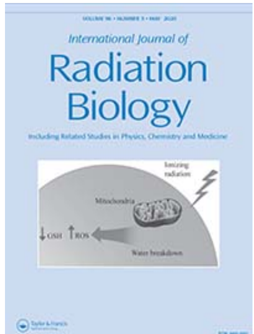

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ORIGINAL ARTICLE

Radiation effects on *Toxoplasma* antigens: different immune responses of irradiated intact tachyzoites or soluble antigens in experimental mice models

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ABSTRACT

Purpose: Purpose: Protein irradiation causes aggregation, chain breakage, and oxidation, enhancing its uptake by antigen-presenting cells. To evaluate if irradiated proteins participate on the protection, we studied the immune response induced in mice immunized with irradiated soluble extracts of *T. gondii* tachyzoites (STag) or irradiated intact *T. gondii* RH tachyzoites ($RH^{0.25\,kGy}$).
Material and Methods: Soluble extracts of *Toxoplasma gondii* tachyzoites (STag) were irradiated at different dose by Cobalt-60 source. By polyacrylamide gel electrophoresis (SDS-Page) we evaluated the effects on primary structures of protein STags induced by irradiation. By Enzyme-linked Immunosorbent Assay (ELISA) we evaluated the difference between humoral immune response induced by irradiated STag or RH tachyzoites in immunized mice from the detection of specific immunoglobulin G (IgG) antibodies in the serum of immunized mice. From challenge with viable RH strain of *T. gondii* we evaluated the protection induced in the immunized animals. By cytometry we performed the phenotyping of T and B lymphocytes in the peripheral blood of the immunized animals.
Results: Irradiation dose of 1.5 kGy induced minimal changes in most proteins, without affecting their antigenicity or immunogenicity. Immunization showed saturation at the dose of 10 µg/mice, with worst response at higher doses. STag irradiated at 1.5 kGy ($STag^{1.5\,kGy}$) induced higher survival and protection similar to *T. gondii* RH strain irradiated at 0.25 kGy ($RH^{0.25\,kGy}$), with higher serum levels of high affinity IgG compared to STag native. Blood immune memory cells of mice immunized with $STag^{1.5\,kGy}$ had higher proportions of $CD19^+$ (cluster of differentiation 19) and $CD4^+$ (cluster of differentiation 14) cells, whereas mice $RH^{0.25\,kGy}$ had high proportion of memory $CD8^+$ (cluster of differentiation 8) cells.
Conclusions: Our data suggest that major histocompatibility complex type I (MHCI) pathway, appears seem to be used by $RH^{0.25\,kGy}$ to generate cytotoxic cells while STag1.5 kGy uses a major histocompatibility complex type II (MHCII) pathway for B-cell memory, but both induce sufficient immune response for protection in mice without any adjuvant. Irradiation of soluble protein extracts enhances their immune response, allowing similar protection against *T. gondii* in mice as compared to irradiated intact parasites.

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KEYWORDS
Gamma irradiation; *Toxoplasma gondii*; vaccines; protection; subcomponent

Introduction

Toxoplasmosis, caused by the protozoan Apicomplexa, *Toxoplasma gondii*, is widely disseminated and little symptomatic. Chronic infection maintains residual cysts throughout life, but protects from reinfection (Jongert et al. 2009). In this complex scenario, vaccines with residual cysts are not feasible and vaccines of subcomponents result in low protection. The use of irradiated intact RH tachyzoites, a non-cyst forming strain, as a vaccine was able to protect animals against challenge with several strains of the agent with diverse virulence. The induced immunity was composed by specific antibodies and cellular immunity, in established mice model studying acute infection mortality or the chronic infection burden as tissue cysts in non-lethal strains (Zorgi et al. 2011). In this model, the effect of ionizing radiation seems to occur at the genomic level, by inducing double DNA breaks in the agent and causing their death, only during mitotic division after the invasion in the host cell, freeing free antigens that induces immunity without residual infection (Hiramoto et al. 2002). This type of vaccine offers the same antigens as the infection, without symptoms or formation of cysts in the tissues, thus reaching the vaccine targets for toxoplasmosis (Hiramoto et al. 2002;

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Zorgi et al. 2011). Alternatively, this protection could involve also other irradiated subcomponents of tachyzoites, as proteins or other soluble components, as irradiated proteins have shown to be potent immunogens (Kusel et al. 1989).

Ionizing radiation acts on proteins, improving its efficiency in inducing immunity (Do Nascimento et al. 1996) or soluble *Toxoplasma gondii* extracts (da Costa et al. 2018) without deep studies in biochemical and structural differences that occur in irradiated proteins that could justify this enhanced immunity. Ionizing radiation has been described as an efficient tool in the elimination of poison toxicity with maintenance of its antigenic and immunogenic characteristics (Abdal Dayem et al. 2017). Venoms are generally low immunogenic epitopes activity after being irradiated they have been more immunogenic activity than the native form (Ferreira Junior et al. 2005). This enhancement of immunogenicity was related to the formation of aggregates (Do Nascimento et al. 1996), but the immunogenicity enhancement could be related to other factors, as the reported increased uptake of irradiated crotoxin by macrophages (Cardi et al. 1998). Studies with ovalbumin irradiation showed change in antigenicity only at doses greater than 1.0 kGy (M.-J. Kim et al. 2014), similar to found by our group where irradiated STag at the 1.5 kGy was more efficient than higher or lower radiation doses in inducing protection and humoral response (da Costa et al. 2018). An irradiated antigen undergoes changes such as protein aggregation, chain breaks and oxidative reactions (Do Nascimento et al. 1996). These alterations are recognized by receptors present in tissue macrophages, which may also act in immune presentation of antigens (Zeng et al. 2003). Adjuvants for enhancing immune response to external antigens are believed to promote inflammation (De Gregorio et al. 2013). Its action seems to be related to the insolubilization of antigenic mixtures and attraction of neutrophils, whose potent extracellular myeloperoxidase promotes oxidative changes in proteins (Biedroń et al. 2015) similar to water radiolysis induced by gamma irradiation. The ionizing radiation could simulate this oxidation and alter the proteins, and leading then to cellular receptors specialized in antigen presentation cells (APCs), with greater uptake and selective presentation in the adaptive immune response (Biedroń et al. 2015).

