Med Microbiol Immunol
DOI 10.1007/s00430-015-0447-5

ORIGINAL INVESTIGATION

Immunity in the spleen and blood of mice immunized with irradiated *Toxoplasma gondii* tachyzoites

Nahiara Esteves Zorgi[1,2] · Andrés Jimenez Galisteo Jr.[2] · Maria Notomi Sato[3] · Nanci do Nascimento[4] · Heitor Franco de Andrade Jr.[1,2,5]

Received: 28 July 2015 / Accepted: 21 December 2015

Abstract *Toxoplasma gondii* infection induces a strong and long-lasting immune response that is able to prevent most reinfections but allows tissue cysts. Irradiated, sterilized *T. gondii* tachyzoites are an interesting vaccine, and they induce immunity that is similar to infection, but without cysts. In this study, we evaluated the cellular immune response in the blood and spleen of mice immunized with this preparation by mouth (v.o.) or intraperitoneally (i.p.) and analyzed the protection after challenge with viable parasites. BALB/c mice were immunized with three i.p. or v.o. doses of irradiated *T. gondii* tachyzoites. Oral challenge with ten cysts of the ME-49 or VEG strain at 90 days after the last dose resulted in high levels of protection with low parasite burden in the immunized animals. There were higher levels of specific IgG, IgA and IgM antibodies in the serum, and the i.p. immunized mice had higher levels of the high-affinity IgG and IgM antibodies than the orally immunized mice, which had more high-affinity IgA antibodies. B cells ($CD19^+$), plasma cells ($CD138^+$) and the $CD4^+$ and $CD8^+$ T cell populations were increased in both the blood and spleen. Cells from the spleen of the i.p. immunized mice also showed antigen-induced production of interleukin-10 (IL-10), interferon gamma (IFN-γ) and interleukin 4 (IL-4). The $CD4^+$ T cells, B cells and likely $CD8^+$ T cells from the spleens of the i.p. immunized mice proliferated with a specific antigen. The protection was correlated with the spleen and blood $CD8^+$ T cell, high-affinity IgG and IgM and antigen-induced IL-10 and IL-4 production. Immunization with irradiated *T. gondii* tachyzoites induces an immune response that is mediated by B cells and $CD4^+$ and $CD8^+$ T cells, with increased humoral and cellular immune responses that are necessary for host protection after infection. The vaccine is similar to natural infection, but free of tissue cysts; this immunity restrains infection at challenge and can be an attractive and efficient model for vaccine development in toxoplasmosis.

Keywords *Toxoplasma gondii* · Vaccine · $CD4^+$ T lymphocytes · $CD8^+$ T lymphocytes · B lymphocytes · Ionizing radiation

✉ Heitor Franco de Andrade Jr.
hfandrad@usp.br

[1] Departamento de Parasitologia, Instituto de Ciências Biomédica, USP, Av. Prof. Lineu Prestes, 1374, Edifício Biomédicas II Cidade Universitária, São Paulo, SP CEP: 05508-000, Brazil

[2] Laboratório de Protozoologia, Instituto de Medicina Tropical de São Paulo, FMUSP, USP, Av. Dr. Enéas de Carvalho Aguiar, 470, 1° Andar, São Paulo, SP CEP: 05403-000, Brazil

[3] Departamento de Dermatologia, Instituto de Medicina Tropical de São Paulo, FMUSP, USP, Av. Dr. Enéas de Carvalho Aguiar, 470, 3° Andar, São Paulo, SP CEP: 05403-000, Brazil

[4] Laboratório de Biologia Molecular, Instituto de Pesquisas Energéticas e Nucleares, IPEN, Rua Travessa 400, Cidade Universitária, São Paulo, SP CEP: 05508-900, Brazil

[5] Department of Pathology, Faculty of Medicine, Universidade de São Paulo, São Paulo, Brazil

Introduction

Toxoplasmosis is an important medical and veterinary zoonosis caused by an obligate intracellular protozoan parasite, *Toxoplasma gondii* [1]. Infection with this agent may occur in several ways, such as congenital transmission [2], consumption of undercooked or raw meat containing parasite cysts [3, 4], consumption of food [5] or water contaminated with oocysts [6], or by blood transfusion or

Published online: 05 January 2016

organ transplant from infected donors [7]. The contamination of meat from cattle, pigs, sheep and goats with tissue cysts of *T. gondii* has become a major source of disease transmission to humans [8]. The infected animals' meat can progress to toxoplasmosis, which can cause abortions, leading to considerable economic losses to the livestock industry. In addition, *T. gondii* infection in sheep and goats is a major cause of reproductive losses [9].

Toxoplasma gondii infection induces strong and long-lasting immune protection to the host [10]. In acute infection, all infecting forms evolve to the rapidly proliferating tachyzoites, with systemic spread in all tissues. The acute disease is usually controlled by a specific immune response, but the slow-growing bradyzoites evolve to long-lived immune-evading cysts. The established, large immune response is not active against cysts; instead, it results in abolishing the disease, but the infection remains in the long-lived cells in many of the host's organs, particularly those in the central nervous system and skeletal, smooth and cardiac muscle [1].

In immunocompetent individuals, the disease is usually asymptomatic, but eye infection with *T. gondii* leads to vision loss or blindness in some cases [11]. During acute infection, congenital toxoplasmosis is transmitted by the mother to the fetus, which can lead to permanent neurological damage with or without hydrocephalus, microcephaly or chorioretinitis with blindness [2]. The toxoplasmosis can severely affect people with an impaired immune system, such as patients infected with human immunodeficiency virus (HIV) [12], transplant patients [13] or patients with neoplasia [14].

Currently, there is no effective vaccine for the prevention of toxoplasmosis in humans or animals; however, there is one commercial live attenuated vaccine to toxoplasmosis, TOXOVAX®. This vaccine is used for the immunization of New Zealand sheep with live tachyzoites of the low cyst-forming strain S48, which prevents 70 % of abortions and reduces toxoplasmosis-induced economic losses in wool production [15]. However, the vaccine has low efficiency as well as logistical problems for distribution due to low stability [16].

There are several studies evaluating new vaccines for toxoplasmosis, including inactivated and attenuated strains [17, 18], recombinant proteins [19], DNA genes or plasmids [20] and ultraviolet-attenuated [21] or gamma-irradiated parasites [18, 22]. These vaccine models were developed and tested in animal models, but none have been extensively tested for cellular immune responses or long-lived memory cells.

Many challenges remain in vaccine development, largely due to the nature of the pathogen. Most licensed viral vaccines suggest that humoral immunity is the key mediator of efficiency, but cellular immunity is also likely critical to increasing the protection against intracellular eukaryote pathogens [23], such as *T. gondii*. The immune response-induced $CD4^+$ or $CD8^+$-specific T cell signals for a particular pathogen have been described for some vaccines, such as those against malaria [24]. For toxoplasmosis, the immune response is mediated by $CD4^+$ T cells, $CD8^+$ T cells and B cells may cooperate to increase the protection of the host, and thus, this specific response to the agent provides efficient long-term immunity [25].

Hiramoto et al. [18] of our group studied a vaccine model for toxoplasmosis with viable radiation-sterilized *T. gondii* tachyzoites. They determined a 200 Gy dose that maintains morphology and physiology of the agent but abolishes its reproduction within host cells or experimental models. Mice immunized by i.p. route with this preparation showed increased survival when challenged with the RH strain and decreased numbers of brain cysts when challenged with the ME-49 strain. Specific IgG humoral immune response as well as cellular response to increased lymphocyte proliferation and blood cytokine production was found. Recently, we had shown that this immunogen could be also used orally for mice immunization with protection after challenge with several *T. gondii* strains, focusing in avidity and IgG production in bone marrow and spleen [22]. In this work, we analyze those two routes looking for activation of immune cells and induction of memory cells in peripheral blood and spleen. We also studied the humoral immune response with production of total and high-affinity IgG, IgA and IgM antibodies and analyzed the protection after longer periods challenged with two cystogenic strains.

Materials and methods

Parasites and animals

The cryopreserved *T. gondii* strains RH (I), ME49 (II) and VEG (III) were maintained in liquid nitrogen and recovered by passage in mice (Protozoology Laboratory, IMTSP). Isogenic, young male BALB/c male mice (20 g) were obtained from our colony (Bioterism Center of School of Medicine of University of São Paulo) and maintained in sterilized cages with commercial food (Nutrients Nuvital®) and water provided ad libitum. The animal manipulations were 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 including euthanasia were submitted to and approved by the Animal Experimentation Ethic Council—Institute of Biomedical Sciences/University of São Paulo (ICB/USP).

Irradiation and immunization

Viable irradiated tachyzoites were produced as previously described [18]. Briefly, the peritoneal cavities of

intraperitoneally (i.p.) infected mice were washed with phosphate-buffered saline (PBS) to obtain *T. gondii* RH strain tachyzoites. The parasite suspensions were filtered through a 5-μm polycarbonate filter and maintained in an ice-cold bath until the moment of irradiation with 255 Gy from a uniform source of Cobalt-60 in Gammacell™ (Atomic Energy of Canada Ltd.). Sham non-irradiated parasites were also produced, and both suspensions were cryopreserved in liquid nitrogen. Groups of at least four mice each assay were immunized with three biweekly doses of 10^7 irradiated tachyzoites; the parasites were administered either by i.p. injection or via oral gavage (v.o.) of 10^7 irradiated tachyzoites suspended 1:1 (v/v) in 6 % aluminum hydroxide.

Toxoplasma gondii antigen preparation, ELISAs and antibody affinity determination

Toxoplasma gondii RH strain tachyzoites were harvested from the peritoneal cavities of previously infected mice using PBS washes. The recovered suspensions 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 $10{,}000 \times g$ for 3 min at 4 °C. The supernatants were harvested and used as the *T. gondii* antigen after determining the protein concentration. ELISA plates were coated overnight at 4 °C with 1 μg protein/mL of the *T. gondii* antigen in 0.05 M carbonate buffer, pH 9.0. The 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, the sample serum diluted in PBST was added for 1 h at 37 °C. For the antibody avidity determination, an additional step of a 15-min incubation with a 6 M urea chaotropic solution was added to remove the low avidity antibodies. Next, the plates were washed and appropriately diluted anti-mouse IgG, IgA or IgM peroxidase-conjugated antibodies were added (Sigma Aldrich®). After further washes, the bound conjugate was revealed with 3,3′,5,5′-tetramethylbenzidine (TMB—Sigma Aldrich®) for 30 min; the reactions were stopped by adding 4 M sulfuric acid (H_2SO_4). The absorbance at 450 nm was determined using multi-mode microplate reader (Filter Max F5—Molecular Devices®).

Surface markers from the spleens and peripheral blood cells from BALB/c mice with irradiated *T. gondii* tachyzoites

The spleens and peripheral blood cells were obtained from the mice that were i.p. and v.o. immunized with the irradiated *T. gondii* tachyzoites at 15 days after the third dose, from mice that were chronically infected with ten cysts of the *T. gondii* ME-49 strain and from non-immunized mice. The organs were dissociated in sterile conditions in a laminar flow in RPMI 1640 culture medium. The peripheral blood was obtained by cardiac puncture, and the mononuclear cells in the blood and spleen were separated using Ficoll–Paque™ Premium 1084 (GE Healthcare®) according to the manufacturer's instructions. After pelleting from the central layer, the cells were suspended in 1 mL of culture medium, counted in a Neubauer chamber to obtain the total cell number and finally adjusted to a concentration of 10^6 cells/mL in RPMI 1640 medium. After this procedure, the cells were subjected to cell surface labeling to phenotype the cell population. Anti-mouse monoclonal antibodies were used, including anti-CD3-Pacific Blue (BD Biosciences®), anti-CD4 V500 Horizon (BD Biosciences®), anti-CD8 APC-H7 (BD Biosciences®), anti-CD45RB FITC (BD Biosciences®), anti-CD69 PE (BD Biosciences®) and anti-CD19 PE-Cy7 (BD Biosciences®). A 1:50 dilution of each antibody was prepared in the BD FACSFlow Sheath Fluid™ (BD Biosciences®) solution, and 20 μL of this solution was added to each sample and was then incubated for 30 min at 4 °C in the absence of light to label the cells. Next, 300 μL of the BD FACSFlow Sheath Fluid™ (BD Biosciences®) solution was added, and the cells were analyzed using a flow cytometer (BD Biosciences® LSRFortessa). We used anti-mouse and anti-hamster CompBeads® (BD Biosciences®) for gain compensation. The data were collected (50,000 events) by the BD FACSDIVA® software and analyzed using the FlowJo X® software.

