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ORIGINAL ARTICLE

Scavenger receptors mediate increased uptake of irradiated *T.gondii* extracts by J774 macrophages

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ABSTRACT

Purpose: Protein extracts developed increased immunogenicity without the aid of adjuvants after gamma irradiation. Gamma irradiation of snake venom increased antivenin production by detoxification and enhanced immunity, probably due preferential uptake of irradiated venoms by macrophage scavenger receptors. We studied this uptake of irradiated soluble *Toxoplasma gondii* extract (STag) by the J774 macrophage cell line similar to antigen presenting cells.

Material And Methods: We labeled STag by biosynthesis in living tachyzoites with radioactive amino acids before purification and irradiation or by adding labels as biotin or fluorescein in stored STag, for quantitative studies or subcellular distribution visualization.

Results: There was enhanced binding and uptake of irradiated STag into the cells compared to non-irradiated STag. Using fluorescein labeled antigens and morphological assays, we confirmed that cells avidly ingested both native and irradiated proteins but native STag were digested after ingestion while irradiated proteins remained in the cell, suggesting diverse intracytoplasmic pathways. Native or irradiated STag present the same in vitro sensitivity to three types of peptidases. Inhibitors of scavenger receptors (SRs) such as Dextran sulfate (SR-A1 blocker) or Probucol (SR-B blocker) affect the specific uptake of irradiated antigens, suggesting its association with enhanced immunity.

Conclusions: Our data suggests that cell SRs recognize irradiated proteins, mainly SRs for oxidized proteins, leading to antigen uptake by an intracytoplasmic pathway with fewer peptidases that prolongs presentation to nascent major histocompatibility complex I or II and enhances immunity by better antigen presentation.

Abbreviations: STag: Soluble Tachyzoite *Toxoplasma* antigen; $STag_B$: Soluble Tachyzoite *Toxoplasma* antigen conjugated with biotin; $STag_F$: Soluble Tachyzoite *Toxoplasma* antigen conjugated with FITC; $STag_{3H}$: Soluble Tachyzoite *Toxoplasma* antigen radiolabeled with ^{3}H-proline; MØJ774: Macrophages cell line; B_{max}: number of maximum specific binding sites estimated by non-linear regression; SCV: Scavenger cell surface receptors; FAU: Fluorescence arbitrary units

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Introduction

Gamma radiation was used to produce vaccines against eukaryotic protozoa, a challenging task due to antigenic variation and specific immune evasion mechanisms of these pathogens (McAllister 2014). The most successful model, *Plasmodium* irradiation, provides protection against malaria that have caused disease in humans for millenniums, was recently revived (Good and Stanisic 2020), probably due to the disappointing results of recombinant protein vaccines (Cockburn and Seder 2018). Toxoplasmosis is a similar apicomplexan disease and affects a large portion of human population as there is no efficient human vaccine toward it. We irradiated live *Toxoplasma gondii* tachyzoites for vaccine development in experimental models with good results (Hiramoto et al. 2002) in several models of protective immune response (Zorgi et al. 2016), but with storage problems due to liquid nitrogen conservation. These results were explained by the clastogenic effects of gamma radiation on parasite nucleic acids, leading to viability without reproduction of intact irradiated agents (Hiramoto et al. 2002). Higher doses of irradiated acellular extracts of *T. gondii* promote the same immunity as irradiated tachyzoites in mouse experimental models, without the storage problems due to refrigerator conservation (da Costa et al. 2018). Irradiated snake venom or its purified proteins are better immunogens for use in antisera production, attributed to scavenger receptor (SR) mediated immune recognition during antigen presentation (Do Nascimento et al. 1996; Cardi et al. 1998). We hypothesized that irradiated *T. gondii* proteins would participate in this protective response, probably by a diverse immune pathway than intact tachyzoites but also providing

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*(In memoriam)

adequate protection (da Costa et al. 2020). This protection must be related to modifications of the irradiated proteins that resulted in enhanced immunity.

Gamma ionizing radiation affects living organisms not only by modifying nucleic acids of the intact agent and mitotic death (Hiramoto et al. 2002) but also alters proteins, with several effects, but maintaining or enhancing immunogenicity (Do Nascimento et al. 1996). After gamma irradiation, an antigen undergoes changes such as protein aggregation, chain breaks, and oxidative reactions, due to water radiolysis (Davies 2016). These changes could lead to recognition of irradiated proteins by receptors present in the surface of "cleansing" cells, macrophages, which are the antigen presenting cells (APCs) in the adaptive immune response (Cardi et al. 1998). These APCs must select antigens in order to present them, in order to improve the yield of immune response. Antigens from normal host apoptotic cells would be completely digested and unselected for immune presentation, but proteins affected by inflammatory reactions could be related to the invader and selectively presented for immune reactions (Biedron et al. 2015). SRs are responsible for tissue "cleansing" and are classified as several types such as SR-A intended for cleaning and reuse of dead tissue cellular remains (Hawkins and Devitt. 2013); or related inflammatory response SR-B2 CD36 (Rhainds et al. 2003) which are responsible for various intracellular processing (Zeng et al. 2003) and binding of oxidized molecules intended for adaptive immune presentation (Witztum and Lichtman. 2014). Adjuvants used in immunogens promote this type of selection and targeting for adaptive immune responses, resulting in a more efficient vaccine (De Gregorio et al. 2013). This action seems to be related to the insoluble nature of antigenic mixtures (Awate et al. 2013) and partially because it attracts neutrophils, whose potent extracellular myeloperoxidase promotes oxidative changes in proteins (Biedron et al. 2015). Ionizing radiation also produces this specific oxidation of proteins and may target antigens for specific cellular receptors in APCs, with increased uptake and selective presentation. Irradiated crotoxin demonstrated selective uptake by macrophages, inhibited by probucol (Cardi et al. 1998), an anti-atherosclerosis drug directed to block the SR-B2 type receptor, associated with the clearing of oxidized lipids and lipoproteins (Zeng et al. 2003).

The study of the binding and uptake of irradiated STags by these cells in vitro could elucidate the early steps of antigen processing, which could result in enhanced immune response of irradiated proteins. This process depends on their recognition by cell surface receptors or uptake pathways. Labeled antigens allowed monitoring and quantification of their processing at the cellular or subcellular level. A better understanding of these steps would demonstrate that gamma irradiation could aid in the production of a safe, non-adjuvant dependent, and efficient toxoplasmosis vaccine capable of preventing human or veterinary disease.