Irradiated tachyzoites vaccine is efficient but have logistic problems as it is conserved in liquid nitrogen, troublesome to use in the field especially for veterinary use, a problem also seen in commercial vaccines (Buxton and Innes 1995). Overcoming these problems must be imperative for the success of a vaccine, and subcomponents preparation must be much more feasible for this aspect (Rappuoli 2007). Radiation improves this vaccine but the effect of irradiation thus could be related to normal antigens delivered by parasites with damaged DNA or to irradiated proteins from tachyzoites, directed to an adequate presentation for the immune response (Hoffman and Chattopadhyay 2008). A better understanding of these steps would make it possible to demonstrate whether these modifications could be the key to the production of a safe, non-adjuvant, efficient toxoplasmosis vaccine capable of preventing human or veterinary disease since it would not impair meat for human consumption by the absence of tissue cysts, a fact relevant in commercial vaccines (Buxton and Innes 1995). We study comparatively the tachyzoites irradiated vaccine and Stag irradiated vaccine in mice models, looking for structural alterations, humoral and cellular immune memory response and protection of immunized animals to challenge with virulent strain.

Material and methods

Experimental animals

For the immunization and challenge experiments were used male BALB/c or C57Bl/6j mice (isogenic), weighing 20 to 22 g, provided by the Central Biotery of the University of São Paulo School of Medicine. All animals were kept in plastic cages with autoclaved pine shavings, receiving Nuvital® commercial feed (Nuvital®, Canguiri, Colombo, PR) and water *ad libitum*. The animals used were euthanized in a CO_2 chamber, with manipulation conducted according to laboratory animal care standards (Institute of Laboratory Animal Resources (U.S.) 1996) and the "Principles of Ethics in Animal Experimentation" (COBEA - Brazilian College of Animal Experimentation). The project was submitted and approved by the Research Ethics Committee of the Tropical Medicine Institute of São Paulo (Project No. 000274A).

Obtainment and irradiation of Toxoplasma gondii tachyzoites soluble antigen

Toxoplasma gondii tachyzoites of RH strain obtained from the peritoneal exudate of previously infected animals was filtered on 3.0 μm polycarbonate membranes (ISOPORE, Millipore®, Burlington, MA, USA) for removal of other mammalian cell types. The purified parasites were sonicated with probe inside solution, in 40 cycles for 5–10 periods of 30 s in an ice bath until complete lysis of the agent. The suspension received one volume of 0.3 M NaCl (LabSynth®, Vila Nogueira, Diadema, Brazil) solution and was cleared by centrifugation at 10,000 *g* for 30 min at 4 °C, as elsewhere described (Camargo et al. 1978) The obtained soluble supernatant was aliquoted and used as soluble antigen (STag) for irradiation, immunizations and also for immunosorbent in ELISA assays. Protein content was determined by fluorimeter Qubit® system (Thermo®, Waltham, Massachusetts, USA). The soluble antigens of *T. gondii* tachyzoites (STag) obtained were subjected homogeneously to irradiation at doses of 0.25, 0.5, 1.0 and 1.5 kGy, with 90% shielding by gamma-ray exposure by a source of Cobalt-60 (Gamma cell, Atomic Energy of Canada), with a dose rate of 1.03 kGy/h, at room temperature in the presence of oxygen. Samples remained on the outside of the source throughout the irradiation time, as sham controls.

Characterization of structural and antigenic modifications induced in irradiated soluble extracts of *T. gondii* tachyzoites

Polyacrylamide gel electrophoresis (SDS-Page) and Western Blot

Samples containing 5–10 μg of protein STags was added in 10 μL of sample buffer (10% glycerol, 5% β-mercaptoethanol (Sigma®), 2% SDS, (LabSynth®) pH 6.8 0.0625 M, Bromophenol blue (Sigma®) 0.001% 50%), heated for 5 min at 100 °C and applied to the gel. The analysis of electrophoretic mobility in a discontinuous and denaturant system was performed according to Laemmli (1970), in Mini-Protean II system (BIO-RAD®, Inc., Hercules, California, USA) using stained molecular weights markers. Native or irradiated STag samples, separated by SDS-PAGE, were stained with Coomassie Blue R-250 or transferred to nitrocellulose membranes (Millipore®, Burlington, MA, USA), in a semi-dry transfer system, Trans-Blot RD (BIO-RAD®) (Towbin et al. 1992). Transferred nitrocellulose membranes free binding sites was blocked with phosphate buffered saline (PBS) containing 5% of dry skim milk (PBSTL) and 0.02% Tween 20 (BIO-RAD®). Antigenicity of transferred proteins was detected by reacting the completely blocked membrane with 1/100 dilution of serum of chronically infected mice with ME-49 *T. gondii* strain. Bound specific IgG at membranes was detected with anti-mouse IgG peroxidase conjugated (Sigma®), which was revealed with insoluble 3,3′,5,5′-tetramethylbenzidine (TMB) commercial reagent for membrane (Thermo®, Waltham, MA, USA).

Protein stained gels was carefully digitalized at high resolution and the structural effect of radiation on STag proteins were evaluated by using ImageJ® (National Institute of Health, Bethesda, MD, USA) and Image Lab™ (BIO-RAD®) imaging software. Calibrated image at 8-bit scale (Image-Type_8-bit), allows measure the area of each detectable protein band. This detection provides the molecular mass of each detectable band, represented by histograms. The values of the masses were acquired (shown in Supplements) and submitted to statistical analysis, looking for the correlation (r squared, r^2) between the detectable bands in the STag native (non-irradiated), or irradiated STags.

Immunization

We evaluated the better protein concentration present in the STag samples to induce humoral immune response and the difference of immunity induced between STag or irradiated intact RH tachyzoites at 0.25 kGy. Groups of 5 BALB/c mice received three biweekly doses with concentrations of 2, 6, 10 and 20 μg–0.1 ml/animal of STag native or irradiated at 0.25 and 1.5 kGy and tachyzoites RH irradiated at 0.25 kGy, subcutaneously, diluted in sterile PBS1x. The irradiated tachyzoites samples were obtained from the repository in the Protozoology laboratory (Zorgi et al. 2011). Blood samples from the animals was obtained by light section of the tail end of the immunized and control mice, collected on filter paper, stored and dried at –20 °C, fifteen days after the last immunization dose.