The cell populations were expressed as the absolute number of cells in the spleen and were calculated as the proportion of identified cells from each phenotype associated with the starting total cell population. To analyze the activation of each lymphocyte population, the cells were reacted with the phenotypic markers as above and using the following activation markers: anti-CD44 APC, anti-CD45RB FITC, anti-CD69 PE and anti-CD23-FITC. Activation was expressed as the mean fluorescence intensity (MFI) in the selected population.

Production of cytokines, IL-10, IL-2, IL4, IL6, IL-17A, IFN-γ and TNF-α by the spleen cells from mice immunized with irradiated *T. gondii* tachyzoites

The spleen cells (2×10^6 cells/well) from immunized, infected and control mice were placed in polystyrene culture plate (TPP®) and stimulated with 10 μg/mL of the total *T. gondii* antigen extract (specific stimulus) or 5 μg/mL concanavalin A (ConA—non-specific stimulus). Cells without stimulation (basal) served as a control. The *T. gondii* antigen extract and ConA used in assays

were previously titrated (data not shown). The cells were maintained in an incubator with 5 % CO_2 at 37 °C for 72 h. After incubation, the cells were centrifuged at 700×*g* for 8 min and the supernatant was used for cytokine determination. The cytokine analysis was performed using a commercial kit for mouse cytokines, the Cytometric Bead Array® (CBA) Th1, Th2 and Th17 (BD Biosciences®), according to the manufacturer's instructions, and the analysis was conducted in a flow cytometer (BD Biosciences® LSRFortessa). The data were collected by the BD FACSDIVA® (BD Biosciences®) software and analyzed using the FCAP Array V 3.0® (BD Biosciences®) software.

Proliferation of B cells ($CD19^+$), TCD4 cells ($CD3^+CD4^+$) and TCD8 cells ($CD3^+CD8^+$) from the spleens of mice immunized with irradiated *T. gondii* tachyzoites

The spleen cells (4 × 10^6 cells/mL) from immunized, infected and control mice were placed in appropriate dilution of 5 μM 5–6 carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes®—Life Technologies™), and the cells were incubated at 37 °C and 5 % CO_2 for 5 min. Next, the cells were shaken to label the homogeneous cells and again incubated for 5 min. After labeling, RPMI 1640 culture medium with 10 % fetal bovine serum was added to the cultures in a fivefold excess. The cells were maintained on ice for 5 min. After incubation, the cells were centrifuged at 700×*g* for 8 min, the supernatant was discarded, and the pellet was dissolved in 10 mL of RPMI 1640 culture medium with 10 % fetal bovine serum. The resuspended pellet was centrifuged again, and the resulting pellet was dissolved in 1 mL of RPMI 1640 culture medium with 10 % fetal bovine serum. Finally, the cells were placed in culture and received specific stimulation with the total *T. gondii* antigen extract (30 μg/mL), non-specific stimulation with concanavalin A (10 μg/mL) or were not stimulated cells (basal); cells not labeled with CFSE were also prepared and maintained in an incubator at 37 °C with 5 % CO_2 for 6 days. After the incubation period, the cells were centrifuged at 700×*g* for 8 min and the pellet dissolved in 200 μL of BD FACSFlow Sheath Fluid™ (BD Biosciences®). After this procedure, the cells were labeling to determine the populations of lymphocytes, as previously described. After labeling, the cells were analyzed by flow cytometry (LSRFortessa). The data were collected (50,000 events) by the FACSDIVA BD software and analyzed using the FlowJo X software. All immunized sample analysis was compensated using basal levels of non-immunized mice cells as standards submitted to all phenotype markers reagents.

Challenge and DNA extraction of the brain tissue from the immunized mice

Infective *T. gondii* ME49 and VEG cysts were obtained from the brains of chronically infected mice, which are routinely maintained at the Protozoology Laboratory, IMTSP. Briefly, after a minimum of 30 days of infection, the mice were euthanized, and their brains were removed and homogenized in 10 mL of sterile saline. Cyst counts were performed using a phase contrast microscope, and the suspension was adjusted to 50 cysts in each mL. The immunized or control mice were challenged with ten cysts of the respective strain administered by oral gavage 90 days after the last immunizing dose; the mice were observed for daily survival determination. After 30 days, the surviving animals were killed and the brains were macerated in sterile saline solution. The solution containing the brain was subjected to DNA extraction to analyze the absolute number of parasites by real-time PCR (real-time PCR System® 7500—Applied Biosystems®). The DNA extraction was performed using the DNA Mini Kit QIAamp® (QIAGEN®) according to the manufacturer's instructions.

Real-time PCR analysis for the absolute quantification of the parasites in the brain tissue from the immunized mice

The analysis of the absolute number of parasites in the brains from mice immunized with irradiated *T. gondii* tachyzoites and challenged with the *T. gondii* ME-49 or VEG strains was performed by real-time PCR to detect the parasite DNA. For this detection, we used the sense B1JW63 (GCACCTTTCGGACCTCAACAACCG) and antisense B1JW62 (TTCTCGCCTCATTTCTGGGTCTAC) primers for the B1 gene [26] and the Power SYBR Green® PCR Master Mix (Applied Biosystems®) according to the manufacturer's instructions in a real-time PCR System® 7500—Applied Biosystems®. The system was programmed for an initial denaturing step at 95 °C for 10 min, followed by 40 cycles of PCR using a 60 °C annealing temperature. In each assay, a standard curve starting with 1.2 × 10^5 *T. gondii* RH strain tachyzoites/mL was serially diluted 1:4 until a concentration of one parasite/mL was obtained. All samples and standards were run in triplicates. The number of parasites was automatically determined by the 7500 software (Applied Biosystems®).

Statistical analysis

The comparisons between the quantitative values, such as the amount of antibody, absolute number of cells, the MFI and the parasite load in the brain, were made using the

ANOVA test after checking the homogeneity of variances. Comparisons were considered significant when the likelihood of equality is <5 % ($p < 0.05$). All statistical estimates were calculated using the statistical package GraphPad Prism 5.0.

Results

Immunization protocol and protection in immunized BALB/c mice

Groups of five BALB/c mice received three doses of irradiated *T. gondii* tachyzoites biweekly by intraperitoneal injection or oral administration with aluminum hydroxide. During and after the immunization period, the animals showed no indication of infection and all animals survived for several months after immunization. To evaluate the induced immunological protection, the BALB/c mice immunized with irradiated *T. gondii t*achyzoites were challenged with ten cysts of the *T. gondii* ME49 (Fig. 1a) or VEG strain (Fig. 1b) by oral administration at 90 days after the last immunization. Thirty days after the challenge, the animals were killed and their brains were removed to quantify the absolute numbers of parasites by real-time PCR. We observed that the challenge with both the ME-49 strain (Fig. 1a) and the VEG strain (Fig. 1b) in the intraperitoneally and orally immunized animals showed a significant decrease in the number of parasites in the brain tissue compared with the infected mice without immunization.

Humoral immune responses in the immunized BALB/c mice

To verify that the humoral immune response was induced by immunization with the irradiated *T. gondii* tachyzoites, we analyzed the production of specific IgG, IgA and IgM antibodies in the serum of the immunized animals by ELISA. The intraperitoneally immunized BALB/c mice showed significantly increased levels of the total specific IgG antibodies compared with the control animals. The orally immunized BALB/c mice had lower specific total IgG antibody production levels compared with the intraperitoneally immunized animals, but this was significantly higher than the control group. Chronically infected mice also showed significantly higher levels of specific total IgG antibodies in the serum. We observed that the specific IgG antibodies induced by immunization with irradiated *T. gondii* tachyzoites showed high affinity for the antigen, and we observed a significant increase in the specific high-affinity IgG antibodies in the intraperitoneally immunized mice and chronically infected animals (Fig. 2a).

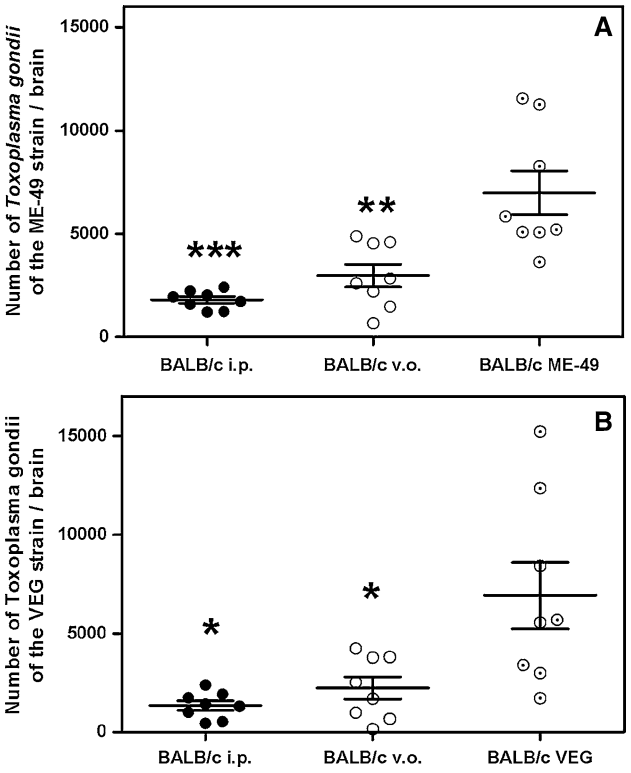


Fig. 1 Absolute quantification of *T. gondii* parasites by real-time PCR in the brain tissue of mice immunized with irradiated *T. gondii* RH strain tachyzoites by intraperitoneal or oral administration that were orally challenged with ten cysts of the ME-49 (**a**) or VEG (**b**) strain. The *bars* represent the means and standard error of the mean, and the presence of an *asterisk* indicates a significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) compared with the control group. *BALB/c i.p.* intraperitoneally immunized BALB/c mice, *BALB/c v.o.* orally immunized BALB/c mice, *BALB/c ME-49* BALB/c mice infected with ten cysts of the *T. gondii* ME-49 strain (**a**) without immunization; *BALB/c VEG* BALB/c mice infected with ten cysts of the VEG strain of *T. gondii* (**b**) without immunization

To determine whether the antibody responses induced by immunization with *T. gondii* irradiated tachyzoites were long lasting, we analyzed the production of specific IgG antibodies in the serum by ELISA after a longer period of immunization. The intraperitoneally immunized BALB/c mice and chronically infected mice showed a significant increase in the specific total IgG antibodies in the control group after 120 days of immunization, and the orally immunized mice did not produce a significant amount of serum IgG antibodies after this period. Immunization with irradiated *T. gondii* tachyzoites induced a permanent humoral immune response in the intraperitoneally immunized mice, with a significant increase in the production of specific high-affinity IgG antibodies after 120 days of immunization compared with the group without immunization. The chronically infected mice also showed a