Material and methods

Cells, parasites, and STag

We used mouse macrophage lineage J774A.1 (ATCC® TIB-67™) and monkey lineage VERO 76 (ATCC® CRL-1587™) cells according to their maintenance protocols from the ATCC (ATCC - American Type Culture Collection, Manassa, VA). Soluble tachyzoites extract was produced by the infection of VERO cells with frozen RH strain tachyzoites suspension as previously described (da Costa et al. 2018). Tachyzoites were seeded on VERO cell monolayers and allowed to infect the cells overnight. The medium was changed to remove the non-infecting tachyzoites, and the infected cells were maintained until lysis of 50% of the monolayer. The medium was removed and filtered through a 3-μm filter to remove host cells, washed in culture medium without serum, and suspended at a concentration of 10^8 parasites/mL in sterile water. The suspension was incubated by 15 min in an ice bath and subjected to five cycles of 30 s each of ultrasonic disruption in conical tubes. Disrupted suspension was cleared by centrifugation at $10{,}000 \times g$ for 10 min, supernatant was removed and an equal volume of 0.3 M NaCl was added. Protein content was determined by the Bradford assay and the sample was adjusted in saline to 100 μg/mL of protein. This fraction was named native STag, stored at −80 °C and used in subsequent experiments. A portion of this fraction was irradiated with 0.25 or 1.5 KGy in a Gamma Cell irradiator (Atomic Energy, Canada Ltd., 1.03 KGy/h) at room temperature at IPEN/CNEN and used as the irradiated fractions, $STag^{0.25KGy}$ and $STag^{1.5KGy}$.

Production of radiolabeled antigens - $STag_{3H}$

Biosynthetically labeled *T. gondii* extracts were produced by infection of VERO Cells with RH strain tachyzoites in the presence of ^{3}H-proline in the medium. Briefly, monolayers of VERO Cells were grown in Dulbecco Modified Minimum Essential Medium with 2% fetal calf serum in 25-cm^2 flasks. When semiconfluent growth was observed, the medium was changed to Dulbecco's Modified Eagle's Medium (DMEM) without serum containing 10 μCi/mL of ^{3}H-proline (100 Ci/μmole, Amersham BioSciences) and 10^6 RH strain tachyzoites, obtained from a previous culture were seeded onto the monolayer. The culture was maintained in 5% CO_2 at 36 °C for parasite growth and infection progressed until 50% cells were lysed, usually 24–48 h. The cells were scraped, and cell suspension was sheered several times through a fine needle to disrupt infected cells and immediately filtered through a 3-μm polycarbonate filter to remove intact cells and nuclei. The filtered suspension containing tachyzoites was centrifuged at $3000 \times g$ for 10 min, and the pellet containing intact labeled tachyzoites was washed twice with sterile phosphate buffered saline (PBS). The labeled tachyzoite pellet was processed for STag production as above described. The protein content was determined by Bradford or Qubit assays and the radioactivity present was determined in a scintillation counter by solving in Omni Mix scintillation cocktail.

STag labeling with biotin ($STag_B$) or fluorescein ($STag_F$)

Native and irradiated STags were submitted to molecular exclusion chromatography in Sephadex G-25 for removal of

low molecular weight amine containing reactants and free labels, selecting the large excluded proteins >5 KDa as targets for labeling. Excluding low molecular weight ligands is important for high specific activity allowing detection. For fluorescein labeling, the excluded proteins were reacted with fluorescein isothiocyanate (FITC) at an estimated rate of four fluorescein molecules for each 30 KDa protein in the STags, at pH 8.0 for 4 h in an ice bath. Free reactive labels were destroyed by adding 0.01 M sodium borohydride for 60 min. For biotin labeling, the excluded proteins were added with Biotinamido-hexanoic acid-N hydroxidesuccinimide ester, at the molar ratio of four biotins to each 30 KDa protein in the STags and incubated at pH 8.0 for 4 h at room temperature. The free labels were eliminated by adding 0.1 M glycine for 60 min. All labeled antigens were submitted to molecular exclusion chromatography in Sephadex G-25 for removal of free reactive markers, and their protein content was determined by Qubit assays and adjusted to 10 μg/mL. All reactions were performed at same time and the same batch of each of the antigens, STag, $STag_0^{.0.25KGy}$, and $STag^{1.5KGy}$. Spectrophotometric analysis of those preparations showed the same fluorescence intensity for the three different fluorescent antigens. Biotin content was measured in microwell plates, adsorbed with dilution of biotin labeled STags, and avidin peroxidase reaction, showing similar biotin content in the different preparations.

J774 macrophages antigen binding and uptake studies

The J774 macrophages were grown in DMEM, supplemented with 10% inactivated fetal bovine serum (FBS) in a humid atmosphere with 5% CO_2 at 37 °C in small culture bottles (Corning®, New York, USA). Cells were seed into 24- or 96-well tissue culture plates and used at same confluency as the binding or uptake studies. For uptake studies, cells were maintained at the same culture conditions and the antigen was added at different intervals, thus at the end of antigen addiction, all wells could be processed at the same time. For next steps, the cell layer was carefully washed twice with ice cold medium without serum. For binding studies, plates were kept in an ice bath and the antigen is added, incubated for 30 min, and carefully washed twice with ice cold media. The cell layer was then fixed with 1% formaldehyde and processed for antigen detection.

Detection of $STag_{3H}$ in J774 cells

The J774 macrophages (2×10^6 cells/well/mL) were grown on 13-mm coverslips and carefully washed by adding pre-heated to 37° C DMEM without serum, and cells were incubated for 0–4 h in the presence of native $STag_{3H}$ or STag irradiated at 0.25 and 1.5 kGy. The cells were carefully washed with PBS five times to remove free antigen, and the washed cells were detached by adding trypsin-versene (Sigma®, St.Louis, USA) to obtain the whole cell suspension. The amount of ^{3}H-proline in the cell suspension was determined by liquid scintillation cocktail Omni Fluor(PerkinElmer®, Waltham, MA), in which the radionuclide is uniformly distributed in a liquid medium capable of converting kinetic energy from nuclear reactions into light energy. All samples were measured twice for 600 s.