Enzyme-linked Immunosorbent assay (ELISA)

Polystyrene plates of 96 or 384-wells (Cornig®, New York, USA) were coated with *T. gondii* tachyzoites total extract antigen (10 μg/ml), suspended in 0.1 M sodium carbonate buffer pH 9.5, overnight at 4 °C in humid chamber. The plates were washed five times with PBS containing 0.2% Tween 20 (PBST) in a microplate washer (Tecan®, Mannedorf, Switzerland), followed by blocking with PBST, containing 3% Molico® (Vevey, Vaud, Switzerland) skimmed milk (PBSTL) for 1 h in an oven at 37 °C. After blocking, serum samples control from mice non-immunized and serum from animals immunized at the appropriate dilution (1:100), were added on the plates and incubated at 37 °C for 1 h. The anti-mouse IgG conjugate, in appropriate dilution, conjugated to peroxidase (Sigma®, St. Louis, MO, USA), with 1-h incubation at 37 °C, was applied after further washing. Reaction development was performed by the addition of TMB (Sure blue Microwell Peroxidase substrate KPL) and stopped after 30 min by the addition of 4 N HCl. Absorbance readings were performed on a Multimode Microplate Reader FilterMax F5 (Molecular Devices®, San Jose, CA, USA) microplate reader in the absorbance of 450 nm. The results were expressed in arbitrary units (AU), determined as the ratio of the absorbance from each sample. The mean of absorbance of the negative control sample was used for intra-test quality control for conjugate variation, following the equation: AU = Abs. of positive sample/mean of Abs. of negative samples. The avidity of the avidity antibodies was performed in the same manner as described above, except for the addition of chaotropic solution (Urea 6 M [LabSynth®], while the rest of the wells were maintained in PBSTL) after washing the serum stage with incubation period of 15 min at 37 °C. The results were expressed arbitrarily by arbitrary units (AU) (AU = Absorbance [Abs.] of positive sample + urea 6 M/Abs. of sample without urea 6 M.

Challenge with lethal RH T. gondii strain

Parasites obtained from the peritoneal exudate of animals previously inoculated and filtered on a 3.0 μm polycarbonate membrane (ISOPORE™, Millipore®) for the removal of other cell types present, were resuspended in saline solution and quantified in Neubauer chamber for adjustment of concentration of parasites. The immunized animals were challenged with 100 μl of saline containing 10^3 viable *T. gondii* tachyzoites intraperitoneally. Control groups of unimmunized mice was inoculated with the same amount of tachyzoites. Mortality was monitored daily.

Phenotype of memory blood cells by flow cytometry

Peripheral blood cells were obtained from mice immunized subcutaneously with STag native or irradiated at 0.25 kGy, 1.5 kGy and tachyzoites RH0.25 kGy and unimmunized mice, via retro-orbital plexus. Mononuclear cells were separated by Ficoll-Paque™ Premium 1084 (GE Healthcare®, Little Chalfont, UK) according to the manufacturer's instructions. After cell separation, the cells were suspended in 1 ml culture medium, counted in Neubauer's camera to adjust a concentration of 2×10^6 cells/ml. After an incubation period of 72 h in the presence of antigen of total *T.*

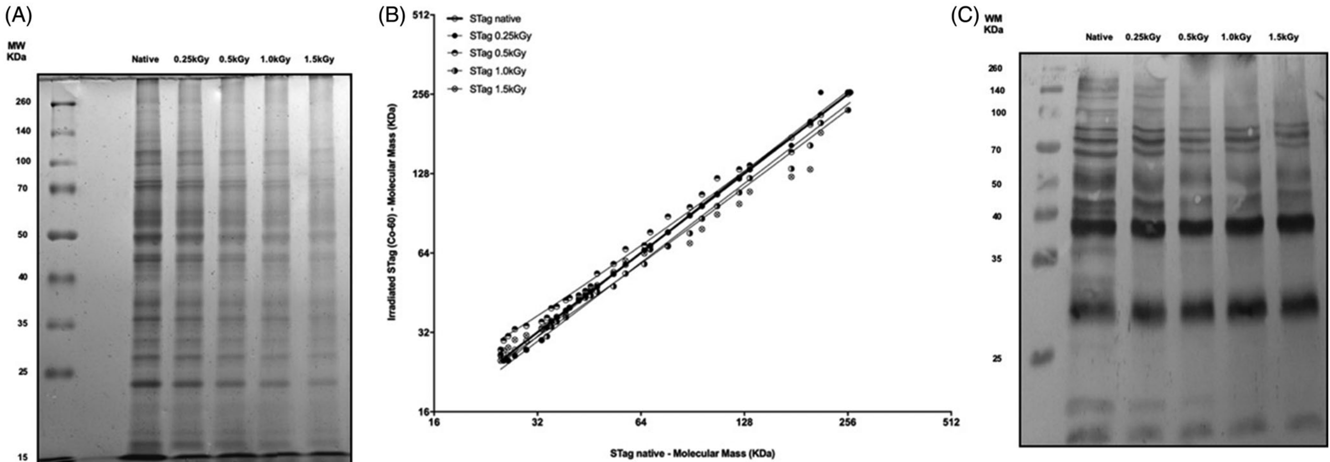

Figure 1. (A) SDS-Page (12.5%). (B) Linear regression showing the difference (r^2) between STag bands from native or irradiated identified in the SDS-Page by Image Lab 6.0.1 software (BIO-RAD®). STag native x STag$^{0.25kGy}$ ($r^2 = 0.9943$); STag native x STag$^{0.5kGy}$ ($r^2 = 0.9886$); STag native x STag$^{1.0kGy}$ ($r^2 = 0.9842$); STag native x STag$^{1.5kGy}$ ($r^2 = 0.9602$). (C) Western blot of STag native or irradiated at different doses of γ-radiation by Co-60 source.

gondii extract (10 μg/mL/well), in an incubator at 37 °C and 5% CO_2, the cells were submitted to cell surface marking for phenotyping of cell populations, as elsewhere reported (Zorgi et al. 2016). Anti-mouse monoclonal antibodies such as: anti-CD3 BV421 (BD Biosciences®, Franklin Lakes, NJ, USA), anti-CD4 BV605 (BD Biosciences®), anti-CD8 APC-Cy7 (BD Biosciences®) were used for labeling; anti-CD44 APC (BD Biosciences®); anti-CD45RB FITC (BD Biosciences®); anti-CD19 PE-Cy7 (BD Biosciences®), anti-CD23 PE (BD Biosciences®) and anti-CD27 PerCP-Cy5-5 (BD Biosciences®). The antibody mix was prepared in a 1:50 dilution in ISOTON® (Brea, CA, USA) solution. Twenty microliters of the mix was added in each sample followed by incubation for 30 min at 4 °C in the absence of light. After the incubation period the samples were acquired on a flow cytometer (LSR Fortessa BD Biosciences®). The compensation was performed with Comp Beads® anti-Rat and anti-Hamster (BD Biosciences®). Data was collected by BD FACSDIVA® (BD Biosciences®) software and analyzed in Flowjo X® software (Franklin Lakes, NJ, USA).

Statistical analysis

All statistical analyzes were performed using GraphPad Prism® 6.0 software (San Diego, CA, USA). Comparisons with a probability of equality of less than 5% ($p < .05$) were considered significant. The comparison between immunizations or phenotyping was done using *t*-test and ANOVA for multiple comparisons and Bonferroni test applied for comparison between the selected groups. For the survival, tests after challenge was applied the Log-Rank test.