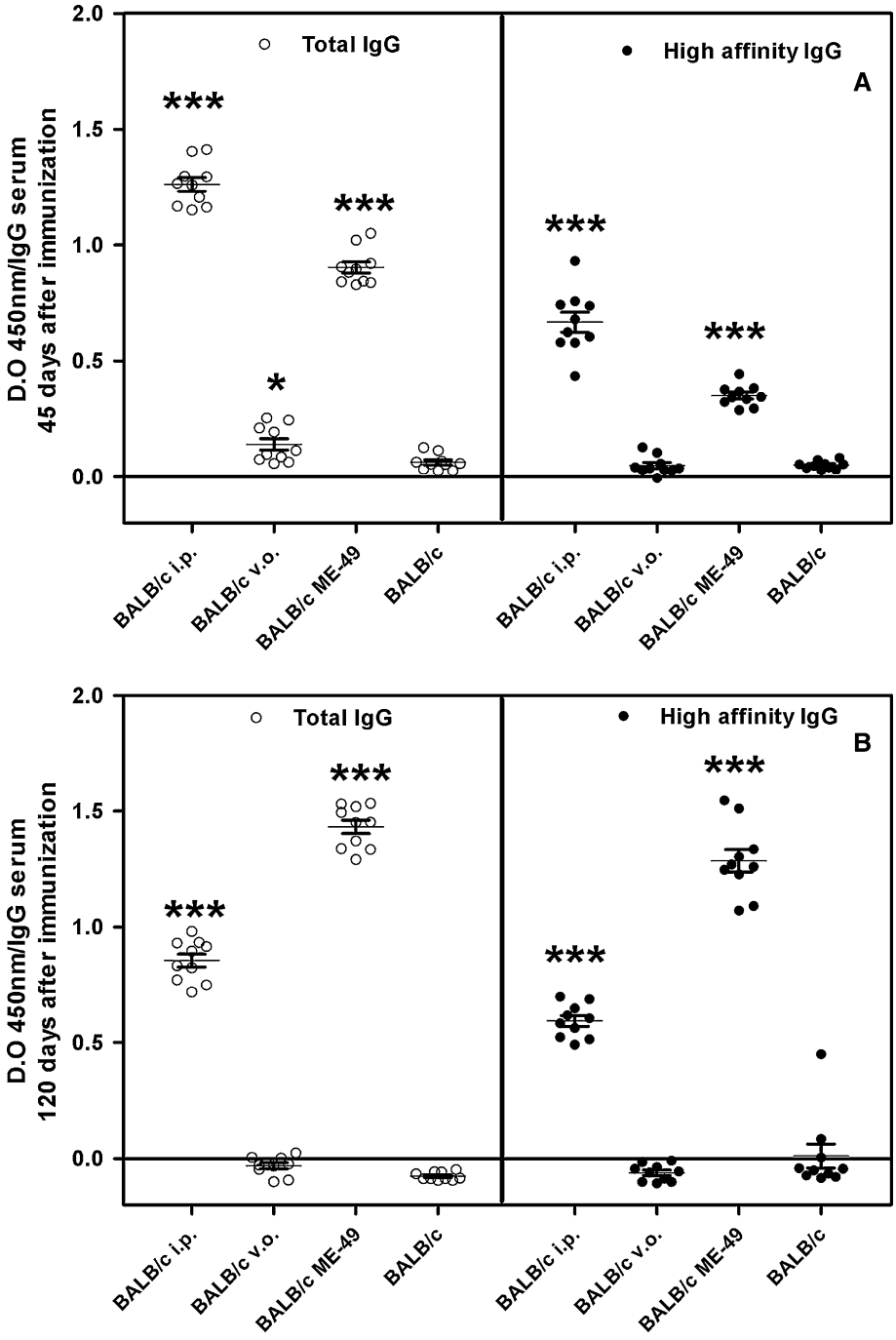


Fig. 2 Detection of total anti-*T. gondii* (*open circles*) and high-avidity (*closed circles*) IgG antibodies in the sera of mice that were intraperitoneally or orally immunized with irradiated *T. gondii* RH strain tachyzoites at 45 days (**a**) and 120 days (**b**) after the immunization. The *bars* represent the means and standard error of the mean, and the presence of an *asterisk* indicates a significant difference (**$p < 0.01$; ***$p < 0.001$) compared with the control group. *BALB/c i.p.* intraperitoneally immunized BALB/c mice, *BALB/c v.o.* orally immunized BALB/c mice, *BALB/c ME-49* BALB/c mice at 30 days after infection with ten cysts of the *T. gondii* ME-49 strain, *BALB/c* BALB/c control without immunization and infection

significant increase in the high-affinity IgG antibodies (Fig. 2b).

Both routes of the immunization produced significantly higher levels of total IgA anti-*T. gondii* antibodies in the mouse serum compared with the control animals. The animals receiving intraperitoneal immunization had lower levels of specific IgA antibodies in the serum. The chronically infected mice also showed significant levels of specific serum IgA antibodies. We noted that immunization with irradiated *T. gondii* tachyzoites induced the production of specific high-affinity IgA antibodies against the antigen, and we observed a significant increase in the specific high-affinity IgA antibodies in the orally immunized mice and chronically infected animals (Fig. 3a).

The intraperitoneally and orally immunized BALB/c mice and chronically infected mice showed significantly higher levels of total IgM anti-*T. gondii* antibodies compared with the control animals. Mice that received the immunogen intraperitoneally showed a significant increase in the production of specific high-affinity IgM antibodies (Fig. 3b).

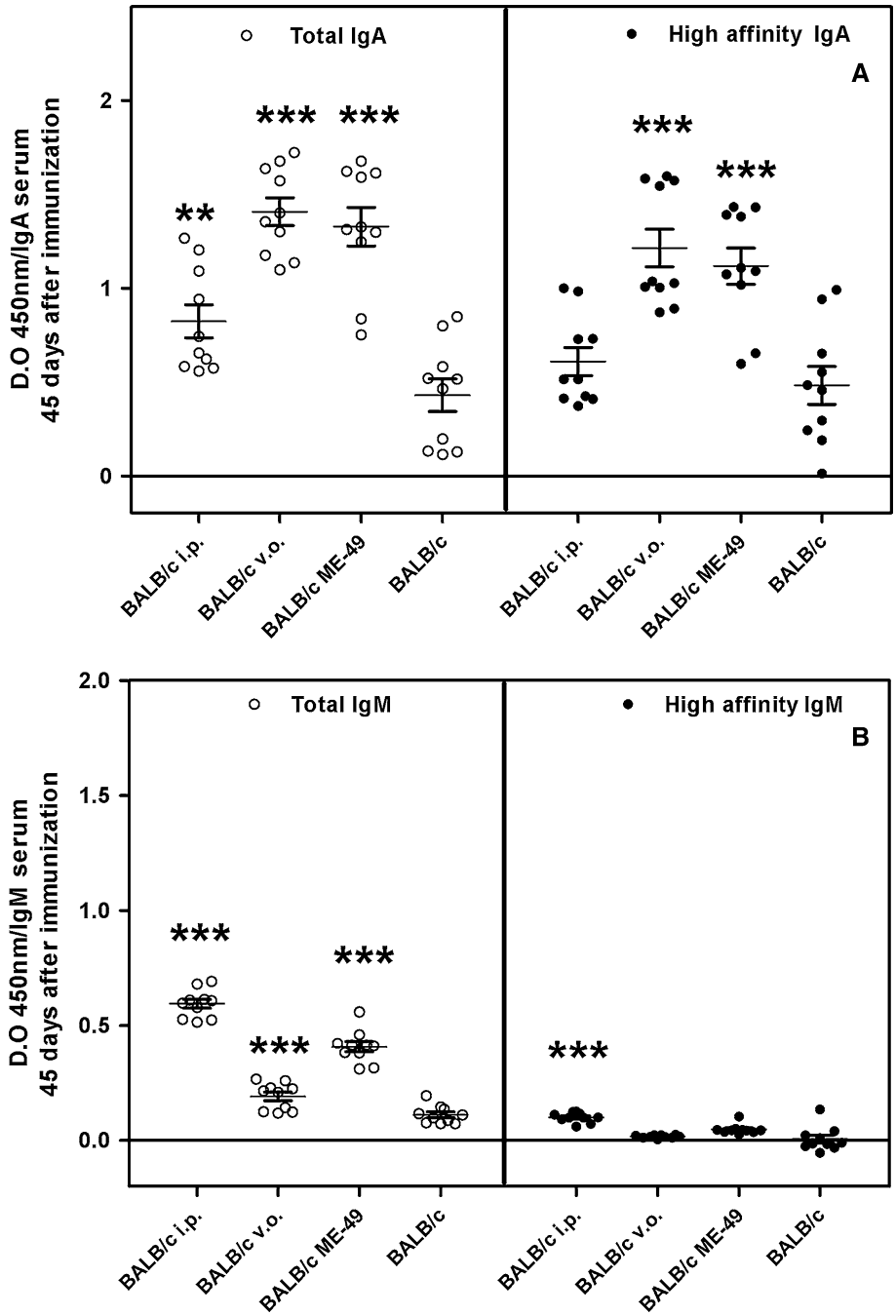


Fig. 3 Detection of total anti-*T. gondii* (*open circles*) and high-avidity (*closed circles*) IgA (**a**) and IgM (**b**) antibodies in the sera of mice that were intraperitoneally or orally immunized with irradiated *T. gondii* RH strain tachyzoites at 45 days after the immunization. The *bars* represent the means and standard error of the mean, and the presence of an *asterisk* indicates a significant difference (**$p < 0.01$; ***$p < 0.001$) compared with the control group. *BALB/c i.p.* intraperitoneally immunized BALB/c mice, *BALB/c v.o.* orally immunized BALB/c mice, *BALB/c ME-49* BALB/c mice at 30 days after infection with ten cysts of the *T. gondii* ME-49 strain, *BALB/c* BALB/c control without immunization and infection

Cell populations in the spleens and blood of immunized mice

The mice immunized with irradiated *T. gondii* tachyzoites by either the i.p. or v.o. route were euthanized 15 days after the last dose, and the blood was collected by cardiac puncture, and the spleens were removed in sterile conditions in a laminar flow hood. The cells were purified as described in "Materials and methods" section. We analyzed the immunization-induced production of cells involved in the immune response by flow cytometry, including B lymphocytes ($CD19^+$), helper T lymphocytes ($CD3^+CD4^+$) and cytotoxic T lymphocytes ($CD3^+CD8^+$), from the spleen and peripheral blood of groups of immunized animals. For a more specific analysis, we chose to report the results as the absolute number of cells of each population and not merely the proportion of these cells, as described in "Materials and methods" section. The flow cytometry results shown are the average of three experiments, with three animals from each group in each experiment.

There was a significant increase in the population of B cells ($CD19^+$) in the spleens from the intraperitoneally