Detection of fluorescent $STag_F$ in J774 cells

The J774 macrophages (2×10^6 cells/well/mL) were grown on 13 mm coverslips on 24 wells plates, were carefully washed by adding pre-heated DMEM without serum, and the cells incubated in the presence of native $STag_F$ or irradiated at 0.25 and 1.5 kGy (1 μg/well/Artificial Fluorescent units(UAF)∼ 1000,000). The wells were carefully washed and cells fixed by adding 1% paraformaldehyde pH 7.5–8.0, for 10 min and subsequently washed 5 times with 1× PBS. Fixed cells containing fluorescein-labeled STag (FITC-green) were labeled with a second marker, Hoechst 33342 (Sigma®-blue), used to label viable cell DNA for a period of 10 min, followed by further washes with 1× PBS. The coverslips were carefully removed from the wells and mounted inverted on microscopy slides in suitable media (90% glycerol, 0.86 M Tris/HCl pH 8.6 and 0.1% p-phenylenediamine, as anti-fading, reducing photo destruction). After assembly, the slides were observed under a microscope BX-51 Olympus® (Shinjuku-ku, Tokyo, Japan) with Optomics photographic system. For fluorescence, we used filters with 490 nm excitation and 525 nm emission for FITC and 355 nm excitation and 465 nm emission filter for Hoechst 33342 (Sigma®). We obtained pictures from a non-exposed field with each fluorophore at the same exposure time for fluorescein for all samples, resulting in variable counterstaining. Each black and white fluorescent image were artificially colored and superposed using Image J software (U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997–2018).

Detection of biotinylated $STag_B$ in J774 cells

Briefly, 96 well plate containing J774 cells ($2x10^5$ cell/well) were challenged with each of the $STag_B$ antigens were washed carefully with PBS at least five times and the cells were fixed by adding 2 volumes of 1% paraformaldehyde for 10 min in each well. Cell layers were subsequently washed five times with PBS containing 0.1% FSA and 0.02% Tween20. Avidin conjugated to peroxidase was added at a 1:10,000 dilution in 1× PBS, and the cultures were incubated for 1 h at 37 °C. After new washes, the reaction was developed by adding liquid TMB commercial reagent and interrupted after 30 min by adding 4 N HCL. Optical densities (OD) readings were performed on a Multimode Microplate Reader FilterMax FS5 microplate reader (Molecular Devices®, Sunnyvale, California) at 450 nm absorbance.

Peptidase action on STags

We conducted tests for proteolytic action on the total extract substrate adjusted to a concentration of 100 μg/mL. Samples of native and irradiated $STag_F$ were added in

quadruplicate to 96-well black plates. Peptidase were added at 5 ng/well (50 ng/mL) in their respective buffer. For trypsin, the buffer contained 0.05 M Tris/HCl, 0,01 M, $CaCl_2$ pH 8.1; for papain, the buffer contained 5.5 M Cysteine, 1 mM EDTA, 0.067 M mercaptoethanol in 0.05 M phosphate buffer pH 6.5; and for pepsin, the solution was 0.01 M HCl pH 3. Samples were incubated for 30 min at 37 °C and 100 μL of 5% trichloroacetic acid (TCA) was added, shaken, and incubated for 30 min in an ice bath. Plates were centrifuged at 2000 ×g for 20 min at 4 °C. The supernatant was discarded, and the wells were washed with 100 μL ice cold 5% TCA and recentrifuged. After removing the supernatant, the precipitate was suspended in 100 μL of 5% $NaHCO_3$. Fluorescence was determined at 488 nm excitation and 524 nm emission using a Filter Max spectrofluorometer. Samples that were incubated without enzymes were considered as controls and the protein integrity was determined as a percent of protein maintained in the well in relation to the mean of the control wells.

Dextran sulphate and probucol interference in the binding of $STag_{3H}$ and $STag_B$ in J774 cells

For $STag_B$ or $STag_{3H}$ binding inhibition assays, macrophage cultures obtained as described above were maintained for 30 min in an ice bath. After this period samples of 0.14 μM dextran sulfate and 0.77 μM probucol (Sigma®) diluted in DMEM at a concentration of 0.4 μg/100 μL/well were added to the wells for 30 min at 37 °C. After this incubation, the wells were washed twice with DMEM preheated without serum. Samples of native or irradiated STag at a concentration of 1 μg/100 μL/well were added and incubated for 1 h at 37 °C. The wells were washed five times with PBS and the antigens were detected as described above for each antigen type.

Statistical Methods

All results presented were analyzed using parametric methods and they were considered significant if the probability of equality was less than 5% ($p < .05$) with a 90% power, using the Graph Pad Prism (version 5; GraphPad Software, La Jolla, CA, USA). Main comparison between mean results of each experimental time or group were performed by parametric ANOVA in the same conditions. Binding sites determination were performed using non-linear regression model of specific binding sites using the equation ($Y = Bmax * X/(Kd + X)$) in scatchard plots (Dahlquist 1978), and the statistical difference of the estimated values was determined using F tests, ANOVA with Bonferroni post-tests.

Results

Cell uptake assays

We evaluated the binding and uptake of native or irradiated STag, labeled with biosynthesized radioactive amino acid precursors in parasite-infected cells ($STag_{3H}$) or biotin-coupled ($STag_B$) for colorimetric detection in J774 macrophage cultures. In Figure 1, the difference in the binding of native or irradiated $STag_{3H}$ at 0.25 and 1.5 kGy (1 μg/well) incubated for different periods (0, 0.5, 1, 2, and 4 h) is shown. The radioactivity counts (cpm) were obtained by liquid scintillation for two counts of 600 s, with a 2% error in the measurement. In Figure 1, the incorporation of the native or irradiated $STag_{3H}$ to the layer of MØJ774 cells. The incorporation of the $STag_{3H}$ native in J774 macrophage cells was increased up to 60 min, after which we observed a decay of incorporation at 4 h, reducing the radioactivity detected in the cell layer. The $STag_{3H}^{0.25kGy}$ demonstrated a slow but increasing incorporation similar to that of native