Results

Structural and antigenic modifications induced in irradiated soluble extracts of T. gondii *(STag)*

STag samples were submitted to different doses of irradiation with γ-rays by homogeneous source of Co-60 (0.25, 0.5, 1.0 and 1.5 kGy). After irradiation of STags, we evaluated the electrophoretic profile of samples in gel with a resolution of 12.5% (Figure 1(A)). The electrophoretic profile of the STag$^{0.25\,kGy}$ showed no changes when compared to the STag (non-irradiated). From the dose of 0.5 kGy, we observed the presence of a drag in proteins of high weight that became more evident with the increase of the doses. Samples irradiated at 1.0 and 1.5 kGy further evidence the drag suggesting breaks and possible junctions of some epitopes. The structural characterization of the extracts by SDS-Page provided us a qualitative evaluation of native and irradiated STag proteins. With the use of ImageJ® software and Image Lab 6.0.1 software (BIO-RAD®), we performed the relative quantification of induced changes in the extracts after irradiation, represented by the run area of each extract (shown in Methods section). We evaluated the difference (r^2) between the bands present in the STag native and irradiated (Figure 1(B)). The off-line points suggest that gamma radiation acted on proteins of high molecular mass, while those of lower mass, appeared to not undergo modifications, while remaining similar across all STag analyzed. The action on proteins of higher molecular mass modified the slope of the correlation line, showing that the radiation has a dose-response effect on proteins of high molecular mass.

After evaluating the effect of radiation on the primary structures of the extract proteins, separate samples in the SDS-Page, were transferred to nitrocellulose membranes and revealed by the addition of specific IgG antibodies anti-*T. gondii*, present in the serum of infected mice with ME-49strain. In Figure 1(C), we show the recognition profile of these specific antibodies to the STag proteins submitted to different doses of gamma radiation. Recognition of the specific antibodies was similar in all STags studied. There was no difference in reactivity between STag native or irradiated at 0.25 and 0.5 kGy. Even at higher doses, 1.0 and 1.5 kGy, the antigenic characteristics were also maintained, but with some lesser reactivity of small molecular weight epitopes.

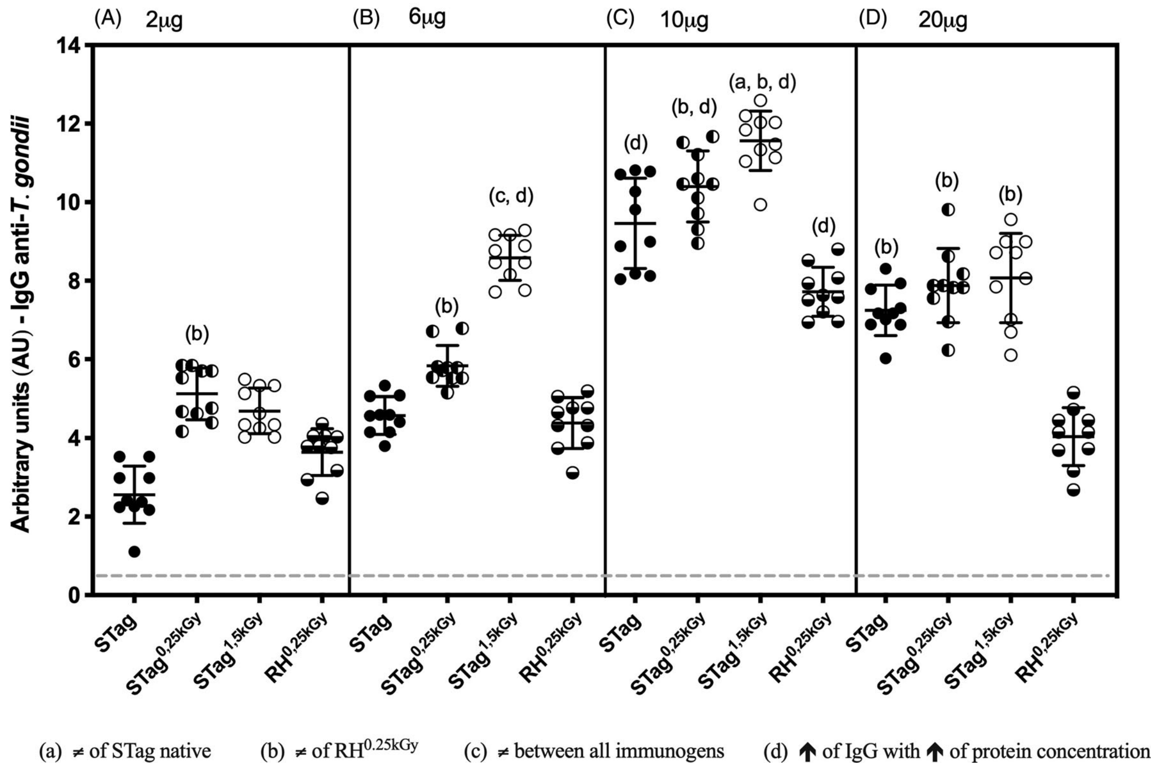

Figure 2. Specific IgG anti-*T. gondii* antibodies in the serum of BALB/c mice immunized with 3 bi-weekly doses of STag or $STag^{0.25kGy}$, $STag^{1.5kGy}$ and $RH^{0.25kGy}$ with different concentrations of protein, detected by ELISA. (A) 2 μg; (B) 6 μg; (C) 10 μg; (D) 20 μg. Asterisks represent the significant difference between the groups (** $= p < .05$; **** $= p < .0001$). Dashed lines demonstrate the arbitrary units of basal IgG antibodies.

Effects of ionizing gamma radiation on the immunogenic properties of irradiated STags, comparison between the immune response induced by STag and irradiated tachyzoites, and effective concentration of STag proteins to improve antibodies production