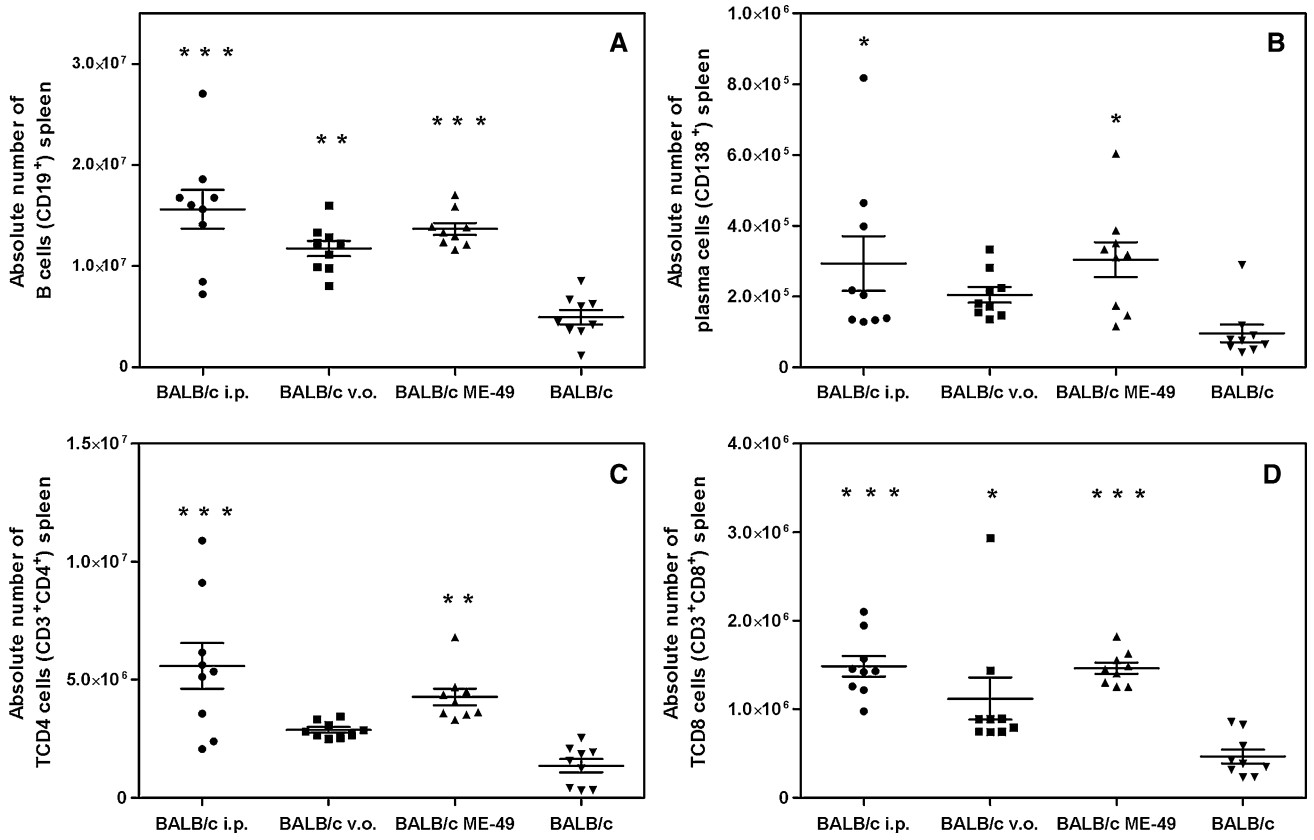


Fig. 4 Absolute number of B cells ($CD19^+$) (**a**), plasma cells ($CD138^+$) (**b**), TCD4 cells ($CD3^+CD4^+$) and TCD8 cells ($CD3^+CD8^+$) in the spleens of mice that were intraperitoneally or orally immunized with irradiated *T. gondii* RH strain tachyzoites at 45 days after the immunization. The *bars* represent the means and standard error of the mean, and the presence of an *asterisk* indicates a significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) compared with the control group. *BALB/c i.p.* intraperitoneally immunized BALB/c mice, *BALB/c v.o.* orally immunized BALB/c mice, *BALB/c ME-49* BALB/c mice at 30 days after infection with ten cysts of the *T. gondii* ME-49 strain, *BALB/c* BALB/c control without immunization and infection

and orally immunized mice (Fig. 4a). There was also an increase in this cell population in the spleens of the animals infected with the *T. gondii* ME-49 strain; this response was similar to the immunization. The increased population of plasma cells ($CD138^+$) was significant in the spleens from the intraperitoneally immunized mice and chronically infected mice (Fig. 4b).

There was a significant increase in the population of T $CD4^+$ cells ($CD3^+CD4^+$) in the spleens of animals that received the immunogen by intraperitoneal injection and animals infected with cysts of the *T. gondii* ME-49 strain compared with the control group (Fig. 4c). Mice from both routes of immunization and the infection models showed a significant increase in the population of T $CD8^+$ cells ($CD3^+CD8^+$) in the spleen compared with the animals without immunization and without infection (Fig. 4d).

Figure 5 shows the percentage of B cells ($CD19^+$), plasma cells ($CD138^+$), helper T cells ($CD3^+CD4^+$) and cytotoxic T cells ($CD3^+CD8^+$) in the peripheral blood of the mice that were intraperitoneally or orally immunized with irradiated *T. gondii* tachyzoites. There was a significant increase in the percentage of B cells (Fig. 5a) and plasma cells (Fig. 5b) in the peripheral blood of the orally immunized mice.

The animals that received the immunogen orally also showed a significant increase in the percentage of T $CD4^+$ cells in the peripheral blood compared with the control group (Fig. 5c). Mice that were immunized by both routes and mice infected with the *T. gondii* ME-49 strain showed a significant increase in the percentage of T $CD8^+$ cells in the peripheral blood compared with the animals without immunization and without infection (Fig. 5d).

Activation of the cell populations in the spleen and peripheral blood of the immunized mice

To analyze cellular activation in the B cells ($CD19^+$), $TCD4^+$ cells ($CD3^+CD4^+$) and $TCD8^+$ cells ($CD3^+CD8^+$)

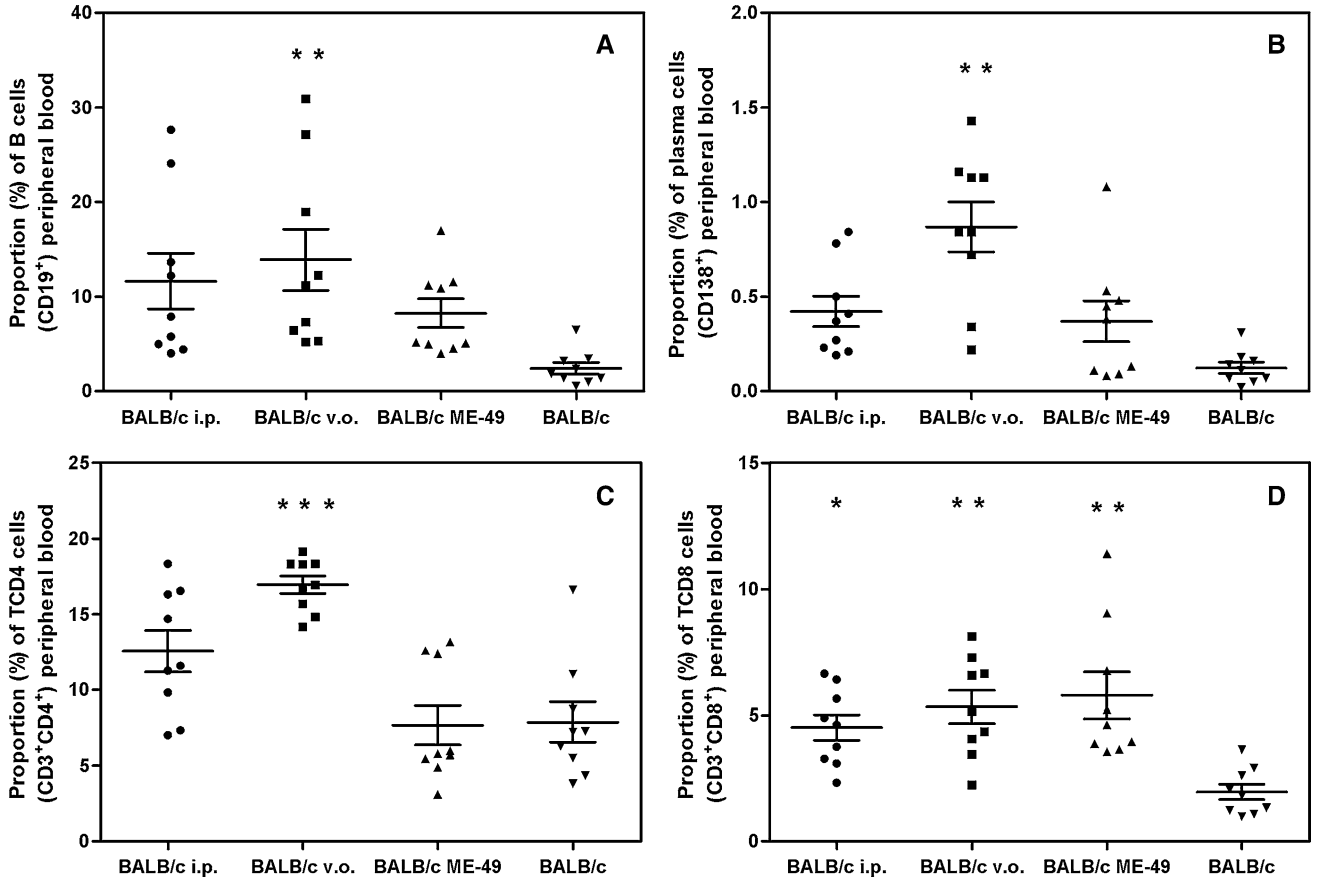


Fig. 5 Proportion (%) of B cells ($CD19^+$) (**a**), plasma cells ($CD138^+$) (**b**), TCD4 cells ($CD3^+CD4^+$) (**c**) and TCD8 cells ($CD3^+CD8^+$) (**d**) in the peripheral blood of mice that were intraperitoneally or orally immunized with irradiated *T. gondii* RH strain tachyzoites at 45 days after the immunization. The *bars* represent the means and standard error of the mean, and the presence of an *asterisk* indicates a significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) compared with the control group. *BALB/c i.p.* intraperitoneally immunized BALB/c mice, *BALB/c v.o.* orally immunized BALB/c mice, *BALB/c ME-49* BALB/c mice at 30 days after infection with ten cysts of the *T. gondii* ME-49 strain, *BALB/c* BALB/c control without immunization and infection

in the spleens and peripheral blood of mice that were intraperitoneally or orally immunized with irradiated *T. gondii* tachyzoites, we analyzed these cells using specific surface markers, such as anti-CD45RB and anti-CD69, by flow cytometry. These activated cell populations were expressed as MFI.

The B cells in the spleens of the intraperitoneally immunized mice showed a significant increase in CD69 expression compared with the B cells from the control animals (Fig. 6a). The T $CD4^+$ cells from the spleens of mice immunized by both routes and animals infected with the *T. gondii* ME-49 strain exhibited a significant increase in CD69 expression (Fig. 6b). The increased CD69 expression on the T $CD8^+$ cells from the spleen was only significant in the intraperitoneally immunized mice (Fig. 6c).

CD45RB expression was significantly decreased on T $CD4^+$ cells from the spleens of the intraperitoneally or orally immunized mice and the infected mice compared with the control group (Fig. 6d). The expression of this receptor in the T $CD8^+$ cells from the spleens only showed a significant decrease in the infected mice (Fig. 6e).

The activated population of B cells, T $CD4^+$ and T $CD8^+$ cells from peripheral blood is shown in Fig. 8. The infected mice showed a significant increase in CD69 expression in the B cells from the peripheral blood compared with animals without infection (Fig. 7a).

The T $CD4^+$ cells from the peripheral blood of the intraperitoneally immunized mice and infected mice displayed an increase in CD69 expression (Fig. 7b). The increased CD69 expression on T $CD8^+$ cells from the peripheral blood of the immunized and infected animals was not significant (Fig. 7c). CD45RB expression was only significant in the T $CD8^+$ cells from the peripheral blood of intraperitoneally immunized mice and mice infected with the *T. gondii* ME-49 strain (Fig. 7e).

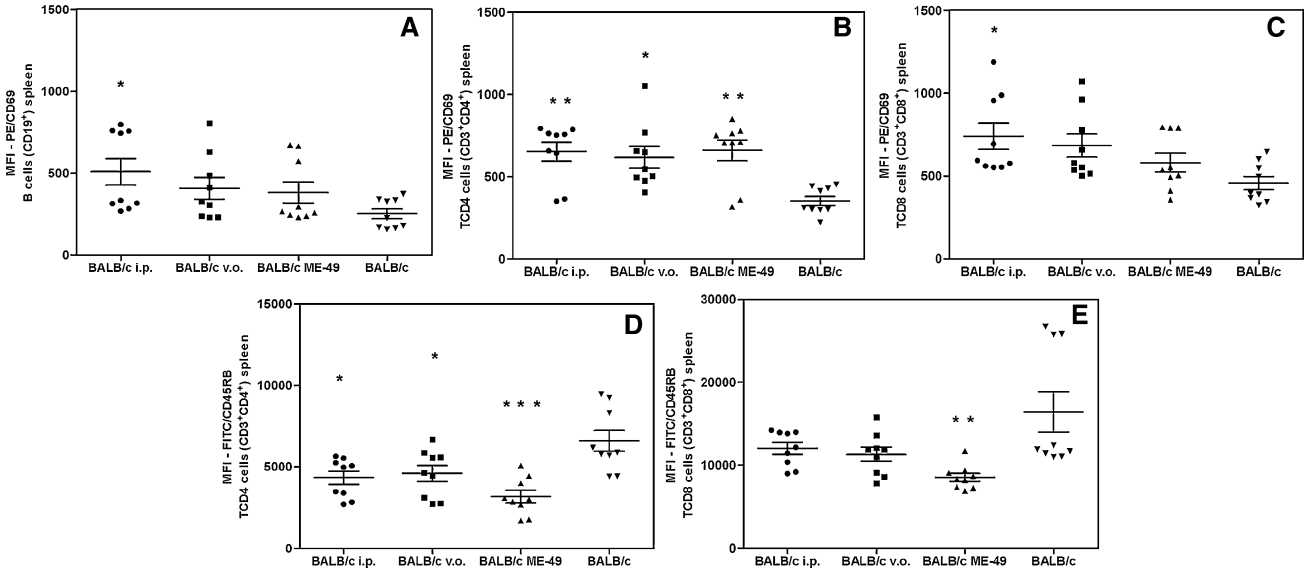


Fig. 6 CD69 expression in B cells ($CD19^+$) (**a**), TCD4 cells ($CD3^+CD4^+$) (**b**) and TCD8 cells ($CD3^+CD8^+$) (**c**), and CD45RB expression in TCD4 cells ($CD3^+CD4^+$) (**d**) and TCD8 cells ($CD3^+CD8^+$) (**e**) in the spleens of mice that were intraperitoneally or orally immunized with irradiated *T. gondii* RH strain tachyzoites at 45 days after the immunization. The *bars* represent the means and standard error of the mean, and the presence of an *asterisk* indicates a significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) compared with the control group. *BALB/c i.p.* intraperitoneally immunized BALB/c mice, *BALB/c v.o.* orally immunized BALB/c mice, *BALB/c ME-49* BALB/c mice at 30 days after infection with ten cysts of the *T. gondii* ME-49 strain, *BALB/c* BALB/c control without immunization and infection

