant direct correlation with serum IgG, serum high-avidity IgG, production of IgG by bone marrow and spleen cells and production of high-avidity IgG by bone marrow cells. Challenge with isolated *T. gondii* strains failed to show significant correlations, but challenge with P and VEG strains showed similar correlations with association to the production of high-avidity IgG by spleens cells. However, there was a lack of association with specific IgA in serum with neither protection against infection nor disease.

The relationship between immunological events studied and qualitative protection (percent of protection) showed a lack of association of immune events with this type of protection if all models were considered. Isolated ME49 challenge resulted in direct relationship with serum specific IgA only, and the joined P and VEG strain challenge models showed the same relationships found in quantitative protection, except for the significance of serum specific IgG levels which was lacking.

4. Discussion

Our model, using irradiated parasites, has ideal characteristics for the study of vaccine-induced immune protection without the use of viable agents. Ionizing radiation maintains the whole structure and cell biology of the parasite without damaging proteins; thus, the host is exposed to the same parasite antigens and the immunity that results is similar to immunity induced by natural infection [15].

We analyzed humoral immune responses using specific enzyme immunoassays to detect serum levels of anti-*T. gondii* IgG and IgA in BALB/c and C57BL/6J mice immunized with irradiated *T. gondii* tachyzoites. Most humoral immune responses studies have evaluated mice immunized with isolated recombinant proteins, such as surface proteins, called SAGs [27], or proteins associated with cell invasion, called ROPs, AMAs, MICs and GRAS [17], which are considered the major *T. gondii* antigens. These immunizations cause different types of protection, according to the strain mouse challenged [9,28]. Mice immunized with isolated recombinant proteins exhibit little protection against reinfection with no reduction in the number of brain cysts [29] or combined with adjuvants that activate the immune system [30]. Our model does not use any adjuvants; irradiated *T. gondii* tachyzoites alone were sufficient to induce immunity similar to a natural infection [15] that provided protection against challenge with different *T. gondii* strains. The production of high-affinity antibodies during primary antigen exposure is important for protection against reinfection and the immunity of the host [31]. Our data also detected specific high-avidity IgG antibodies in the serum in immunized mice that correlates well with protection, suggesting that these antibodies may be a good marker for protection.

We demonstrated that the secretion of anti-*T. gondii* IgA in the stools of mice immunized with irradiated tachyzoites was greater in BALB/c mice immunized by oral gavage. Immunization via the oral route is of great importance for toxoplasmosis vaccination studies, as this is the main route of infection and parasites first contact the host intestinal mucosa [32]. S-IgA antibodies have been shown to reduce tachyzoite infection of human enterocytes *in vitro*, thus suggesting that IgA secretion is important in defending the intestinal mucosa from pathogen invasion [33]. Studies of mucosal immunity report that often antigen alone is not sufficient to induce an effective humoral immune response; however, this immunity can be increased significantly with the use of adjuvants [34]. Cholera toxin (CT) and labile enterotoxin are considered strong mucosal adjuvants and are therefore used for strengthen an immune response [35]. Mice immunized intranasally with purified SAG1 protein associated with non-toxic labile enterotoxin induced both a mucosal and systemic response with the production of antigen specific IgG and IgA [36]. Our model of oral immunization with aluminium hydroxide maintains the immunogenicity of the antigen [37], while protecting the parasites from the destructive effect of gastric acid environment preserving them to reach the intestinal mucosa. Our results also showed intestinal mucosal immune responses in immunized animals through the detection of antigen specific S-IgA in stools; importantly, antigen-specific S-IgA is produced both by i.p. injection or oral gavage immunized mice.

Our data showed that both strains of mice immunized by both routes of inoculation showed antigen experienced cells that produced anti-*T. gondii* antibodies 15 days after the last immunization, probably memory B cells. The direct *ex vivo* production of antibodies demonstrates the presence of plasma cells producing antibodies directed against *T. gondii* specific antigens in the spleen. Our data showed significant levels of direct *ex vivo* anti-*T. gondii* antibody production from bone marrow cells from immunized mice by either route; these data indicate that *T. gondii* humoral responses also occur in the bone marrow. Humoral immune responses in the bone marrow can be observed in splenectomised mice and mice deficient in lymphotoxin-alpha that lack lymph nodes and Peyer's patches. These animals were challenged with *Salmonella typhimurium* and were found have specific antibody-producing cells in their bone marrow [38]. Other authors suggest that the events inducing immunological memory take place in secondary lymphoid organs, but that there is a recirculation of these cells to the bone marrow, which is considered of great importance in the induction of humoral immune responses [21]. Our data suggest that the bone marrow, aside from being a primary lymphoid organ, may also be an important secondary lymphoid organ with the presence of antigen-specific memory B cells.