Groups of 05 BALB/c mice were immunized with three biweekly doses of different protein concentrations of STag native or irradiated at 0.25 and 1.5 kGy and intact RH tachyzoites irradiated at 0.25 kGy (2, 6, 10 and 20 μg/animal). In general, it is possible to observe that the increase in protein concentration of the immunogens evaluated increased progressively the concentration of specific IgG antibodies up to 10 μg/mice (Figure 2). The concentration of 2 μg/mice of $STag^{0.25\,kGy}$ (Figure 2(A)), induced higher production of IgG anti-*T. gondii* antibody in relation to all immunogen evaluated ($p < .05$). With increasing of protein concentration to 6 μg/mice (Figure 2(B)), it is observed that $STag^{0.25\,kGy}$ and $STag^{1.5\,kGy}$ induced higher indices of antibodies IgG anti-*T. gondii* than non-irradiated STag ($p < .0001$). Animals immunized with $STag^{0.25\,kGy}$ had higher concentrations of specific IgG antibodies than the group immunized with irradiated intact tachyzoites ($RH^{0.25\,kGy}$). In addition, among all the immunogens evaluated, $STag^{1.5\,kGy}$ was the immunogen that induced higher concentrations of specific IgG antibodies ($p < .0001$). The protein concentration of 10 μg/mice (Figure 2(C)) of STags induced an increase in production of specific IgG in all groups evaluated. Among STag, we can observe that the production of IgG anti-*T. gondii* antibodies in animals immunized with STag and $STag^{0.25\,kGy}$ was similar. Animals immunized with 10 μg of $STag^{1.5\,kGy}$ showed higher concentrations of IgG anti-*T. gondii* among all groups evaluated ($p < .0001$), and the $STag^{0.25\,kGy}$, induced higher concentrations of IgG antibodies than immunization with $RH^{0.25\,kGy}$ ($p < .0001$). Immunization with the concentration of 20 μg/mice of both STag and total tachyzoites did not increase the production of IgG anti-*T. gondii* antibodies (Figure 2(D)).

The same samples were assayed for avidity of antibodies using 6 M urea as a chaotropic agent, (Figure 3). Figure 3(A) shows the AU of IgG avidity induced by the immunization of mice with 2 μg masses of immunogens, which was low ($\leq$5) in all, indicating decrease of antibodies maturation. Groups immunized with a mass of 6 μg of immunogens (Figure 3(B)) showed intermediate avidity ($5 < IA < 6$), whereas immunization with 10 μg (Figure 3(C)) induced antibodies of high avidity (>6) in all immunogens except $RH^{0.25\,kGy}$. When we analyzed the avidity of antibodies induced by immunization with 20 μg of STag, $STag^{0.25\,kGy}$, $STag^{1.5\,kGy}$ and $RH^{0.25\,kGy}$, we observed less avidity than with the previous mass of immunization, although higher in the group immunized with $STag^{1.5\,kGy}$.

Immunized groups with different concentration of protein STag or $RH^{0.25\,kGy}$ were challenged with 10^3 viable RH tachyzoites of the *T. gondii* 30 days after the last vaccine dose for evaluation of the protective response induced after immunizations. In Figure 4, we present the survival induced in mice where independent of the mass of protein used, all the

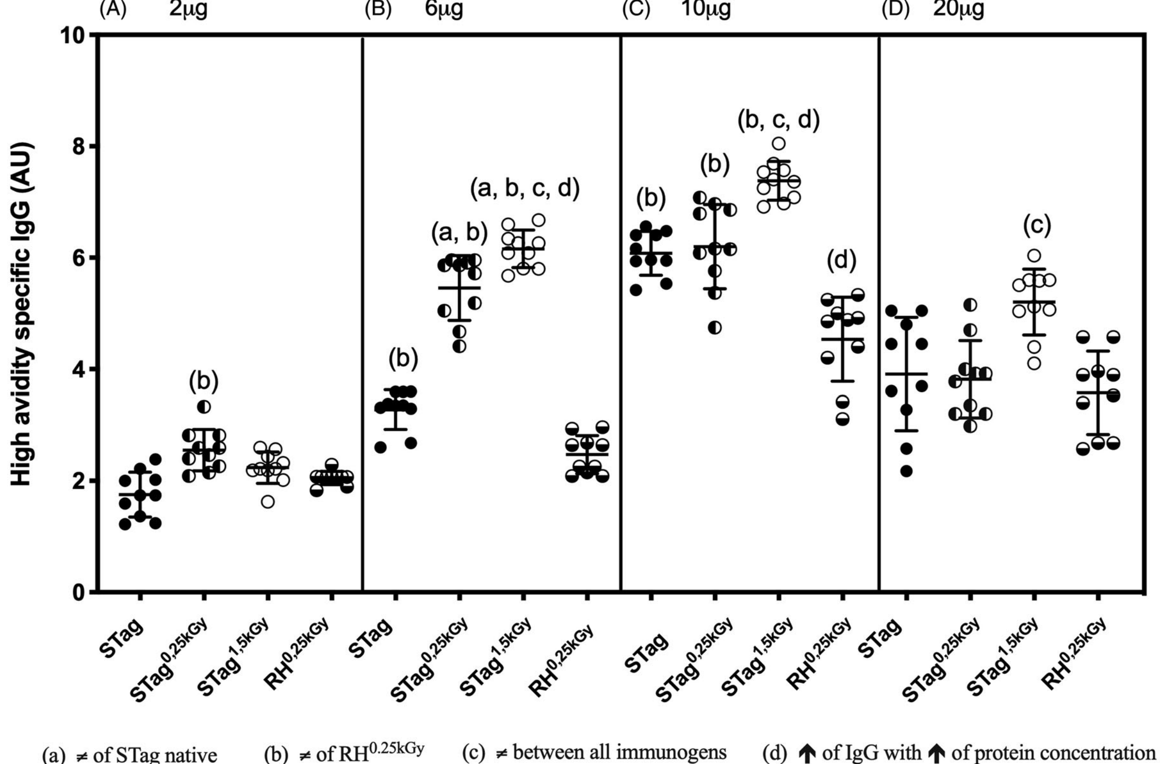

Figure 3. Comparison between high avidity specific IgG antibodies anti-*T. gondii* in the serum of BALB/c mice immunized with three bi-weekly doses of STag or STag$^{0.25kGy}$, STag$^{1.5kGy}$ and RH$^{0.25kGy}$ with different concentrations of protein, detected by ELISA. (A) 2 μg; (B) 6 μg; (C) 10 μg; (D) 20 μg. Asterisks represent the significant difference between the groups (*$p < .01$; **$p < .05$; ***, ****$p < .0001$).

immunizations were able to increase the survival of the animals compared to the unimmunized control group, which survived for three days post challenge. Figure 4(A) shows the survival of the immunized groups with 2 μg of STag native, STag$^{0.25\,kGy}$ 250 Gy and RH$^{0.25\,kGy}$ that survived for 5 days after challenge with a discreet and insignificant greater protection for 7 days in immunized with STag$^{1.5\,kGy}$. The groups immunized with 6 μg of immunogen, shown in Figure 4(B), showed 6-day survival in STag native, less than 10–11 days observed in animals immunized with STag$^{0.25\,kGy}$, STag$^{1.5\,kGy}$ and RH$^{0.25\,kGy}$. In immunized animals with 10 μg of immunogen, we observed a greater increase in the survival of the immunized animals compared to other masses of protein studied (Figure 4(C)), similar to those immunized with 20 μg immunogen. In these groups, STag native immunization increased to six days survival, while the groups immunized with STag$^{0.25\,kGy}$ and RH$^{0.25\,kGy}$ survived for 12 days. The survival of the group immunized with STag$^{1.5\,kGy}$ was greater than 14 days post challenge. Some groups showed animals (1 animal/group) that survived for more than 20 days after the challenge.