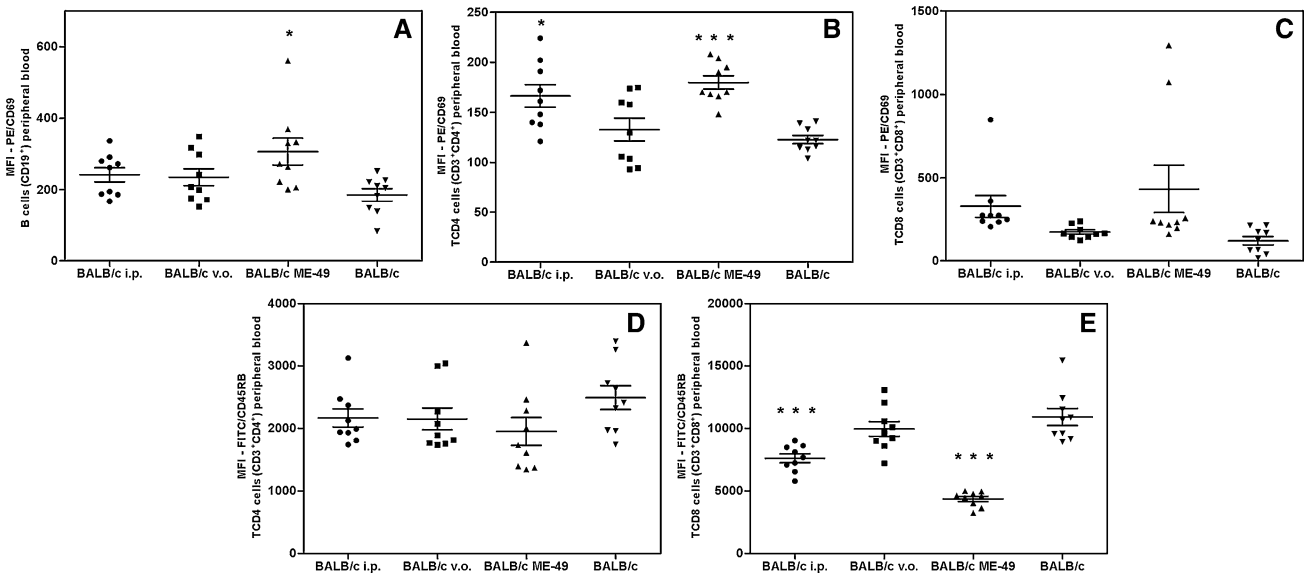


Fig. 7 CD69 expression in B cells ($CD19^+$) (**a**), TCD4 cells ($CD3^+CD4^+$) (**b**) and TCD8 cells ($CD3^+CD8^+$) (**c**), and CD45RB expression in TCD4 cells ($CD3^+CD4^+$) (**d**) and TCD8 cells ($CD3^+CD8^+$) (**e**) in the peripheral blood of mice that were intraperitoneally or orally immunized with irradiated *T. gondii* RH strain tachyzoites at 45 days after the immunization. The *bars* represent the means and standard error of the mean, and the presence of an *asterisk* indicates a significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) compared with the control group. *BALB/c i.p.* intraperitoneally immunized BALB/c mice, *BALB/c v.o.* orally immunized BALB/c mice, *BALB/c ME-49* BALB/c mice at 30 days after infection with ten cysts of the *T. gondii* ME-49 strain, *BALB/c* BALB/c control without immunization and infection

Cellular immune responses in the immunized BALB/c mice

To evaluate the cellular immune responses induced by immunization with irradiated *T. gondii* tachyzoites, we analyzed the production of IL-10, IL-17A, IL-6, IL-4, TNF alpha, IFN gamma and IL-2 by cytometric bead array (CBA). The production of these cytokines was examined in spleen cells cultured in the presence of a non-specific stimulus (ConA, positive control) or a specific stimulus (*T. gondii* protein antigen). Unstimulated cells (basal) were used as a negative control. There was an increase in the production of all cytokines in the non-specifically stimulated cells compared to the basal cytokine production cytokine (data not shown). The spleen cells from the intraperitoneally immunized mice showed a significant increase in the production of cytokines, such as IL-10 (Fig. 8a), IL-4 (Fig. 8f) and IFN gamma (Fig. 8d), after the stimulus with the *T. gondii*-specific antigen. The chronically infected mice showed a significant increase in the production of cytokines, such as IL-6 (Fig. 8e), TNF alpha (Fig. 8b) and IFN gamma (Fig. 8d), from the splenic cells after specific stimulation.

Proliferation of spleen cells from the immunized BALB/c mice

To verify the *T. gondii*-specific antigen-induced proliferation of B cells and CD4 and CD8 T cells, the spleen cells from mice immunized intraperitoneally or orally with irradiated tachyzoites of *T. gondii*, mice infected with ten cysts of the *T. gondii* ME-49 strain, and uninfected and non-immunized mice were analyzed. After 45 days of immunization, two animals from each group were euthanized and the spleens were removed in a sterile manner to dissociate the cells. After purifying the cells, the spleen cells were subjected to labeling with CFSE to quantify the stimulation-induced cell proliferation. The cells stimulated with the non-specific stimuli (ConA) responded with a 60 % increase in proliferation (data not shown). To quantify the proliferation of each population, the cells were stimulated with the specific antigen (*T. gondii* antigen) and then labeled with CFSE and antibodies specific for the cell type, as described in "Materials and methods" section. The mice that were intraperitoneally immunized with irradiated *T. gondii* tachyzoites and the infected mice showed a significant increase in T $CD4^+$ cells ($CD3^+CD4^+$) proliferation compared with the animals without immunization (Fig. 9a). The T $CD8^+$ cell ($CD3^+CD8^+$) proliferation was only significant in the infection models (Fig. 9b). In addition, the mice that received the immunogen intraperitoneally showed a significant increase in the B cell ($CD19^+$) population compared with the control group (Fig. 9c).

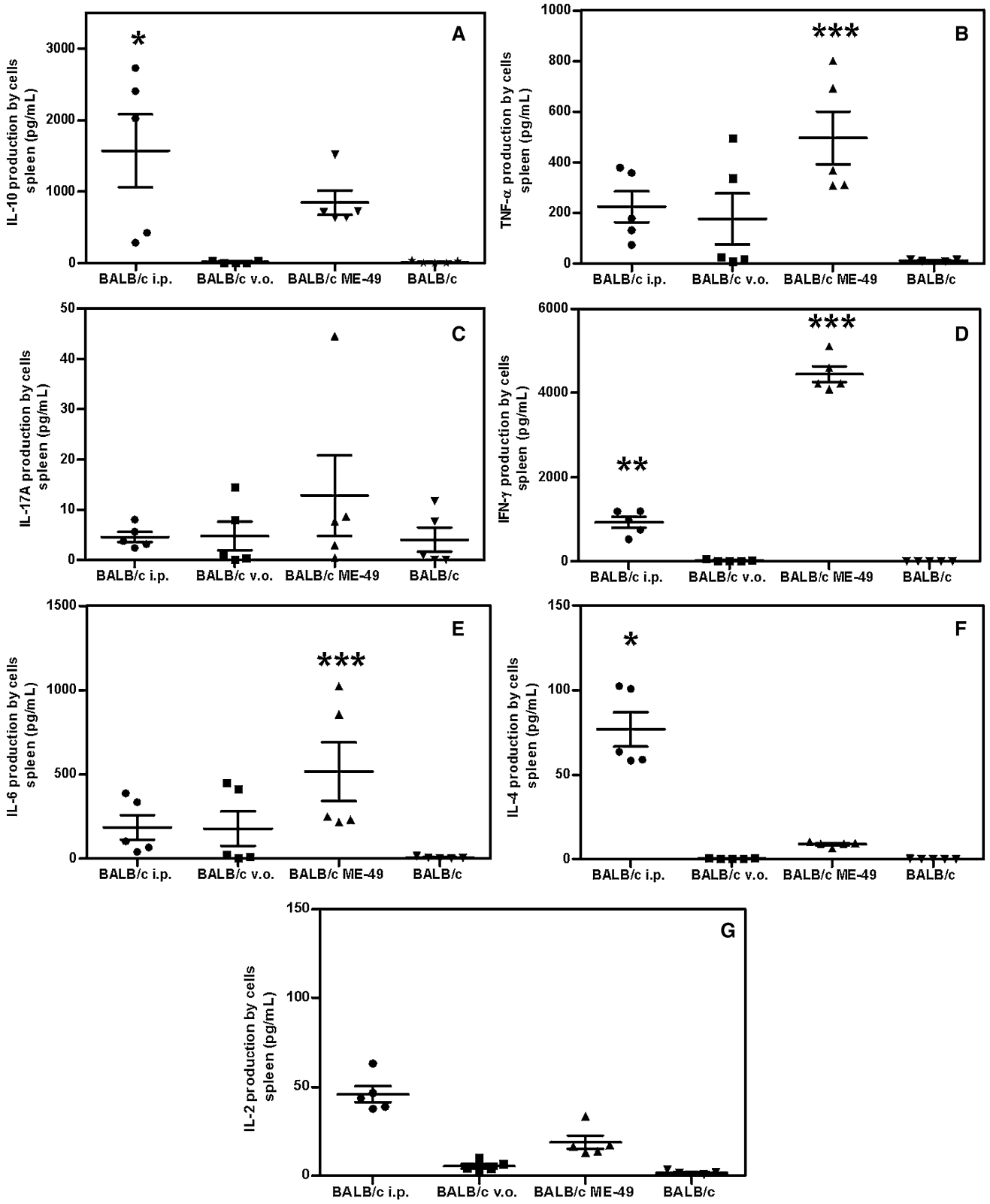
Discussion

Our data demonstrated that the intraperitoneally or orally immunized BALB/c mice showed immune protection after challenge with different strains of *T. gondii*. This immune response was mediated by T $CD4^+$ cells, T $CD8^+$ cells and B cells, resulting in avidity maturation of antibodies and memory cells that were responsive to the antigen. Our vaccine model used irradiated *T. gondii* tachyzoites to induce sterilization of the parasite without cell death, and it maintained an intact structure, physiology and intact and unaltered proteins, but it induced mitotic death at reproduction, abolishing cyst production [18]. This immunogen induced immunity to the agent in the host similar to the natural infection, which is usually protective against reinfection [27].

Oral vaccines would be ideal for an effective vaccine for the prevention of toxoplasmosis, as oral ingestion is the major route of infection. The bradyzoites and tachyzoites of *T. gondii* survived after treatment with pepsin, and these parasites still active, which can infect mice and rats [28]. Tachyzoites are relatively resistant to gastric juice of host [29]. In our studies, the vaccine model orally with irradiated viable tachyzoites together with aluminum hydroxide clearly shows that the tachyzoites survive in the digestive tract of the animal and induce a specific immune response to agent.