Our data showed antigen high-avidity IgG production by bone marrow cells and spleen by IVIAP of mice immunized by both routes of inoculation. Antibody production by these cells was similar to antibody production by spleen cells and bone marrow of mice infected with *T. gondii* strain ME49; these results suggest that the humoral immune response in vaccination is similar but lower to the response to infection.

Protection against toxoplasmosis infection can be measured in a qualitative form (pathogen clearance) or against disease in a quantitative form (decreased parasite burden) as previously described [39]. Quantitative protection of immunized BALB/c mice was higher when challenged with ME49 strain and lower when challenged with P and VEG strains. ME49, a type II strain, showed higher virulence in relation to other *T. gondii* strains than demonstrated by other authors [40], however, the proportion of protection was also higher with ME49 compared to less virulent strains. Immunized C57Bl/6J mice exhibited similar results but with less quantitative protection. The amount of quantitative protection is associated to the *T. gondii* susceptibility of different mouse strains; as expected; BALB/c mice were more resistant to infection [41]. Other authors also reported that C57BL/6J mice are more susceptible to *T. gondii* infection and exhibit a larger number of brain cysts as compared to BALB/c mice [42].

Qualitative protection against infection showed similar results, with immunized BALB/c mice showing greater protection; both routes of immunization resulted in 80% protection to the ME49 strain, which was similar to the P strain. Immunized C57BL/6J mice showed total susceptibility to infection with ME49 strain, while i.p. immunization resulted in partial protection (80% and 75%) when challenged with P and VEG strains, respectively.

Several studies have assessed immunization with vaccines for toxoplasmosis protection through challenge with less virulent

strains [9]. To prepare a model vaccine for toxoplasmosis, it is necessary to assess strategic points, not just survival or minor infections, but total protective immunity. The challenge of simulating oral infection, the use of more than one type of parasite strain for challenge and the use of different mouse strains is recommended to verify the efficacy of immunization in different genetic situations [9]. Our vaccination model with the use of irradiated *T. gondii* tachyzoites induced protective immunity against different *T. gondii* strains as mice showed reduced numbers of brain cysts. Additionally, in immunized BALB/c mice challenged with strain ME49, there was a total reduction of cysts by conventional microscopy. Vaccination induced a memory immune response in the spleen and the bone marrow, with the production of high-affinity antibodies similar to natural infection.

We tried to correlate immune events with these two forms of protection against *Toxoplasma*, prevention of the infection or the disease. Determination of the correlation between protection and events is often the first step toward developing a vaccine against a disease and current vaccines are usually assessed by measuring the induction of antibodies in the serum or mucosa, where the objective is to block or interfere with the infection, with few studies looking for neutralizing effect on the agent [18]. When we analyzed the correlation of quantitative protection of the three genetically diverse strains of *T. gondii* used in challenge with all immune events, we observed that specific immune memory events are important for associated models; however, serum IgA does not seem to be important, except for protection against the virulent ME49 strain. These results suggest that the presence of antibodies and specific memory cells are important to prevent disease but not infection. Qualitative protection showed similar results as quantitative protection, and also related to producing high-affinity antibodies in the serum and antibody production by bone marrow and spleen cells.

We studied in our models oral immunization, which is rarely used in *T. gondii* vaccination [9]. Other approaches targeting mucosal immunity use the nasal route [43]. Prevention of infection requires mucosa immunity. Low inflammatory activity of IgA prevents the entry of bacteria and other pathogens without causing inflammatory damage at the site [44]. Other authors also evaluated the importance of IgA present in mucosal surface in other diseases such as poliomyelitis [45], rotavirus [46] and influenza [47].