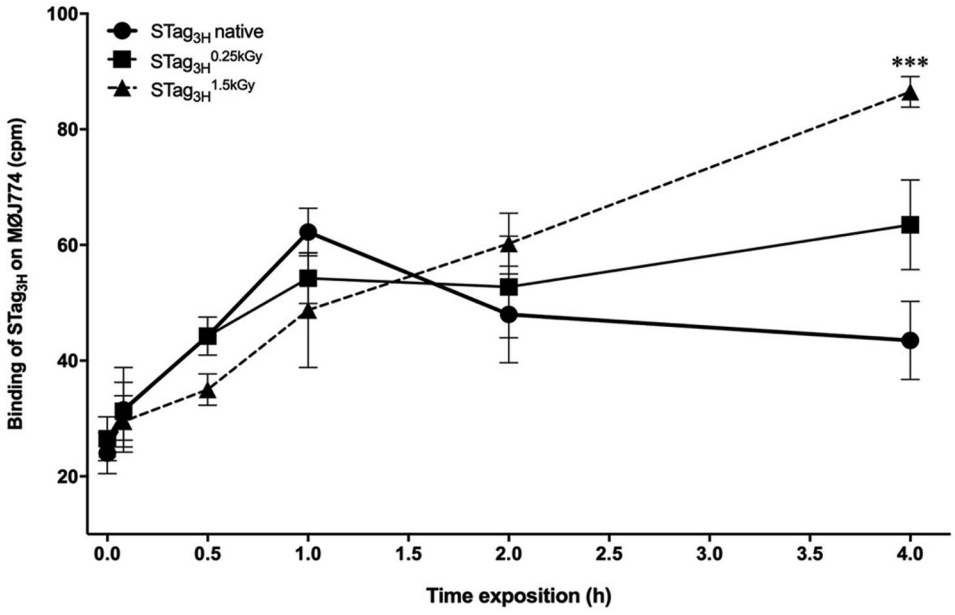

Figure 1. Evolution of the total binding of native $STag_{3H}$ and $STag_{3H}$ irradiated at 0.25 or 1.5 kGy (1 μg/well) in J774 macrophages (2×10^6 cells/well) at different exposure periods (0, 0.5, 1, 2, and 4 h). Bars represent the standard error (SEM) of the mean of 6 samples. *** Significant difference between the values of the slope in linear regression ($p < .001$).

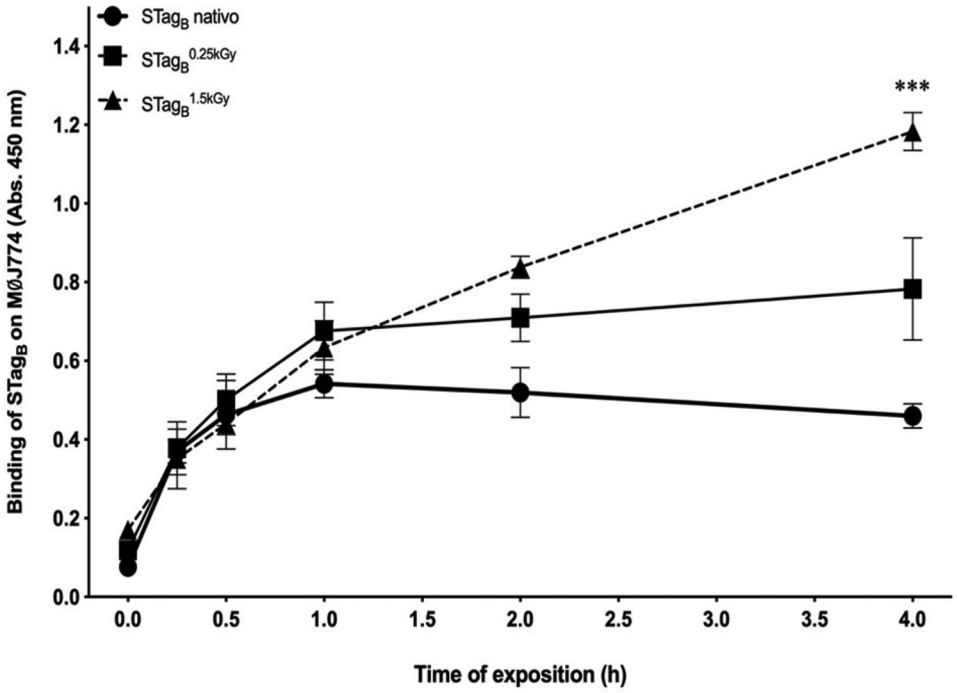

Figure 2. Evolution of the total binding of native $STag_B$ and $STag_B$ irradiated at 0.25 and 1.5 kGy (1 μg/well) in J774 macrophages (2×10^5 cells/well) at different exposure periods (0, 0.5, 1, 2, and 4 h). Bars represent the standard error of the mean (SEM) of 8 samples. *** Significant difference between the values of the slope in linear regression ($p < .001$).

$STag_{3H,}$ up to 30 min. The incorporation of $STag_{3H}^{1.5kGy}$ into the J774 macrophages was slower and increasing compared to the native and 0.25 kGy $STag^{3H}$, and with longer times, we observed that the binding to the cell layer was cumulative. From the non-linear regression of these values, it was possible to estimate the maximum number of specific binding sites (B_{max}) of each $STag_{3H}$ in the cell layer, which was 51.4 ± 4.0 in native $STag_{3H}$, 58.3 ± 4.3 in $STag_{3H}^{0.25kGy}$, and 96.14 ± 13.7 in $STag_{3H}^{1.5kGy}$. STag 1.5 kGy has a higher number of binding sites in the cell ($p < .005$). These binding sites are probably the sum of several surface receptors and appears independent of antigen processing as the radioactive tracer proline is maintained in the cell after protein degradation.

We conducted assays with improved sensitivity biotin labeled STags, to validate the results obtained with less sensitive $STag_{3H}$ in the binding of native or irradiated extracts in J774 macrophage cells. The J774 cells were grown in 96-well plates (2×10^6 cells/well) as described in the Methods section and incubated in the presence of the native or irradiated extracts (1 μg/well) for 0, 0.5, 1, 2, and 4 h. In Figure 2, the difference in the binding between native and irradiated $Stag_B$ in the permeated cell layer are presented. In spite of the difference in the kinetic profile of the binding between the $STag_{3H}$ and $STag_B$ assays, it was possible to demonstrate a greater detection of the $STag^{1.5kGy}$ at the end of the assay with different kinetics given the type of enzymatic reaction involved in biotin detection. It was noted that the binding pattern was similar to that observed with the use of ^{3}H-proline incorporated antigens, following the $STag_B$ native decay pattern after a certain period and the linear growth of the $STag_B^{1.5kGy}$. The three antigens showed similar binding to J774 macrophages within 30 min. After 60 min, native $STag_B$ reached its highest peak and began to decay as determined in $STag_{3H}$. $STag_B^{1.5kGy}$ showed an increased binding profile and increased binding to J774 cells after incubation. $STag_B^{0.25kGy}$ maintained a hybrid response between the two as seen in $STag_{3H}$. This data was analyzed with the non-linear regression model of specific binding sites using the equation ($Y = Bmax * X/(Kd + X)$) with Graph Pad Prism (version 5; GraphPad Software, La Jolla, CA, USA), and the statistical difference of the estimated values was determined. The number of binding sites was 1.466 ± 0.085 for $STag_B^{1.5kGy}$ ($p < .001$), which was higher than the 0.8414 ± 0.033 for $STag_B^{0.25kGy}$ and 0.5271 ± 0.019 for native $STag_B$. Our data shown that irradiated proteins are recognized by additional receptors other than the regular receptors used for native proteins. The quantitative data are directly correlated with the data obtained with the biosynthetic labeled radioactive antigen ($r^2 = 0.96$), when analyzed with the same non-linear model adapted for the different quantification method. The difference in the numbers of binding sites resulted from those diverse methods employed for its determination, but the binding sites between each STag was proportional, resulting in high correlation.