The efficiency of the immunization model using irradiated *T. gondii* tachyzoites was assessed by challenge with different strains of the parasite 90 days after the last immunizing dose. Mice that were immunized by both inoculum routes showed increased levels of protection against both the ME-49 strain (type II) and with the VEG strain (type III), with low parasite burden in the brain. Previous work by our group has also shown that mice immunized with the same vaccine model showed increased quantitative and qualitative protection following challenges with different strains 15 days after the last dose [22]. Mice immunized with other model vaccines, such as a DNA vaccine (SAG5), and challenged with 20 cysts of the *T. gondii* PRV strain showed a reduction of cysts in the brain compared with the controls [30]. Most of the studies that developed a vaccine for toxoplasmosis assessed immune protection after immunization using challenges assays [31, 32], but these tests are performed in a short period after immunization and are usually used only one strain of the agent. Vaccination with irradiated parasites exhibits ideal characteristics for vaccine development, due to high levels of protection to the host against both type II and type III strains at long periods after challenge.

Our data showed that mice immunized with irradiated *T. gondii* tachyzoites by both routes of immunization and mice chronically infected with the ME-49 strain showed a



significant increase in specific IgG, IgA and IgM antibodies in the serum. The infection or immunization with different *Toxoplasma* immunogens promotes a significant humoral immune response by producing specific antibodies, such as IgG, IgA and IgM [33, 34]. As reported, this response provides a first line of defense against infection, acting through

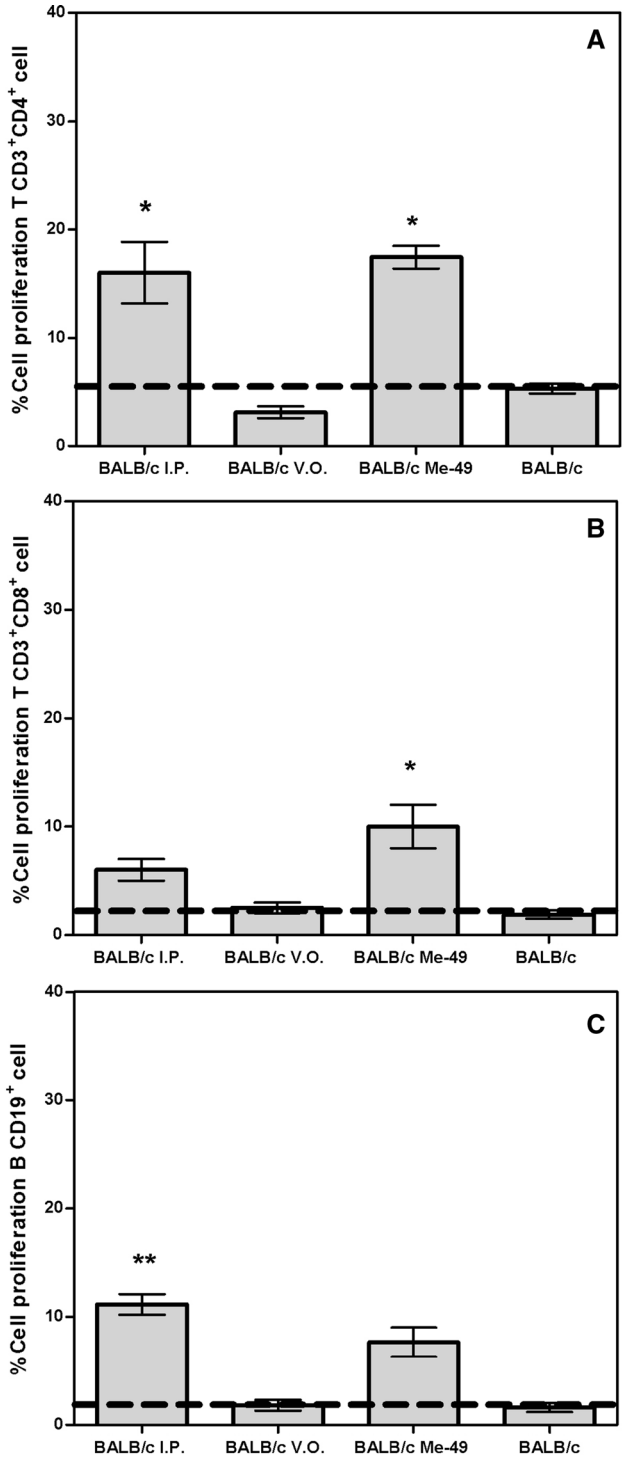
◀**Fig. 8** Production of IL-10 (**a**), TNF-α (**b**), IL-17A (**c**), IFN-γ (D), IL-6 (**e**), IL-4 (**f**) and IL-2 (**g**) by stimulated spleen cells from mice that were intraperitoneally or orally immunized with irradiated *T. gondii* RH strain tachyzoites at 45 days after immunization. The cells were stimulated with the *T. gondii* antigen for 72 h. The *bars* represent the means and standard error of the mean, and the presence of an *asterisk* indicates a significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) compared with the control group. *BALB/c i.p.* intraperitoneally immunized BALB/c mice, *BALB/c v.o.* orally immunized BALB/c mice, *BALB/c ME-49* BALB/c mice at 30 days after infection with ten cysts of the *T. gondii* ME-49 strain, *BALB/c* BALB/c control without immunization and infection

various mechanisms to mitigate or even eliminate the agent or infected cell [35]. The presence of specific antibodies in the serum may be a means to assess the effectiveness of an immune effector for a particular pathogen, because antigen-specific antibodies have been associated with vaccine protection against many diseases [36]. Antibody function in toxoplasmosis is a neglected research field. *T. gondii* infection is commonly diagnosed by specific IgG and IgM detection in the serum [37], and IgM presence is useful as a hallmark of acute infection, which is crucial in prenatal screening [38]. The immune functions of these antibodies, such as in protection assays, have been studied less frequently [39], although some studies have shown the importance of B cells and their products in toxoplasmosis [40]. The detection of antibodies is usually reported as an efficiency marker in vaccine models; for example, a DNA vaccine encoding a glutathione S-transferase antioxidant *T. gondii* protein delivered by intramuscular injection also showed an increase in specific IgG and IgM antibodies in the serum [41]. In our immunization models, the irradiated *T. gondii* tachyzoites induced a strong IgG and IgM response 45 days after immunization, and these antibodies levels negatively correlated with the parasite burden after challenge, reinforcing the importance of these two classes of antibodies for protection.

Mice immunized by both routes of inoculation and the chronically infected mice also had specific IgA antibodies in the serum. This antibody class is produced at higher levels in the v.o. immunized mice, with evidence of affinity maturation. IgA is the most abundant antibody isotype produced in mammals and has few inflammatory properties. Its primary function is to maintain homeostasis in mucosal surfaces and play an important role in protecting the gut against invading pathogens [42]. A higher IgA response was observed in the serum of v.o. immunized mice, and these antibodies are likely related to the mucosal immune response, as IgA synthesis occurs primarily in the gut lymphoid tissues and it is excreted by a secretory component in the gut [43]. Some of these antibodies are secreted into the blood as monomeric IgA, while dimeric IgA (secretory IgA, S-IgA) is predominantly directed to secretions [44]. Complex antigen processing at several sites results in monomeric IgA or secretory IgA or even a combination of the two forms [45]. Other immunization models also demonstrated the increase in IgA in the serum of mice immunized by intramuscular injection with a DNA vaccine [46]. The production of secretory IgA together with serum IgA was observed in mice that were intranasally immunized with different *T. gondii* peptides (AMA1, RON1, RON4) [47]. A previous study by our group demonstrated both serum IgA or S-IgA in the feces of animals immunized with irradiated *T. gondii* tachyzoites [22]. Our data show that higher IgA levels were produced by v.o. immunization, but this antibody appears to be ineffective after invasion, as higher blood IgA levels are ineffective at preventing increased parasite invasion in the brain. Despite this fact, more detailed studies on this antibody production in the mucosa of the v.o. vaccinated animal are needed due to the observed partial protection that could be effective in the early steps of mucosal invasion, resulting in the partial protection observed in the presence of low concentrations of other antibody classes.

Our data showed that immunization with irradiated *T. gondii* tachyzoites induced a long-lasting humoral immune response, with circulating specific IgG antibodies present for a period of 120 days after immunization. This long-term response was achieved without any immune adjuvants, on which most vaccine subcomponents are dependent, but may promote autoimmunity [48]. It is likely that the long-term response is associated with the migration of antigen-specific plasma cells from the spleen or lymph nodes to survival niches, mainly in the bone marrow [49]. Some antibody-producing plasma cells can persist for years in the bone marrow stromal cell compartment [50]. Previous studies performed by our group reported that upon specific stimulation, specific antibodies are produced by the bone marrow cells in vitro [22]. These data suggest that the immunization with irradiated *T. gondii* tachyzoites induces an immune response with the activation of plasma cells and the maintenance of long-term specific antibody levels in the blood.

The analysis of cell phenotypes in our models showed increased numbers of the $CD19^+$, $CD3^+$ subsets $CD4^+$ and $CD8^+$ cells, which were also activated, in the blood and spleen of immunized mice. We found higher levels of memory B cells in the blood of the v.o. immunized mice with higher IgA levels, while memory B cells were more frequent in the spleen of the i.p. immunized mice, correlating with high-affinity IgG in those mice. Memory B cells could be generated in the gut by both T cell-dependent or T cell-independent pathways, while the spleen-generated B cells are usually T cell-dependent [51]. When reexposed to antigen, these cells are reactivated and the rapid process results in increased titers of high-affinity antibodies [52]. In our model, the two immunization schedules could use



◀**Fig. 9** Proliferation of TCD4 cells ($CD3^+CD4^+$) (**a**), TCD8 cells ($CD3^+CD8^+$) (**b**) and B cells ($CD19^+$) (**c**) from the spleens of mice that were intraperitoneally or orally immunized with irradiated *T. gondii* RH strain tachyzoites at 45 days after the immunization following stimulation with the *T. gondii* antigen for 6 days. The *bars* represent the means and standard error of the mean of the two animals, and the presence of an *asterisk* indicates a significant difference ($*p < 0.05$; $**p < 0.01$) compared with the control group. *BALB/c i.p.* intraperitoneally immunized BALB/c mice, *BALB/c v.o.* orally immunized BALB/c mice, *BALB/c ME-49* BALB/c mice at 30 days after infection with ten cysts of the *T. gondii* ME-49 strain, *BALB/c* BALB/c control without immunization and infection. Cell proliferation was detected using the carboxyfluorescein succinimidyl ester (CFSE) assay

different pathways to induce memory B cells and high-affinity antibodies, with T cell-independent pathway used in the v.o. immunized mice. B cells are essential for an immune effector response induced by the vaccine, as memory cells are activated and differentiated into plasma cells that produce specific antibodies that recognize the toxin or agent [53]. The presence of the CD69 activation signal indicated a cellular commitment to the B lymphocyte and plasma cell populations in the spleens of the i.p. immunized animals, while its absence in the spleens of the v.o. immunized mice suggested that B and plasma cells are committed outside the spleen. In other models, such as *Francisella tularensis* infection, B cells are activated and subsequently increase CD69 expression in this cell population [54]. Mice immunized with an attenuated *T. gondii* TS4 strain through intracameral eye injection showed that the B cells in the regional lymph nodes are essential for protecting the host eye [55]. B cell-deficient mice are reported to be susceptible to *T. gondii*, with increased mortality and a high CNS parasite burden after infection with cysts of the ME-49 strain [40]. B lymphocytes, plasma cells and the development of cellular activation are key elements of the immune response and the protection of the host against a variety of pathogens [53, 56]. Previous work by our group showed a significant correlation between the antibodies produced by spleen cells and host protection against different strains of *T. gondii* [22]. Our toxoplasmosis vaccine model induced an increased population of plasma cells and activated B lymphocytes, with diverse maturation sites and antibody class production, showing that different immune pathways cooperate for immune protection in toxoplasmosis.