The production of anti-*T. gondii* antibodies by memory cells in the spleen and bone marrow was shown to be important in both quantitative and qualitative in protection. Other studies have shown that mice vaccinated with non-virulent *T. gondii* tachyzoites had a higher survival rate after challenge with virulent strain, suggesting that B cell production of specific antibodies protects from *in vivo* infection by blocking the entry of tachyzoites into host cells [48]. The immune system is complex and redundant, and a vaccine to protect the individual should induce a variety of mechanisms to defend against the invading agent.

In toxoplasmosis, cats are responsible for contaminating the environment [49], and the development of an oral vaccine for these animals could interrupt the transmission of disease to humans or other animals. Such a vaccine could access free-living felids that are important in disease dissemination. Wild animal immunization with oral bait has already been used successfully for immunization against rabies (Raboral VR-G®, Merial Inc., Georgia, USA); it is effective in the immunization of dogs, foxes, cats and other animals that are located in large inaccessible areas [50]. Our studies show that immunization with irradiated *T. gondii* tachyzoites may be an effective vaccine for oral use in cats and other animals, reducing disease dispersion in the environment and consequently reducing contamination of man and livestock. Our vaccination approach has proven effective in determining various aspects of humoral immunological memory, opening prospects for the use of vaccines with irradiated *T. gondii* tachyzoites in prevention models for animal and perhaps human toxoplasmosis.

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References

[1] Dubey JP, Lindsay DS, Speer CA. Structure of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and developmentof tissue cysts. Clin Microbiol Rev 1998;11(2):267–99.
[2] Derouin F, Pelloux H. Prevention of toxoplasmosis in transplant patients. Clin Microbiol Infect 2008;14(12):1089–101.
[3] Petersen E. Toxoplasmosis. Semin Fetal Neonal Med 2007;12(3):214–23.
[4] Dubey JF. Status of toxoplasmosis in sheep and goats in the United States. J Am Vet Med Assoc 1990;196(2):259–62.
[5] Kijlstra A, Jongert E. Control of the risk of human toxoplasmosis transmitted by meat. Int J Parasitol 2008;38(12):1359–70.
[6] Parker SJ, Roberts CW, Alexander J. CD8+ T cells are the major lymphocyte subpopulation involved in the protective immune response to *Toxoplasma gondii* in mice. Clin Exp Immunol 1991;84(2):207–12.
[7] Yamamoto JH, Vallochi AL, Silveira C, Filho JK, Nussenblatt RB, Cunha-Neto E, et al. Discrimination between patients with acquired toxoplasmosis and congenital toxoplasmosis on the basis of the immune response to parasite antigens. J Infect Dis 2000;181(June (6)):2018–22.