Binding assays

Based on the results of the previous experiments, it was decided to test the binding of the different STags to the cell surface of J774 macrophages using the cell temperature to prevent internalization of the antigen into the cell. This experiment was performed on ice cold chilled J774 cell monolayers using biotin labeled antigens, due to the high signal intensity. To avoid the exponential enzymatic reaction pattern, we estimated antigen mass bound to the monolayer,

using a standard curve with a known mass of antigen bound to an ELISA high binding microplate. This approach allowed for the estimation of the antigen mass related to the A_{450} of the final reaction, performed in chilled cells with minimal internalization of the antigens. These data are shown in Figure 3. Native $STag_B$ had a lower cell surface binding capacity compared to other antigens, indicating that the receptor for this antigen had the low frequency of its receptor on the cell surface. $STag_B^{1.5kGy}$, exposed to greater radiation than $STag_B^{0.25kGy}$, demonstrated a higher binding capacity to the cell surface of the monolayer, suggesting recognition by an alternative receptor that is more frequently presented on the cell surface, in addition to binding to the common receptor(s) for native proteins. The less irradiated $STag_B^{0.25kGy}$ presented intermediate levels of binding, probably due to less quantitative binding to the alternative receptor for irradiated proteins in a dose dependent process.

Subcellular determination of irradiated antigen uptake

The J774 cells. were seeded on sterile covers slips in 24 well plates (2×10^6 cells/well) as described in the Methods section. An adequate field at 40× magnification of each coverslip was randomly selected, and each field was photographed with the same illumination and digital exposition for fluorescein filters using this fixed time also for Hoeschst staining pictures. In Figure 4 the composed images of each antigen at each time point are shown. There was no nuclear staining of the STags, demonstrating the absence of unspecific uptake. At 0 time, binding of antigens to the cell layer is presented. After this, staining was concentrated in the cytoplasm of the cells, suggesting an active uptake. Native $STag_F$ shows a high early uptake peaking at 15 min with subsequent decay until it was almost no longer observed at 60 min. $STag_F^{0.25kGy}$ presented a pattern similar to native antigen but some cells maintain the green fluorescence stain at 60 min. There was a constant or progressive fluorescence of $STag_F^{1.5kGy}$ content in the cells peaking at 60 min, showing antigen storage or accumulation. Taken together, this preliminary morphological data confirms the data demonstrated in the quantitative assays, using both radiolabeled and biotinylated antigens. Proteolysis of native fluorescent antigens after ingestion explains the disappearance of fluorescence in the monolayer cells after fixation, which allows the movement of small molecular weight proteins. Discrepancies in counterstaining digital images are caused by the constant exposure of digital imaging selected for green fluorescence.

Peptidase resistance of antigens

We tested whether the radiation could induce a peptidase resistance in antigens that could explain the accumulation by reducing digestion in the cells. Using fluorescein isothiocyanate (FITC) STags and its precipitation by 5% trichloroacetic acid (TCA), we quantitatively evaluated the action of three peptidases against the antigens. Peptidases with three different modes of action; trypsin, a serine peptidase; pepsin, an acid peptidase, and papain a cysteine peptidase. The assays were conducted as described in the Methods section and their results are shown as the remaining arbitrary units of fluorescence (FAU), supplied by the fluorimeter, (Figure 5(A)) and the percentage of susceptibility of $STag_F$ to peptidases, determined by the ratio of remaining FAU to starting FAU in the sample (Figure 5(B)). The maintenance of fluorescence in $STag_F$ samples, native and irradiated at 0.25 or 1.5 kGy, in the absence of the peptidases, demonstrated the absence of endogenous peptidases in the samples. When peptidases are added, intact insoluble proteins decayed ($p < .05$), demonstrating the action of all peptidases in the three STags, in comparison to

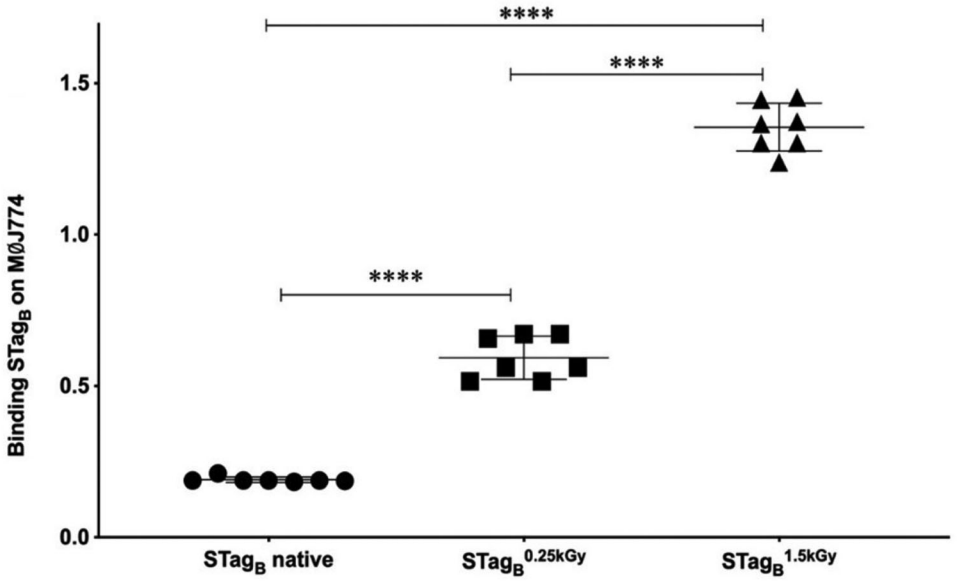

Figure 3. Cell surface receptor binding of biotin labeled STag in chilled J774 macrophages. Bound antigen was expressed in pg/mL as mass of antigen from a standard curve. Data are expressed as mean and error bar (standard deviation, SD). The data was analyzed using ANOVA with Bonferroni post-tests and *** indicates $p < .001$ in isolated comparisons between antigens.