By analyzing helper and cytotoxic T lymphocytes, we also found higher levels of the $CD3^+$, $CD4^+$ and $CD8^+$ subsets in the blood and spleens of the immunized mice. T $CD4^+$ cells are important in the generation and maintenance of both B cell responses and T $CD8^+$ cells by supplying by growth factors or activating cytokines for specific cells [57]. The increase in T $CD4^+$ lymphocytes ($CD3^+CD4^+$) was higher in the blood from the v.o. immunized mice or in the spleens from the i.p. immunized mice. The cytotoxic $CD3^+CD8^+$ T lymphocytes were increased in the peripheral blood and spleens from mice immunized by both routes of inoculation and from chronically infected mice. $CD4^+$ or $CD8^+$ T lymphocytes are important for the control of latent infection in chronic toxoplasmosis [58]. Other studies, such as those using the Mic8 DNA vaccine, reported an increase in these cells in the spleen, which was

considered important for induced immunity [59]. T $CD8^+$ cells are crucial to the host during infection, because they respond to an intracellular pathogen [60, 61] together with T $CD4^+$ lymphocytes, as demonstrated by the high mortality rate in $CD4^+$-deficient mice that were challenged with the non-lethal ME 49 strain [62]. In attenuated live vaccines, the induction of the T $CD8^+$ cytotoxic cells response was considered important and similar to the protection induced by infection [63, 64]. Several reports of with genetic modified non-replicating csp1 strain are similar to our results, using an uracil auxotroph of non-cyst form Tst4 strain [65, 66]. This immunization uses 10^6 viable parasites in two doses, different from our three 10^7 viable tachyzoite doses in our models, showing similar long-term protection but without sterilization of brain cysts [67]. This is the expected protection for parenteral immunization, which was achieved with several systems including ours, and results in high CD8 counts in tissue inflammation [68], as we also demonstrated in our data. We found an increase in $CD4^+$ or $CD8^+$ T cells with low CD45RB expression but high CD69 expression after immunization that could be linked to the reported effector cytotoxic activity [69]. The induction of T $CD8^+$ cells in our models was evident in both schedules, but their activation was evident in the i.p. immunized mice. Our data suggest that this response could be important for tissue cyst control after infection, as the numbers of T $CD8^+$ cells are inversely associated with the number of parasites in the host brain.

Cooperation between immune cells is mediated by cytokines, and we detected several products in the supernatant of antigen-stimulated spleen cells from the immunized mice. The subsets of cell populations were defined by the secretion of specific cytokines; IFN-γ is produced by Th1 cells, IL-4 is produced by Th2 cells and IL-17 by Th17 cells [70]. Our vaccine induced cellular immune response in an animal model, with increased production of IFN-γ, IL-4 and IL-10 by splenic cells after specific stimulation, likely indicating an integrated Th1–Th2 immune response in the i.p. immunized mice. The Th17 subset does not appear to participate in this process. Oral immunization resulted in the spleen cell production of most cytokines, which was similar to the non-immunized mice, showing that the cooperation of cells in this model occurs outside the spleen. IFN-γ production during acute *T. gondii* infection induces a significant and effective cellular immune response [58]. Both $CD4^+$ and $CD8^+$ T cells can produce IFN-γ during intracellular infections, and the response induced by T $CD4^+$ cells is necessary for macrophages to produce potent antimicrobial responses [71]. In addition, the response-induced T $CD8^+$ cells have a critical role in the control of infection by specifically killing the infected cells [72]. This cellular immune response induced after immunization with irradiated *T. gondii* tachyzoites may be related to the increase of $CD4^+$ and $CD8^+$ T cell populations in the spleens of the immunized mice, which assist in the protection observed here.

Increased memory T $CD4^+$ cell and B cell proliferation was observed in the spleens of i.p. immunized mice, but not in v.o. immunized mice, while T $CD8^+$ memory cells only proliferated during the ME49 infection. Several studies for toxoplasmosis use older cell proliferation assays, most of them without cell phenotyping. The spleen cells from mice immunized with *T. gondii* peptides showed an increase in cell proliferation compared with the control group [47]. Mice immunized with the compound SAG1–SAG3 DNA vaccine also showed an increase in spleen cell proliferation [72]. Previous reports from our group using irradiated tachyzoites also show spleen cell-specific proliferation [18]. The importance of cell phenotyping with combined cell proliferation determines the responsive cell that is involved in the stimulus- or immunogen-induced response [73]. Our data reinforced the idea that our vaccine induced TCD4 cell and B cell proliferation, likely memory cells, in the spleens of i.p. but not v.o. immunized mice.

Vaccine models using radiation to sterilize the agent induce an immune response in the host that can develop high levels of protection, because the irradiated agent cannot increase in number or mature as is normally associated with the disease [63]. Currently, ionizing radiation has been used as a tool for the production of immunogens from malaria, where irradiated sporozoites of *Plasmodium falciparum* induce 90 % protection in the host after challenge with virulent sporozoites; this model is used as the vaccine for prevention of disease in endemic countries [74]. Vaccines have been developed for vermin that occur mainly in Japan, China and Southeast Asia, such as opisthorchiasis, where irradiated *Opisthorchis viverrini* metacercariae induced a protective immune response in the host after challenge [75]. The production of a vaccine for toxoplasmosis using irradiated *T. gondii* tachyzoites has ideal characteristics for the study of the immune response in the host, as this model immunogen lacks the development of the pathogen, and thus does induce the disease.

Our data suggest that a vaccine against toxoplasmosis using irradiated *T. gondii* tachyzoites shows immunological characteristics that are ideal for the development of an effective immunogen for the disease. Regardless of the inoculation route, immunization with irradiated *T. gondii* tachyzoites provides long-lasting immunity in the host. The vaccine induces a similar immune response in the host model as infection, with an increase in the total and high-affinity specific antibodies, such as IgG, IgM and IgA, an increase in key cell populations, such as T $CD4^+$, T $CD8^+$ and B lymphocytes, and the induction of a cellular immune response with increased IFN-γ and IL-4. Our model vaccine also induced T $CD4^+$ and B cell proliferation following

specific stimulation. This specific immune response presents a significant correlation for immune protection after challenge of intraperitoneally or orally immunized animals. The v.o. immunization route shows promising, unexplained protection against *T. gondii*, but it is necessary to study the immunology outside the spleen by analyzing the cell populations induced in the mucosa-associated lymphoid tissue to better understand these results.

Our vaccine preparation is easily produced, and it is devoid of any adjuvant, with a significant protection in experimental models associated with specific humoral and cellular memory immune responses. This vaccine must be tested in other protocols to enhance protection, but we can imagine that it could be a great alternative for veterinary use to reduce oocyst production in felids, resulting in low cyst levels in the meat of dairy animals.

Acknowledgments We thank R.P.A. Cardoso and N.M. Orii for their reliable and available technical assistance. We thank our collaborators P.O. Rigatto, Ph.D., for assistance in the flow cytometry analysis and L.M.S Oliveira for assistance in cytokine detection. N.E. Zorgi used this work as a part of her Ph.D. program and was supported by CNPq. H.F. Andrade Jr. is a CNPq and FFM fellow. This work was supported by grants from FAPESP (2013/04676-9) and LIMHCFMUSP.

References

1. Dubey JP (2008) The history of *Toxoplasma gondii*—the first 100 years. J Eukaryot Microbiol 55(6):467–475
2. Torgerson PR, Mastroiacovo P (2013) The global burden of congenital toxoplasmosis: a systematic review. Bull World Health Organ 91(7):501–508
3. Vitale M, Tumino G, Partanna S, La Chiusa S, Mancuso G, Giglia ML, Presti VD (2014) Impact of traditional practices on food safety: a case of acute toxoplasmosis related to the consumption of contaminated raw pork sausage in Italy. J Food Prot 77(4):643–646
4. Hotop A, Buschtöns S, Bangoura B, Zöller B, Koethe M, Spekker-Bosker K, Hotop SK, Tenter AM, Däubener W, Straubinger RK, Groß U (2014) Humoral immune responses in chickens and turkeys after infection with *Toxoplasma gondii* by using recombinant antigens. Parasitol Res 113(4):1473–1480
5. Smith G (2013) Food- and water-borne disease: using case control studies to estimate the force of infection that accounts for primary, sporadic cases. Epidemics 5(2):77–84
6. De Moura L, Bahia-Oliveira LM, Wada MY, Jones JL, Tuboi SH, Carmo EH, Ramalho WM, Camargo NJ, Trevisan R, Graça RM, da Silva AJ, Moura I, Dubey JP, Garrett DO (2006) Waterborne toxoplasmosis, Brazil, from field to gene. Emerg Infect Dis 12(2):326–329
7. Dubey JP, Jones JL (2008) *Toxoplasma gondii* infection in humans and animals in the United States. Int J Parasitol 38(11):1257–1278
8. Jones JL, Dubey JP (2012) Foodborne toxoplasmosis. Clin Infect Dis 55(6):845–851
9. Innes EA, Bartley PM, Rocchi M, Benavidas-Silvan J, Burrells A, Hotchkiss E, Chianini F, Canton G, Katzer F (2011) Developing vaccines to control protozoan parasites in ruminants: dead or alive? Vet Parasitol 180(1–2):155–163
10. Dupont CD, Christian DA, Hunter CA (2012) Immune response and immunopathology during toxoplasmosis. Semin Immunopathol 34(6):793–813
11. Escoffier P, Jeanny JC, Marinach-Patrice C, Jonet L, Raoul W, Behar-Cohen F, Paris L, Danis M, Dubremetz JF, Mazier D (2010) *Toxoplasma gondii*: flat-mounting of retina as a new tool for the observation of ocular infection in mice. Exp Parasitol 126(2):259–262
12. Luma HN, Tchaleu BC, Temfack E, Doualla MS, Ndenga DP, Mapoure YN, Njamnshi AK, Djientcheu VD (2013) HIV-associated central nervous system disease in patients admitted at the Douala General Hospital between 2004 and 2009: a retrospective study. AIDS Res Treat 2013:709810
13. Derouin F, Pelloux H (2008) Prevention of toxoplasmosis in transplant patients. Clin Microbiol Infect 14(12):1089–1101
14. Alvarado-Esquivel C, Liesenfeld O, Torres-Castorena A, Estrada-Martínez S, Urbina-Alvarez JD, Ramos-de la Rocha M, Márquez-Conde JA, Dubey JP (2010) Seroepidemiology of *Toxoplasma gondii* infection in patients with vision and hearing impairments, cancer, HIV, or undergoing hemodialysis in Durango, Mexico. J Parasitol 96(3):505–508
15. Buxton D (1993) Toxoplasmosis: the first commercial vaccine. Parasitol Today 9(9):335–337
16. Hiszczyńska-Sawicka E, Gatkowska JM, Grzybowski MM, Długońska H (2014) Veterinary vaccines against toxoplasmosis. Parasitology 141(11):1365–1378
17. Mévélec MN, Ducournau C, Bassuny Ismael A, Olivier M, Sèche E, Lebrun M, Bout D, Dimier-Poisson I (2010) Mic1-3 knockout *Toxoplasma gondii* is a good candidate for a vaccine against *T. gondii*-induced abortion in sheep. Vet Res 41(4):49
18. Hiramoto RM, Galisteo A Jr, Do Nascimento N, Andrade HF Jr (2002) 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 20(16):2072–2081
19. Liu MM, Yuan ZG, Peng GH, Zhou DH, He XH, Yan C, Yin CC, He Y, Lin RQ, Song HQ, Zhu XQ (2010) *Toxoplasma gondii* microneme protein 8 (MIC8) is a potential vaccine candidate against toxoplasmosis. Parasitol Res 106(5):1079–1084
20. Parthasarathy S, Fong MY, Ramaswamy K, Lau YL (2013) Protective immune response in BALB/c mice induced by DNA vaccine of the ROP8 gene of *Toxoplasma gondii*. Am J Trop Med Hyg 88(5):883–887
21. Zhao Y, Huang B, Huang S, Zheng H, Li YQ, Lun ZR, Shen J, Wang Y, Kasper LH, Lu F (2013) Evaluation of the adjuvant effect of pidotimod on the immune protection induced by UV-attenuated *Toxoplasma gondii* in mouse models. Parasitol Res 112(9):3151–3160
22. Zorgi NE, Costa A, Galisteo AJ Jr, do Nascimento N, de Andrade HF Jr (2011) Humoral responses and immune protection in mice immunized with irradiated *T. gondii* tachyzoites and challenged with three genetically distinct strains of *T. gondii*. Immunol Lett 138(2):187–196
23. De Rosa SC (2012) Vaccine applications of flow cytometry. Methods 57(3):383–391
24. Teo WH, Nurul AA, Norazmi MN (2012) Immunogenicity of recombinant BCG-based vaccine expressing the 22 kDa of serine repeat antigen (SE22) of *Plasmodium falciparum*. Trop Biomed 29(2):239–253
25. Munoz M, Liesenfeld O, Heimesaat MM (2011) Immunology of *Toxoplasma gondii*. Immunol Rev 240(1):269–285
26. Grigg ME, Boothroyd JC (2001) Rapid identification of virulent type I strains of the protozoan pathogen Toxoplasma gondii by PCR-restriction fragment length polymorphism analysis at the B1 gene. J Clin Microbiol 39(1):398–400
27. Filisetti D, Candolfi E (2004) Immune response to *Toxoplasma gondii*. Ann Ist Super Sanita 40(1):71–80