[8] Carvalho FR, Silva DA, Cunha-Júnior JP, Souza MA, Oliveira TC, Béla SR, et al. Reverse enzyme-linked immunosorbent assay using monoclonal antibodies against SAG1-related sequence, SAG2A, and p97 antigens from *Toxoplasma gondii* to detect specific immunoglobulin G (IgG), IgM, and IgA antibodies in human sera. Clin Vaccine Immunol 2008;15(August (8)):1265–71.
[9] Jongert E, Roberts CW, Gargano N, Förster-Wald E, Petersen E. Vaccines against *Toxoplasma gondii*: challenges and opportunities. Mem Inst Oswaldo Cruz 2009;104(March (2)):252–66.
[10] Buxton D, Innes EA. A commercial vaccine for ovine toxoplasmosis. Parasitology 1995;110(s1):S11–6.
[11] Sayles PC, Johnson LL. Intact immune defenses are required for mice to resist the ts-4 vaccine strain of *Toxoplasma gondii*. Infect Immun 1996;64(8):3088–92.
[12] Fox BA, Bzik DJ. Avirulent uracil auxotrophs based on disruption of orotidine-5′-monophosphate decarboxylase elicit protective immunity to *Toxoplasma gondii*. Infect Immun 2010;78(9):3744–52.
[13] Wales A, Kusel JR. Biochemistry of irradiated parasite vaccines: suggested models for their mode of action. Parasitol Today 1992;8(11):358–63.
[14] Hoffman SL, Doolan DL. Malaria vaccines-targeting infected hepatocytes. Nat Med 2000;6(11):1218–9.
[15] Hiramoto RM, Galisteo Jr AJ, Do Nascimento N, Andrade Jr HF. 200 Gy sterilized *Toxoplasma gondii* tachyzoites maintain metabolic functions and mammalian cell invasion, eliciting cellular immunity and cytokine response similar to natural infection in mice. Vaccine 2002;20(16):2072–81.
[16] Wastling IM, Harkins D, Buxton D. Western blot analysis of the IgG response of sheep vaccinated with S48 *Toxoplasma gondii* (Toxovax). Res Vet Sci 1994;57(3):384–6.
[17] Dlugonska H. *Toxoplasma* rhoptries: unique secretory organelles and source of promising vaccine proteins for immunoprevention of toxoplasmosis. J Biomed Biotechnol 2008;2008:632424.
[18] Plotkin SA. Vaccines: correlates of vaccine-induced immunity. Clin Infect Dis 2008;47(August (3)):401–9.
[19] Tangye SG, Tarlinton DM. Memory B cells: effectors of long-lived immune responses. Eur J Immunol 2009;39(August (8)):2065–75.
[20] Cariappa A, Mazo IB, Chase C, Shi HN, Liu H, Li Q, et al. Perisinusoidal B cells in the bone marrow participate in T-independent responses to blood-borne microbes. Immunity 2005;23(October (4)):397–407.
[21] Pillai S, Cariappa A. The bone marrow perisinusoidal niche for recirculating B cells and the positive selection of bone marrow-derived B lymphocytes. Immunol Cell Biol 2009;87(January (1)):16–9.
[22] Tumanov AV, Grivennikov SI, Kruglov AA, Shebzukhov YV, Koroleva EP, Piao Y, et al. Cellular source and molecular form of TNF specify its distinct functions in organization of secondary lymphoid organs. Blood 2010;116(Nov (4)):3456–64.
[23] Mamani-Matsuda M, Cosma A, Weller S, Faili A, Staib C, Garçon L, et al. The human spleen is a major reservoir for long-lived vaccinia virus-specific memory B cells. Blood 2008;111(May (9)):4653–9.
[24] Amanna IJ, Slifka MK. Mechanisms that determine plasma cell lifespan and the duration of humoral immunity. Immunol Rev 2010;July (236):125–38.