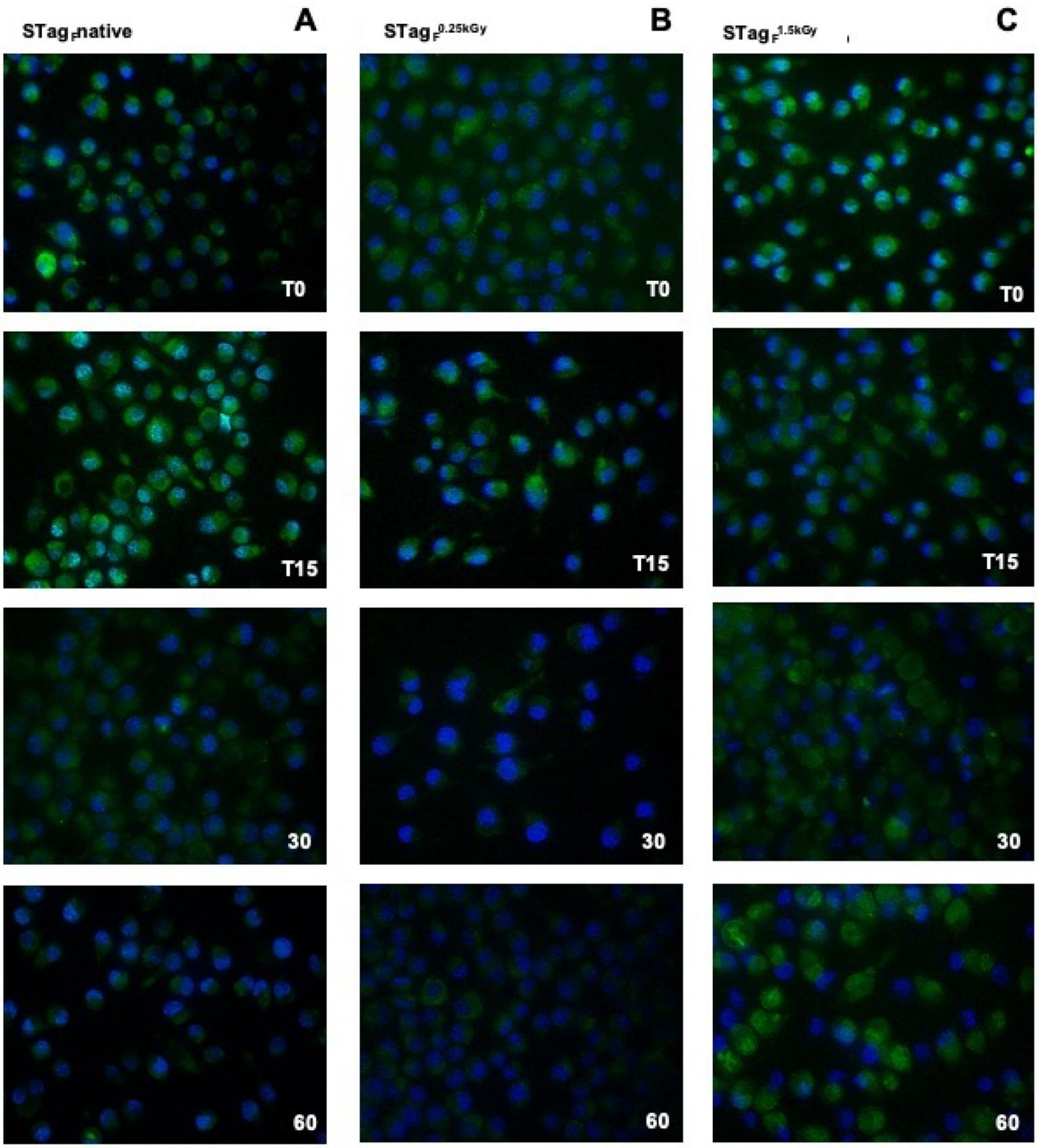

Figure 4. Optical fluorescence microscopy indicating the location of the $STag_F$: **A-** native; **B-** irradiated at 0.25 kGy; **C-** irradiated at 1.5 kGy in J774 macrophage cultures at different exposure periods (0, 15, 30, and 60 min). Green: conjugates of native and irradiated Stag to the fluorescein isothiocyanate (FITC); blue: labeling of the cell nucleus by Hoechst dye. Objective 40× magnification. Micrographs with the same exposition time from the same field were combined with Image J software.

control samples. The peptidase destruction of STags was the same between the antigens, regardless of irradiation. Trypsin acted on 17% of the native $STag_F$ proteins, 22% of the $STag_F^{0.25kGy}$ proteins, and 28% of the proteins of $STag_F^{1.5kGy}$, while papain removed 20% of $STag_F$, 23% of $STag_F^{0.25kGy}$ and 29% of $STag_F^{1.5kGy}$ proteins. The more active pepsin acted on 32% of $STag_F$ and $STag_F^{0.25kGy}$ proteins and in 37% of the proteins of $STag_F^{1.5kGy}$, demonstrating the most disruptive isolated peptidase activity against the extracts.

Scavenger receptor blocking in binding assays

To determine cell surface receptor specificity, we evaluated the effect of cells pretreated with specific SR blocking agents, such as Dextran sulfate, specific for SR group 1 (SCV1) against anionic antigens; and probucol, specific for SR group II (SCVII), related to oxidized molecules, shown in Figure 6. We cultured J774 macrophages on microplates at semi-confluency and chilled the cells in an ice bath. For blocker treatment, the cells were incubated with each blocker at the