The proportion of cellular antigen-responsive cells presented in the peripheral blood of animals submitted to immunizations is shown in Figure 5. Figure 5(A), shows the percentage of B cells (CD19$^+$) detected in the immunized groups. We observed a proportion of 14% of B lymphocytes (CD19$^+$) in animals immunized with STag, less than 19% in those immunized with STag$^{0.25\,kGy}$; and both less than the proportion of 24% in those immunized with STag$^{1.5\,kGy}$ and 23% in those immunized with RH$^{0.25\,kGy}$. In Figure 5(B), we show the percentage of T helper lymphocytes (CD3$^+$CD4$^+$) detected in the lymphocytes of immunized groups stimulated with *T. gondii* antigen. Lymphocytes from animals immunized with STag had 7% T lymphocytes (CD3$^+$CD4$^+$), greater than 4% found in lymphocytes from animals immunized with STag$^{0.25\,kGy}$ but much less than 23% lymphocytes from animals immunized with STag$^{1.5\,kGy}$. In lymphocytes of animals immunized with RH$^{0.25\,kGy}$, there was a 10% ratio of cells (CD3$^+$CD4$^+$), discrepant of STag immunized models. Figure 5(C) shows the percentage of lymphocytes T (CD3$^+$CD8$^+$) where we observed 10% of lymphocytes T (CD3$^+$CD8$^+$) in the lymphocytes of animals immunized with native, 13% of those immunized with STag$^{0.25\,kGy}$ and 14% in immunized with STag$^{1.5\,kGy}$ with apparent dose response effect, but with no statistical significance. In lymphocytes of animals immunized with RH$^{0.25\,kGy}$, there was a 28% higher (and CD3$^+$CD8$^+$) ratio of STag-immunized models.

Discussion

Our data showed that the immunity induced by different forms of irradiated antigens resulted in protection against toxoplasmosis, but with diverse immune mechanisms, according to immunogen, structure and doses used. In the soluble antigen, gamma irradiation induced disseminated minor alterations in most proteins, but without affecting the antigenic properties. This effect was similar to that found in irradiated bovine albumin, where the authors describe that only higher doses of irradiation may cause significant changes in the structure of the proteins (Moon and Song 2001). We reported that radiation doses over

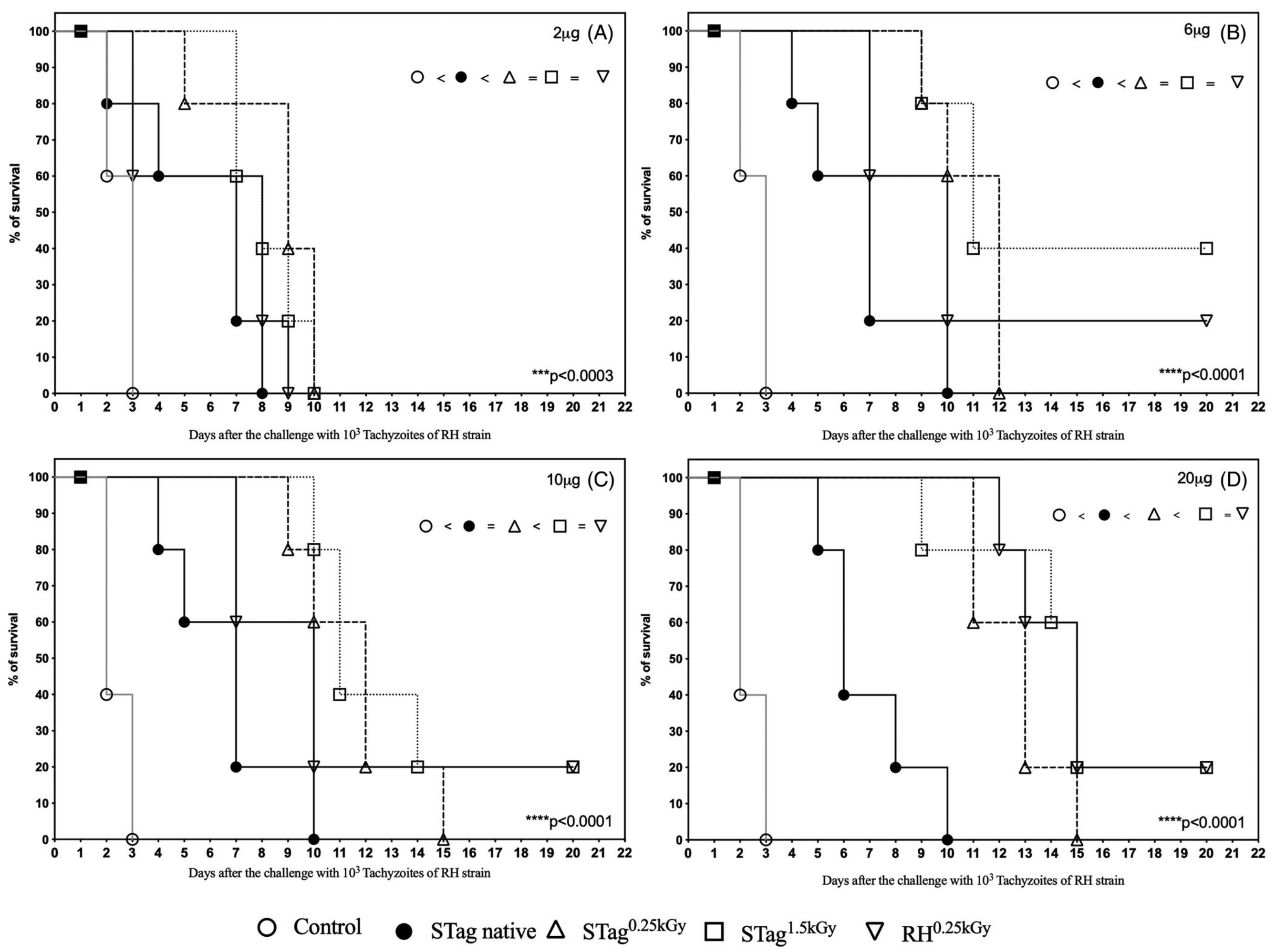

Figure 4. Percentage (%) of the survival of BALB/c control (○) mice or immunized with different masses of STag (●) or (△) STag$^{0.25kGy}$, (□) STag$^{1.5kGy}$ and (▽) RH$^{0.25kGy}$, challenged with 10^3 viable tachyzoites of the virulent RH strain 30 days after the last dose of immunization. (A) 2 μg; (B) 6 μg; (C) 10 μg; (D) 20 μg. Asterisks represent the difference between survival curves between the control groups and immunized by the Log-Rank test (Mantel-Cox).