28. Dubey JP, Lindsay DS, Speer CA (1998) Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clin Microbiol Rev 11(2):267–299
29. Dubey JP (2005) Unexpected oocyst shedding by cats fed *Toxoplasma gondii* tachyzoites: in vivo stage conversion and strain variation. Vet Parasitol 133(4):289–298
30. Lu G, Wang L, Zhou A, Han Y, Guo J, Song P, Zhou H, Cong H, Zhao Q, He S (2015) Epitope analysis, expression and protection of SAG5A vaccine against *Toxoplasma gondii*. Acta Trop 146:66–72
31. Li XZ, Wang XH, Xia LJ, Weng YB, Hernandez JA, Tu LQ, Li LT, Li SJ, Yuan ZG (2015) Protective efficacy of recombinant canine adenovirus type-2 expressing TgROP18 (CAV-2-ROP18) against acute and chronic *Toxoplasma gondii* infection in mice. BMC Infect Dis 15(1):114
32. Tao Q, Fang R, Zhang W, Wang Y, Cheng J, Li Y, Fang K, Khan MK, Hu M, Zhou Y, Zhao J (2013) Protective immunity induced by a DNA vaccine-encoding *Toxoplasma gondii* microneme protein 11 against acute toxoplasmosis in BALB/c mice. Parasitol Res 112(8):2871–2877
33. Hassan IA, Wang S, Xu L, Yan R, Song X, XiangRui L (2014) Immunological response and protection of mice immunized with plasmid encoding *Toxoplasma gondii* glycolytic enzyme malate dehydrogenase. Parasite Immunol 36(12):674–683
34. Chen J, Li ZY, Huang SY, Petersen E, Song HQ, Zhou DH, Zhu XQ (2014) Protective efficacy of *Toxoplasma gondii* calcium-dependent protein kinase 1 (TgCDPK1) adjuvated with recombinant IL-15 and IL-21 against experimental toxoplasmosis in mice. BMC Infect Dis 14:487
35. Vinuesa CG, Chang PP (2013) Innate B cell helpers reveal novel types of antibody responses. Nat Immunol 14(2):119–126
36. Casadevall A (2004) The methodology for determining the efficacy of antibody-mediated immunity. J Immunol Methods 291(1–2):1–10
37. Kotresha D, Noordin R (2010) Recombinant proteins in the diagnosis of toxoplasmosis. APMIS 118(8):529–542
38. Petersen E (2007) Toxoplasmosis. Semin Fetal Neonatal Med 12(3):214–223
39. Denkers EY, Gazzinelli RT (1998) Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. Clin Microbiol Rev 11(4):569–588
40. Kang H, Remington JS, Suzuki Y (2000) Decreased resistance of B cell-deficient mice to infection with Toxoplasma gondii despite unimpaired expression of IFN-gamma, TNF-alpha, and inducible nitric oxide synthase. J Immunol 164(5):2629–2634
41. Wang L, He LY, Meng DD, Chen ZW, Wen H, Fang GS, Luo QL, Huang KQ, Shen JL (2015) Seroprevalence and genetic characterization of *Toxoplasma gondii* in cancer patients in Anhui Province, Eastern China. Parasites Vectors 8:162
42. Macpherson AJ, Geuking MB, McCoy KD (2012) Homeland security: IgA immunity at the frontiers of the body. Trends Immunol 33(4):160–167
43. Woof JM, Kerr MA (2006) The function of immunoglobulin A in immunity. J Pathol 208(2):270–282
44. Mkaddem SB, Christou I, Rossato E, Berthelot L, Lehuen A, Monteiro RC (2014) IgA, IgA receptors, and their anti-inflammatory properties. Curr Top Microbiol Immunol 382:221–235
45. Pabst O (2012) New concepts in the generation and functions of IgA. Nat Rev Immunol 12(12):821–832
46. Wang HL, Pang M, Yin LT, Zhang JH, Meng XL, Yu BF, Guo R, Bai JZ, Zheng GP, Yin GR (2014) Intranasal immunisation of the recombinant *Toxoplasma gondii* receptor for activated C kinase 1 partly protects mice against *T. gondii* infection. Acta Trop 137:58–66
47. Zhang TE, Yin LT, Li RH, Wang HL, Meng XL, Yin GR (2015) Protective immunity induced by peptides of AMA1, RON2 and RON4 containing T-and B-cell epitopes via an intranasal route against toxoplasmosis in mice. Parasites Vectors 8(1):15
48. Pellegrino P, Clementi E, Radice S (2015) On vaccine's adjuvants and autoimmunity: current evidence and future perspectives. Autoimmun Rev 14(10):880–888
49. Shapiro-Shelef M, Calame K (2005) Regulation of plasma-cell development. Nat Rev Immunol 5(3):230–242
50. Minges Wols HA, Underhill GH, Kansas GS, Witte PL (2002) The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity. J Immunol 169(8):4213–4221
51. Fagarasan S, Kawamoto S, Kanagawa O, Suzuki K (2010) Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis. Annu Rev Immunol 28:243–273
52. McHeyzer-Williams LJ, McHeyzer-Williams MG (2005) Antigen-specific memory B cell development. Annu Rev Immunol 23:487–513
53. Plotkin SA (2008) Vaccines: correlates of vaccine-induced immunity. Clin Infect Dis 47(3):401–409
54. Plzakova L, Kubelkova K, Krocova Z, Zarybnicka L, Sinkorova Z, Macela A (2014) B cell subsets are activated and produce cytokines during early phases of *Francisella tularensis* LVS infection. Microb Pathog 75:49–58
55. Lu CY, Ni YH, Chiang BL, Chen PJ, Chang MH, Chang LY, Su IJ, Kuo HS, Huang LM, Chen DS, Lee CY (2008) Humoral and cellular immune responses to a hepatitis B vaccine booster 15–18 years after neonatal immunization. J Infect Dis 197(10):1419–1426
56. Pieper K, Grimbacher B, Eibel H (2013) B-cell biology and development. J Allergy Clin Immunol 131(4):959–971
57. Igietseme JU, Eko FO, He Q, Black CM (2004) Antibody regulation of T cell immunity: implications for vaccine strategies against intracellular pathogens. Expert Rev Vaccines 3(1):23–34
58. Gazzinelli R, Xu Y, Hieny S, Cheever A, Sher A (1992) Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with *Toxoplasma gondii*. J Immunol 149(1):175–180
59. Li ZY, Chen J, Petersen E, Zhou DH, Huang SY, Song HQ, Zhu XQ (2014) Synergy of mIL-21 and mIL-15 in enhancing DNA vaccine efficacy against acute and chronic *Toxoplasma gondii* infection in mice. Vaccine 32(25):3058–3065
60. Grover HS, Chu HH, Kelly FD, Yang SJ, Reese ML, Blanchard N, Gonzalez F, Chan SW, Boothroyd JC, Shastri N, Robey EA (2014) Impact of regulated secretion on antiparasitic CD8 T cell responses. Cell Rep 7(5):1716–1728
61. Moore T, Ekworomadu CO, Eko FO, MacMillan L, Ramey K, Ananaba GA, Patrickson JW, Nagappan PR, Lyn D, Black CM, Igietseme JU (2003) Fc receptor-mediated antibody regulation of T cell immunity against intracellular pathogens. J Infect Dis 188(4):617–624
62. Johnson LL, Sayles PC (2002) Deficient humoral responses underlie susceptibility to *Toxoplasma gondii* in CD4-deficient mice. Infect Immun 70(1):185–191
63. Bickle QD (2009) Radiation-attenuated schistosome vaccination—a brief historical perspective. Parasitology 136(12):1621–1632
64. Hanekom WA (2005) The immune response to BCG vaccination of newborns. Ann NY Acad Sci 1062:69–78
65. Fox BA, Bzik DJ (2002) De novo pyrimidine biosynthesis is required for virulence of *Toxoplasma gondii*. Nature 415(6874):926–929
66. Gigley JP, Fox BA, Bzik DJ (2009) Cell-mediated immunity to *Toxoplasma gondii* develops primarily by local Th1 host immune responses in the absence of parasite replication. J Immunol 182(2):1069–1078
67. Gigley JP, Fox BA, Bzik DJ (2009) Long-term immunity to lethal acute or chronic type II Toxoplasma gondii infection is

effectively induced in genetically susceptible C57BL/6 mice by immunization with an attenuated type I vaccine strain. Infect Immun 77(12):5380–5388
68. Landrith TA, Harris TH, Wilson EH (2015) Characteristics and critical function of CD8+ T cells in the Toxoplasma-infected brain. Semin Immunopathol 37(3):261–270
69. Ciabattini A, Pettini E, Andersen P, Pozzi G, Medaglini D (2008) Primary activation of antigen-specific naive CD4+ and CD8+ T cells following intranasal vaccination with recombinant bacteria. Infect Immun 76(12):5817–5825
70. Zhu J, Paul WE (2008) CD4 T cells: fates, functions, and faults. Blood 112(5):1557–1569
71. Cohen SB, Maurer KJ, Egan CE, Oghumu S, Satoskar AR, Denkers EY (2013) CXCR3-dependent $CD4^+$ T cells are required to activate inflammatory monocytes for defense against intestinal infection. PLoS Pathog 9(10):e1003706
72. Kasper LH, Khan IA, Ely KH, Buelow R, Boothroyd JC (1992) Antigen-specific (p30) mouse CD8+ T cells are cytotoxic against *Toxoplasma gondii*-infected peritoneal macrophages. J Immunol 148(5):1493–1498
73. Quah BJ, Wijesundara DK, Ranasinghe C, Parish CR (2013) Fluorescent target array T helper assay: a multiplex flow cytometry assay to measure antigen-specific CD4+ T cell-mediated B cell help in vivo. J Immunol Methods 387(1–2):181–190
74. Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, Holman LA, James ER, Billingsley PF, Gunasekera A, Richman A, Chakravarty S, Manoj A, Velmurugan S, Li M, Ruben AJ, Li T, Eappen AG, Stafford RE, Plummer SH, Hendel CS, Novik L, Costner PJ, Mendoza FH, Saunders JG, Nason MC, Richardson JH, Murphy J, Davidson SA, Richie TL, Sedegah M, Sutamihardja A, Fahle GA, Lyke KE, Laurens MB, Roederer M, Tewari K, Epstein JE, Sim BK, Ledgerwood JE, Graham BS, Hoffman SL, VRC 312 Study Team (2013) Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. Science 341(6152):1359–1365
75. Papatpremsiri A, Junpue P, Loukas A, Brindley PJ, Bethony JM, Sripa B, Laha T (2014) Immunization and challenge shown by hamsters infected with *Opisthorchis viverrini* following exposure to gamma-irradiated metacercariae of this carcinogenic liver fluke. J Helminthol 90(1):39–47