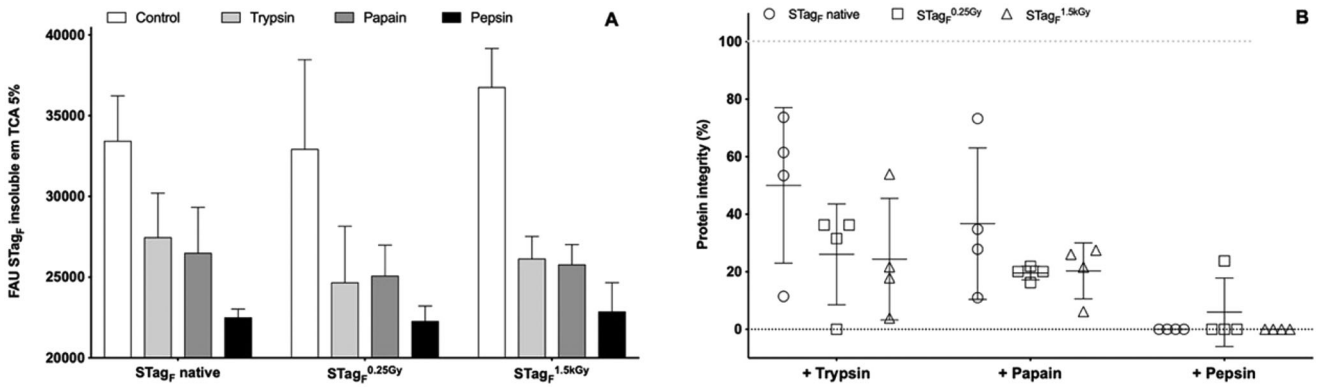

Figure 5. Susceptibility of $STag_F$, native and irradiated at 0.25 or 1.5 kGy, to the action of peptidases trypsin, papain and pepsin, after incubation for 30 min at 37 °C. **A-** Fluorescence arbitrary units (FAU) of native or irradiated TCA insoluble $STag_F$ products; **B-** percentage of protein integrity of $STag_F$ after peptidase treatment. Bars represent the mean and standard error of the mean (SEM). No significant differences were found between the mean of extracts (ANOVA).

concentration described in the Methods section for one h and carefully washed the plate with PBS. Antigens at 10 μg/mL (1 μg/well) were added to both the pretreated and controls cells. We use both biosynthetically labeled radioactive STags or biotin labeled STags. The binding continued for 1 h in an ice bath and the radioactive content or biotin presence was determined as previously described. The results for radioactive STags are shown in Figures 6(A,B). The pretreatment with blockers had no effect on native STag binding, which is a native protein usually not recognized by SRs. However, irradiated antigens demonstrated the significant effect of pretreatment ($p < .001$), 1.5 kGy irradiated STag showed substantial uptake with the SCVII probucol blocker and was also clearly seen in the less irradiated 0.25 kGy STag. The basal uptake was similar between the antigens in the presence of the blocker, showing that a residual uptake occurs independent of the receptors, which are similar to native STag. These data were confirmed using biotinylated antigens, as shown in Figure 6(B) In this improved sensitivity assay, the effect of SCVII blocker, probucol, is more evident and produced a greater effect than that of the SCVI blocker, dextran sulfate, but this blocker also induced a degree of blockage. This data indicates that SCVs appear to participate in cellular uptake of irradiated proteins, but not native antigens. The quantification by two diverse labeled antigens suggests that mainly antigens oxidized by irradiation are recognized in J774 cells, thus this pathway must affect all the subsequent steps of irradiated antigens. In a very preliminary experiment, we failed to block the binding of the irradiated $STag_B$ antigens by using a preincubation of the chilled J774 cells with a commercial monoclonal antibody against CD36, in a very preliminary results (data not shown). As CD36 had several domains and a cleft for fatty acid transport, the antibody could be directed against other exposed domains, without affecting antigen binding to the cleft of CD36 protein.

Discussion

Our data show that irradiated STags presented higher uptake and longer persistence inside a J774 macrophagic cell line in a dose-dependent manner. This finding could explain the enhanced immunogenic effect of irradiated *T.gondii* antigens reported in earlier work (da Costa et al. 2020). Macrophagic cell line increased uptake was demonstrated in several ways, either by biosynthesis of radioactive precursors identical to native proteins or labeling performed extracts. The biosynthesis process using radioactive precursors maintains the integrity and structure of parasite extracts after irradiation without any label or process chemical addiction that could alter its uptake after labeling. This is especially important when the labeling molecule was added to the protein, such as biotin or fluorescein. We found a similar uptake pattern with all the labels used in the experiments indicating that the labeling process did not affect the ligand of the receptor of gamma irradiated extracts.

Binding assays using chilled J774 macrophage cells showed that STag irradiated at 1.5 kGy had a linear and increasing dose binding response, with higher specific binding rates than native (non-irradiated) STag, probably due to additional and more frequent surface receptor binding. The uptake curve in active cells showed no decrease suggesting that the irradiated antigens were not being degraded intracellularly unlike the native antigen, which shows an evident degradation. This suggests different cytoplasmic processing pathways for irradiated antigens. We demonstrated that irradiated antigens are mainly recognized by a frequent cell surface receptor and are internalized in the cell to a pathway with low peptidase activity, whereas native antigens are quickly internalized by receptors with the intracellular route leading to lysosomes and rapid degradation (Staudt et al. 2016). A similar effect of increased uptake and cell distribution was observed with irradiated crotoxin (Do Nascimento et al. 1996). If radiation blocks some targets for proteolytic action, enhanced immunogenicity could occur due to increased proteolytic resistance, independent of intracytoplasmic pathways. Resistance to intracellular peptidases was obtained by the elimination of asparagine endopeptidases sites in the toxin C fragment of tetanus toxin, with high resistance to proteolysis and increased immune presentation (Antoniou et al. 2000); thus, persistence inside the cell without proteolysis could enhance immunity. Gamma irradiation