1.5 kGy induced evident degradation of STag proteins (da Costa et al. 2018). By densitometry, it was possible to confirm that with the increase of the <1.5 kGy dose irradiation, discrete modifications in the area profile of the proteins relative to STag native (non-irradiated) control occur. These modifications were characterized by the increase of the area of the runway area and enlarge of the peaks, a fact already observed in irradiated venoms (Do Nascimento et al. 1996). Densitometry provided detection of subtle and discrete alterations on individual irradiated proteins, using an usual low resolution technique as SDS-PAGE, as describe elsewhere (M.-J. Kim et al. 2014), without those major effects of destruction or aggregation usually found in higher radiation doses (Do Nascimento et al. 1996; da Costa et al. 2018).

All antigens used were efficient in inducing antibodies against *T. gondii*, as expected, but irradiated ones were more efficient in this production and in high affinity antibodies. We obtained a better immunological response with 10 μg/mice, and the immunization with 20 μg/mouse was less efficient in the induction and maturation of specific antibodies, showing a saturation effect, that could be related to exhaustion of antigen presenting cells and excess of antigens inducing a worst immune response. All the pathways of immune response appear saturated at the higher amount of immunogen used, independent of the antigen. The excess of antigen could induce immunological tolerance and reduction of the immune response (Nemazee 2017) as found in our data. We clearly show that the immune response obtained with irradiated soluble antigens depend on an ideal dose, rather than the excess of immunogen mass. The extract irradiated at lower dose STag$^{0.25\,kGy}$, induce a mature immune response compared to native protein at lower immunogen doses but at lower extent than STag$^{1.5\,kGy}$. They also induced maturation of IgG affinity, which was absent in the response induced by intact irradiated tachyzoites, suggesting a different pathway of immune response. It's interesting note that both antigens had induced good protection and humoral and cellular response, but only soluble antigen induced higher affinity maturation. The difference in response must be related to a different cell cooperation for the humoral immune response, suggesting that irradiated antigens or subcomponents in the irradiated tachyzoites preparation may not responsible for the humoral immune response observed, as STag irradiated in the same dose induced affinity maturation.

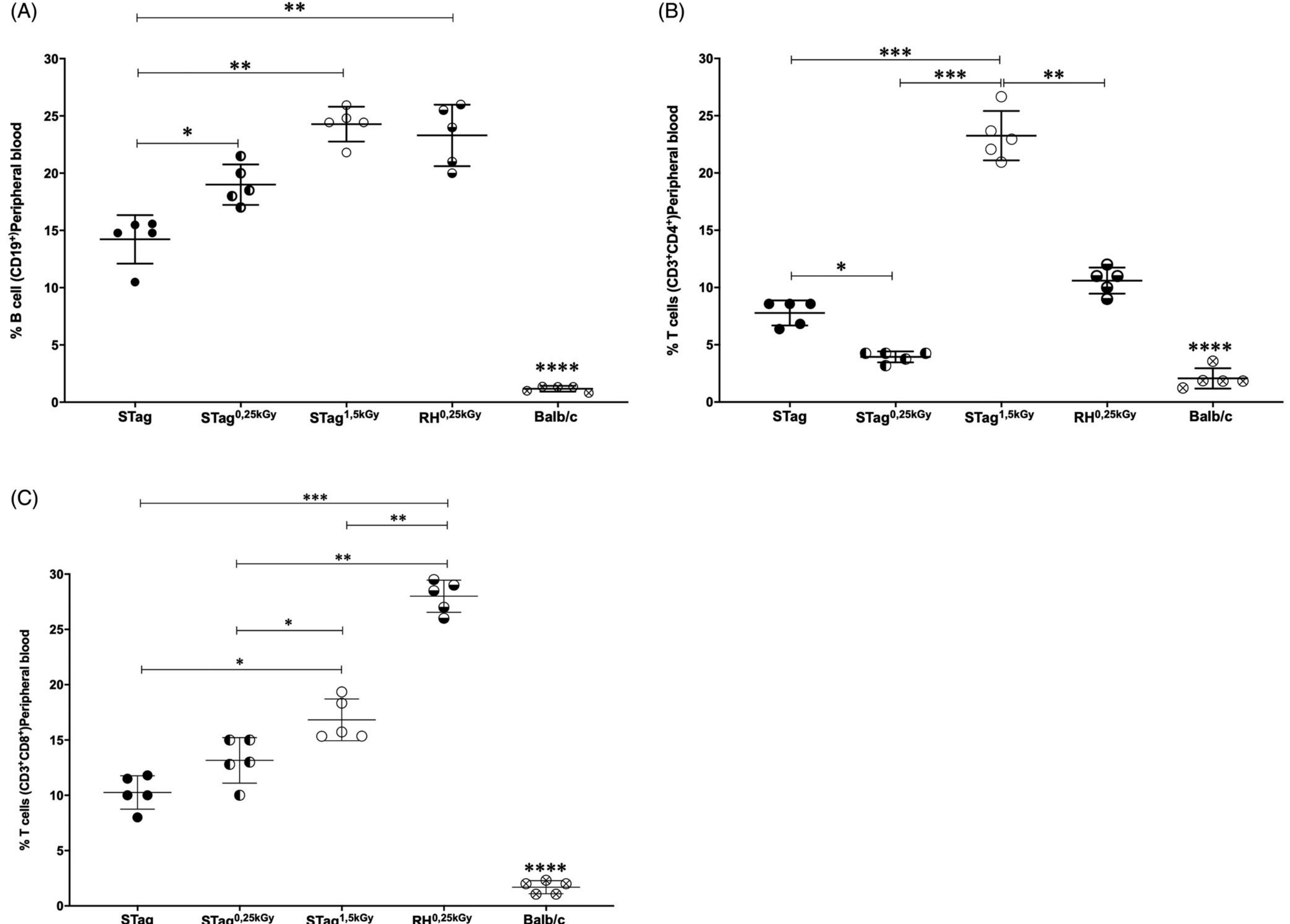

Figure 5. Percentage (%) of lymphocytes B and T present in the peripheral blood of BALB/c mice immunized with 10 μg of STag, STag$^{0.25\ kG}$, STag$^{1.5kGy}$ and 10^7 RH$^{0.25kGy}$ subcutaneously. (A) B cells (CD19$^+$); (B) CD3$^+$CD4$^+$ T cells; (C) CD3$^+$CD8$^+$ T cells. Bars represent the mean and standard error of the mean. Asterisks represent the significant difference between the groups (*$p < .01$; **$p < .05$; ***, ****$p < .0001$).