[25] Lappalainen M, Hedman K. Serodiagnosis of toxoplasmosis. The impact of measurement of IgG avidity. Ann Ist Super Sanita 2004;40(1):81–8.
[26] Oliveira Jr EB, Ferra MLG, Perez RM, Silva AEB, Lanzoni VP, Granato CF. *In vitro*-induced antibody production in chronic hepatitis C virus infection. Braz J Med Biol Res 2003;36(3):361–8.
[27] Letscher-Bru V, Pfaff AW, bou-Bacar A, Filisetti D, Antoni E, Villard O, et al. Vaccination with *Toxoplasma gondii* SAG-1 protein is protective against congenital toxoplasmosis in BALB/c mice but not in CBA/J mice. Infect Immun 2003;71(November (11)):6615–9.
[28] Igarashi M, Kano F, Tamekuni K, Machado RZ, Navarro IT, Vidotto O, et al. *Toxoplasma gondii*: evaluation of an intranasal vaccine using recombinant proteins against brain cyst formation in BALB/c mice. Exp Parasitol 2008;118(3):386–92.
[29] Jongert E, Verhelst D, Abady M, Petersen E, Gargano N. Protective Th1 immune responses against chronic toxoplasmosis induced by a protein-protein vaccine combination but not by its DNA–protein counterpart. Vaccine 2008;26(41):5289–95.
[30] Golkar M, Shokrgozar MA, Rafati S, Musset K, Assmar M, Sadaie R, et al. Evaluation of protective effect of recombinant dense granule antigens GRA2 and GRA6 formulated in monophosphoryl lipid A (MPL) adjuvant against *Toxoplasma* chronic infection in mice. Vaccine 2007;25(May (21)):4301–11.
[31] Brink R, Phan TG, Paus D, Chan TD. Visualizing the effects of antigen affinity on T-dependent B-cell differentiation. Immunol Cell Biol 2008;86(January (1)):31–9.
[32] Buzoni-Gatel D, Schulthess J, Menard LC, Kasper LH. Mucosal defences against orally acquired protozoan parasites, emphasis on *Toxoplasma gondii* infections. Cell Microbiol 2006;8(4):535–44.
[33] Mack DG, McLeod R. Human *Toxoplasma gondii*-specific secretory immunoglobulin A reduces *T. gondii* infection of enterocytes *in vitro*. J Clin Invest 1992;90(December (6)):2585–92.
[34] Reed SG, Bertholet S, Coler RN, Friede M. New horizons in adjuvants for vaccine development. Trends Immunol 2009;30(January (1)):23–32.
[35] Debard N, Buzoni-Gatel D, Bout D. Intranasal immunization with SAG1 protein of *Toxoplasma gondii* in association with cholera toxin dramatically reduces development of cerebral cysts after oral infection. Infect Immun 1996;64(June (6)):2158–66.
[36] Bonenfant C, Dimier-Poisson I, Velge-Roussel F, Buzoni-Gatel D, Del Giudice G, Rappuoli R, et al. Intranasal immunization with SAG1 and nontoxic mutant heat-labile enterotoxins protects mice against *Toxoplasma gondii*. Infect Immun 2001;69(March (3)):1605–12.
[37] Ulanova M, Tarkowski A, Hahn-Zoric M, Hanson LA. The common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. Infect Immun 2001;69(February (2)):1151–9.
[38] Cariappa A, Chase C, Liu H, Russell P, Pillai S. Naive recirculating B cells mature simultaneously in the spleen and bone marrow. Blood 2007;109(March (6)):2339–45.
[39] Dziadek B, Gatkowska J, Brzostek A, Dziadek J, Dzitko K, Dlugonska H. *Toxoplasma gondii*: the immunogenic and protective efficacy of recombinant ROP2 and ROP4 rhoptry proteins in murine experimental toxoplasmosis. Exp Parasitol 2009;123(September (1)):81–9.
[40] Pena HF, Gennari SM, Dubey JP, Su C. Population structure and mouse-virulence of *Toxoplasma gondii* in Brazil. Int J Parasitol 2008;38(April (5)):561–9.
[41] Williams DM, Grumet FC, Remington JS. Genetic control of murine resistance to *Toxoplasma gondii*. Infect Immun 1978;19(February (2)):416–20.
[42] Fux B, Rodrigues CV, Portela RW, Silva NM, Su C, Sibley D, et al. Role of cytokines and major histocompatibility complex restriction in mouse resistance to infection with a natural recombinant strain (type I–III) of *Toxoplasma gondii*. Infect Immun 2003;71(November (11)):6392–401.
[43] Stanley AC, Buxton D, Innes EA, Huntley JF. Intranasal immunisation with *Toxoplasma gondii* tachyzoite antigen encapsulated into PLG microspheres induces humoral and cell-mediated immunity in sheep. Vaccine 2004;22(29–30):3929–41.
[44] Cerutti A, Immunology. IgA changes the rules of memory. Science 2010;328(June (5986)):1646–7.
[45] Ogra PL, Karzon DT. Distribution of poliovirus antibody in serum, nasopharynx and alimentary tract following segmental immunization of lower alimentary tract with poliovaccine. J Immunol 1969;102:1423–30.
[46] Matson DO, O'Ryan ML, Herrera I, Pickering LK, Estes MK. Fecal antibody responses to symptomatic and asymptomatic rotavirus infections. J Infect Dis 1993;167:577–83.
[47] Ennis FA, Yi-Hua Q, Schild GC. Antibody and cytotoxic T lymphocyte responses of humans to live and inactivated influenza vaccines. J Gen Virol 1982;58:273–81.
[48] Sayles PC, Gibson GW, Johnson LL. B cells are essential for vaccination-induced resistance to virulent *Toxoplasma gondii*. Infect Immun 2000;68(March (3)):1026–33.
[49] Dubey JP. Toxoplasmosis – a waterborne zoonosis. Vet Parasitol 2004;126(December (1–2)):57–72.
[50] Mackowiak M, Maki J, Motes-Kreimeyer L, Harbin T, Van Kampen K. Vaccination of wildlife against rabies: successful use of a vectored vaccine obtained by recombinant technology. Adv Vet Med 1999;41:571–83.