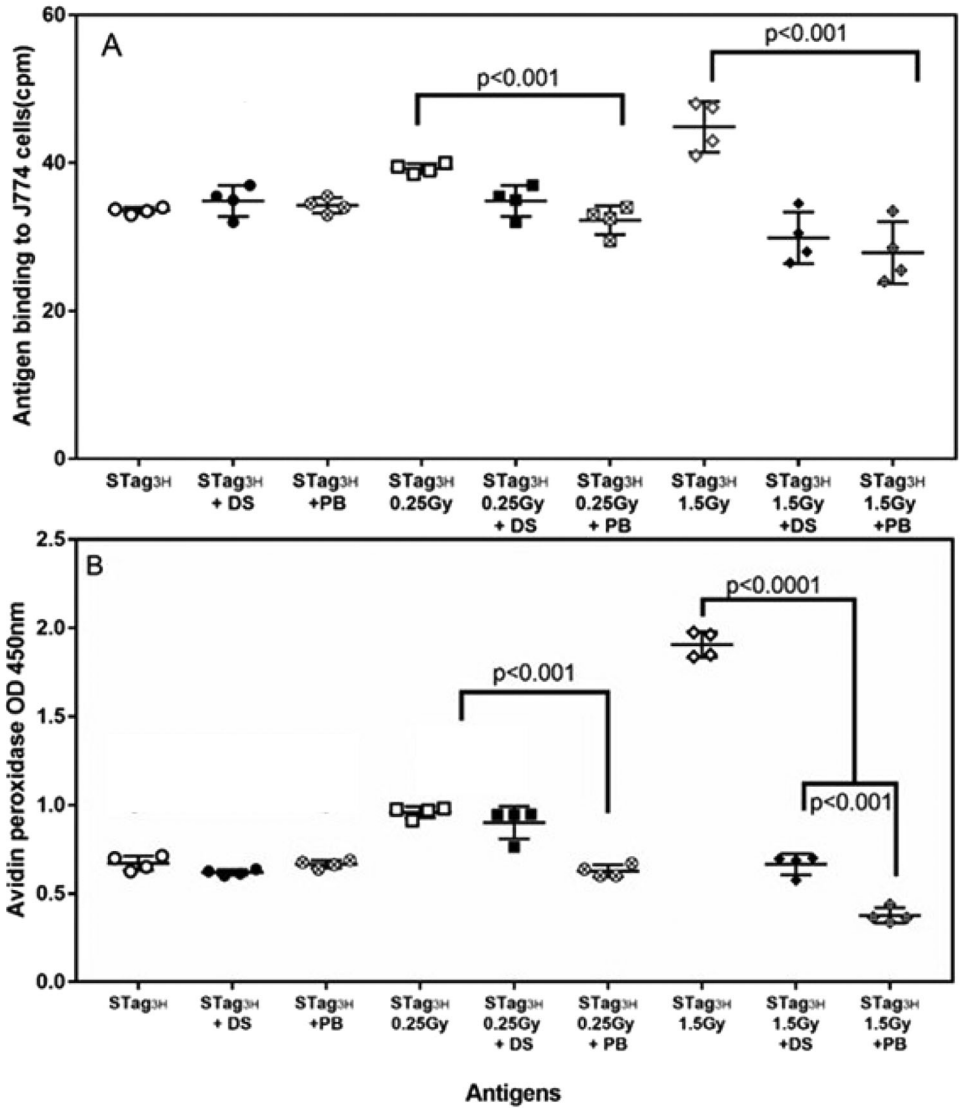

Figure 6. Effect of scavenger receptor blocking agents on the binding of STags to chilled J774 cells. Monolayers were pretreated with blockers for 1 h, washed, and challenged with antigens. **A**- Challenged with irradiated biosynthetically labeled $STag_{3H}$. **B**- Challenged with irradiated biotin labeled antigens. +DS:pretreatment with dextran sulfate SCV1 blocker; +PB: pretreatment with probucol SCV2 receptor blocking; Error bar: standard deviation (SD). Open symbols are without treatment, solid symbols are with Dextran sulfate pretreatment, and crossed symbols are with probucol pretreatment. Statistical analysis was performed by ANOVA with Bonferroni posttests. NS : non-significant; *p*-values are expressed on linking bars.

was reported to induce breaks in protein structure but without specific locations or fragile points of increased proteolysis (Adams et al. 2015). Our data of similar proteolytic sensitivity of native or irradiated antigens clearly shows that alteration of proteolytic susceptibility is not involved in the enhanced immunogenicity caused by gamma radiation of parasite extracts.

Several pathways are involved in the binding and uptake of proteins as STags into macrophages. Scavenger receptors are a family of cell surface proteins that were proposed as an explanation to preferential uptake of irradiated crotoxin, mainly blocked by probucol, a blocker of oxidized protein's SR-B (Cardi et al. 1998). We studied the blocking effect of two SRs on the uptake of STags and the binding of the irradiated STag was affected by both dextran sulfate and probucol, which are negative SR-A SRs and oxidized products LOX CD36 receptor blockers, respectively. Dextran sulfate was less efficient but did affect the native STag binding in macrophages. SR-A is a receptor of negative charges on the surface of apoptotic cells (Fukasawa et al. 1996) or vacuoles of the cells and promotes their intracellular binding to the lysosomes by promoting rapid degradation of the phagocytized products into reusable monomers (Balasubramanian et al. 2012).

In APCs, peptidases are responsible for generating peptide fragments of protein antigens that bind to major histocompatibility complex (MHC) molecules during the immune response, especially those mediated by T lymphocytes (Roche and Furuta 2015). In antigenic extracts, multiple enzymes, and multiple processing sites as proteasomes must be required for the release of epitopes (Blum et al. 2013). Our results showed that the irradiation of the extracts does not make them less susceptible to proteolysis but rather directs them to a different intracellular route with low peptidase activity. The route with less peptidase activity generates peptides of adequate size for the initial immunological

synapse, involving products of considerable molecular weight that induce spatial reactions to multiple receptors (Alarcón et al. 2011). This route offers larger peptides that bind inside the cell for the construction of MHC molecules capable of antigen presentation when exposed on the cell surface (Blander 2018), but those systems were reported as intercommunicated (Ho et al. 2021), and we believe that the antigen presentation must rely on several mechanisms in order to be effective.

There are several intracellular routes involved in antigen presentation, with diverse efficiency including some pathways that result in the complete and nonselective degradation of antigens, resulting in the absence of immunogenicity because of the autodigestion processes. Probucol is involved in blocking the uptake of oxidized products and works for atherosclerosis by decreasing the formation of plaques by oxidized products (Li et al. 1998). This blocker was the most efficient at blocking irradiated STag uptake in macrophages and had previously been described as more efficient in blocking irradiated crotoxin uptake (Cardi et al. 1998). Ionizing radiation induces the oxidation of proteins via hydroxyl radicals (OH•), induced by water radiolysis similar to the product produced by peroxidases (Reisz et al. 2014), similar to neutrophil myeloperoxidase (Kettle and Winterbourn 1997). This association of oxidation of molecules, SRB2 SRs, and adaptive immune response was putatively proposed as a link between innate and adaptive immune response (Witztum and Lichtman. 2014). This protein oxidation may be an approach to increase the presentation of antigens involved in areas with neutrophil inflammation, being a selection for extraneous antigens (Biedron et al. 2015), and ionizing radiation can mimic this biological reaction, inducing a pathway to connect innate-adaptive immunity (Rosales 2020). Our data indicate that irradiated proteins must be mainly recognized by SRs for oxidized proteins, such as CD36, and to a lesser extent by other SRs, promoting the uptake of soluble molecules to macrophages and APCs, without the need for adjuvants. This specific binding in the early stages of adaptive immune response explains the immunity induced by irradiated proteins, and in this case, the protection induced by irradiated extracts of *T. gondii*. Gamma irradiation of antigens proteins is not a guaranteed process for all antigens. It depends on the primary structure of the antigen that allows oxidation for recognition by scavenger receptors in APCs, and extracts from parasites had multiple antigens which allow enhancement of the immunity easily and quickly produced from natural antigens for future vaccines.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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