Challenge tests with virulent strain are of complex analysis, since we are evaluating two joined processes, the infection immunity, that blocks the invasion of the parasite in host cells and the disease immunity, eliminating infected cells, as previously defined in vaccine models of *Toxoplasma* (Zorgi et al. 2011; da Costa et al. 2018). Thus, an immunogen may be effective in preventing infection by inducing protection with antibodies and others may be more important for the prevention of disease by inducing CD8$^+$ cells, which eliminate infected host cells and prevent disease without interfering in early steps of the infection (Rodrigues et al. 2003). The challenge model that we present uses intraperitoneal infection, which implies that we are mainly observing the inhibition of the disease, here represented as the death of the host. Although the antibody cooperate for parasite death by inducing its phagocytosis by leukocytes (Gilbert 2012), the protection found described in the literature is more associated with cellular immunity and the production of agent responsive CD8$^+$ cells which eliminate the infected cells (Dupont et al. 2012). One conclusion is that a significant amount of all the irradiated immunogens is necessary for protection, since the protection induced in all the groups with 2 μg of antigens was detectable but very low and all the challenged animals died. All doses of native STag induced low protection, as usually reported in immunization in the absence of adjuvants, similar to that found in numerous vaccine model (Jongert et al. 2009). Our antigens preparation was free of any adjuvants and promoted immunity. It is usually reported that a high and lasting immune response with lower antigen concentration implies the addition of immunological adjuvants (Moyer et al. 2016). The greatest difficulty with using these adjuvants is toxicity and local side effects, so there is a requirement to have an acceptable low level of those effects (Jongert et al. 2009; Liu et al. 2017). This is not a problem in our vaccine preparation. Protein presentation of the intact tachyzoites must be driven to another immune pathway helping the protection reported (Zorgi et al. 2011, 2016) and also shown here. The radiation had modified selectively the soluble extract components, probably directing it to a specific pathway of antigen presentation easily saturable but effective to induce humoral and cellular immune response. This is expected, as adaptive humoral response with affinity maturation needs three cell-to-cell interaction with soluble antigens as we found, eliminating low avidity antigens specific cells and promoting good immune memory (Chappell et al. 2014).

Memory cells are very important on vaccine evaluation by showing the immune status of the host without challenge

with disease (Furman and Davis 2015). We study those cells in blood of immunized animals and growth induced by antigen a typical feature of memory cells. We observed a high proportion of B and $CD4^+$ memory lymphocytes in $STag^{1.5\,kGy}$ immunization while a high proportion of $CD8^+$ memory lymphocytes were present in mice immunized with irradiated intact tachyzoites. In toxoplasmosis, $CD4^+$ and $CD8^+$ T cells are essential in the control of latent infection (Halonen and Weiss 2013). Lymphocytes T $CD8^+$ participate in the host immune response during infection by limiting the multiplication of intracellular parasites such as *T. gondii* (Gazzinelli et al. 1991), attacking the infected cells. The response by $CD4^+$ and $CD8^+$ cells also induce IFN-γ production in intracellular infection, and this response by $CD4^+$ cells is essential for macrophage activation (Splitt et al. 2018). Our data show that diverse pathways could induce protection and vaccine development in toxoplasmosis, interfering in the several steps of the infection. Vaccine had to induce: (a) protection against disease, eliminating infected cells and preventing the spread of intracellular parasites tissue damage; and (b) protection against infection, preventing the invasion of new cells, by antibodies (Dupont et al. 2014; Zorgi et al. 2016). Our immunization with irradiated intact tachyzoites induces greater production of $CD8^+$ cells, due to and a possible particulate fraction derived to the cellular penetration of this immunogen (Zorgi et al. 2011), that could be presented more efficiently and directly by MHC class I pathway for selection $CD8^+$ cells (Nicoli et al. 2018). The $STag^{1.5\,kGy}$ induces both high concentrations of antibodies and significative numbers of $CD8^+$ cells, probably using the MHC II pathway and indirectly promoting B and $CD8^+$ cells through $CD4^+$ helper cells or specific dendritic cells in lymph nodes (Gerner et al. 2017). Another explanation for the higher proportion of $CD8^+$ cells in blood of immunized mice with $STag^{1.5\,kGy}$, is the fact that proteins altered by oxidation or carbonylation tend to be presented by cells via MHC class I with associated $CD8^+$ response as reported (Y.-C. Kim et al. 2014). Antigens presented for MHC-II $CD4^+$ T cells can be derived from the extra or intracellular soluble antigens in the host cell (Blum et al. 2013). In this context, irradiated soluble antigens appear to be presented and processed by B cells or macrophages and presented via MHC class II to $CD4^+$ T cells, which would induce effector cells and antibodies that would prevent infection and disease (Chorny et al. 2012), a fact clearly identified in the immunization with $STag^{1.5\,kGy}$. Irradiation of a protein or extracts mimics the environment of acute inflammation, with action of neutrophil products, to target an antigen for a better adaptive immune response (Sørensen and Borregaard 2016). This is probably similar to the effect produced by the various types of adjuvants that are used in immunizations, whether the alum or lipid emulsions, which retain the antigen with a suspension of low solubility at one site, with an intense local inflammatory reaction with activated neutrophils (Awate et al. 2013). Radiation-induced water radiolysis functions as a diffusely dispersed disposal of energy radicals (Kempner 2001), which promotes the same type of radicals that are produced by myeloperoxidase and halides in inflammatory environment, transforming gently the protein without adding new molecules, unlike drastic chemical reactions (Biedroń et al. 2015).

In our model, the whole process was performed without the addition of adjuvants to the immunogen, obtaining a humoral, cellular and protective immune response in both the protein extracts and the irradiated intact tachyzoites. Modifications caused in proteins after ionizing gamma irradiation seems modify antigens making them more efficiently recognized by the immune response, generating both antibody and cytotoxic cell production and easily stored and preserved. This report clearly shows that gamma radiation mimics biological processes for immune cooperation, aside to nucleic acids effects, and certainly these effects are potent tools in vaccine development. Radiation is an important tool for vaccine design and production both by its genomic sterilization effects, but also for their gentle antigen immunogenicity enhancement.

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Ethics approval

All animal procedures were performed with the approval of the Research Ethics Committee of the Institute of Tropical Medicine of São Paulo (Project 000274A) and in agreement with the Guidelines for the Care and Use of Laboratory Animals from the National Academy of Sciences.

Disclosure statement

The authors declare that they have no competing interests.

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