

Stimulatory Effect of Synthetic Carbohydrate-Bearing Molecules on Nitric Oxide Production and Enhancing Macrophages Viability upon Exposure of *T. Gondii*

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Abstract—Toxoplasmosis is a parasitic disease caused by *Toxoplasma gondii*, an intracellular parasite that affects most types of warm-blooded vertebrate's cells. Individuals at risk for toxoplasmosis include fetuses, newborns, and immunologically impaired patients. Therefore, finding methods for early boosting immune responses cells would help protect against toxoplasmosis. Synthetic carbohydrate bearing moieties (SCs) were used to treat the infected macrophages and to decolonize the tachyzoites. The current research studied the SCs effect on macrophage phagocytosis after 22-24 hours exposure to the *Toxoplasma gondii* tachyzoites (Tg). Macrophage functional analyses were performed, using nitric oxide (NO) release, upon exposure to Tg treated by SCs. The effect of on macrophage viability (MV) and resistance to *T. gondii* during phagocytosis were studied using fluorescence-activated cell sorting (FACS).

NO release was dependent on the type and the time of SCs exposure to infected macrophages. FACS analysis demonstrated that SCs affected macrophage survival upon *T. gondii* exposure. Murine macrophages exposed to SC-treated tachyzoites (M+SC+Tg) increase macrophage viability (MV) from 52.6% of macrophages infected with tachyzoites (M+Tg) up to 65%-75%. The present study provides new SCs candidates that can be used in the development of new therapeutics and immunostimulants against *T. gondii*.

Keywords—FACS, Nitric Oxide, Synthetic carbohydrate, *T. gondii*.

I. INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite that infects nucleated cells [1]. This wide-ranging host cell specificity proposes that adhesion should involve the recognition of abundant surface-exposed host molecules or, alternatively, the presence of numerous parasite attachment molecules capable of distinguishing different host cell receptors.

T. gondii is responsible for the zoonotic infection, named toxoplasmosis, in birds, mammals, and humans [2]. Even though toxoplasmosis is asymptomatic in healthy people, the infection is life-threatening to neonates and in

immunocompromised patients [3], [4].

The tachyzoites stage (Tg) is the most important stage because they propagate fast inside the host cell, is responsible for distribution the infection from cell to cell and is responsible for many of the disease symptoms [5]. In addition, macrophages can be easily recognized by Tg and restrict the propagation of *T. gondii* [6].

It was shown that macrophages are important effectors of resistance and restrict parasite replication during toxoplasmosis [7], [8]. The ability of monocytes and macrophages to kill intracellular pathogens depends in part on the capacity of these cells to generate potentially toxic oxygen metabolites [7]. Moreover, the major anti-parasitic effector mechanism was reported to be the production of nitric oxide (NO) by activated macrophages [9], [10]. NO is the product of arginine metabolism and one of the most effective bullets against Tg. It was previously reported that NO levels increased during toxoplasmosis [10], [11] and decreased *T. gondii* replication [10]-[12]. It has been shown that NO provided protection from the damaging effects of Tg [13]. *T. gondii* was capable of partially inhibiting NO production by host cells [14].

Natural carbohydrate-bearing molecules mediate cell adhesion, trafficking, recognition, immune responses, inflammation, and signaling [15] - [17].

Several of Tg receptors are Natural carbohydrate-bearing molecules including, but not limited to, N-linked [18] and O-linked sugars [19] and anchored glycosyl-phosphatidylinositol [20]. All these receptors are important which allow the Tg to interact with and respond to the external environment [21]. These receptors can identify different antigenic structures [22]. Numerous vital cell-surface proteins can bind with carbohydrate epitopes by carbohydrate-bearing molecules [23].

Glycoconjugate-polyacrylamide-fluorescein (Glyc-PAA flu) is a synthetic polymer comprised of carbohydrate moieties (Glyc), a polyacrylamide polymer backbone (PAA), and a fluorescein group (flu). Synthetic carbohydrate-bearing moieties (SCs) are suitable ligands for the recognition of pathogens due to the fact that Glyc-PAA-flu can be immobilized in a solid phase and used for binding and recognition [28]. SCs stimulate cell-cell adhesion and subsequent recognition and activation cells' receptors of living organisms [15], [16]. It was reported that SPCs selectively recognize, bind [24], and inhibit other pathogens [25], bacterial spores [26], and toxins [27].

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The objectives of the present study is) employ selective and specific SCs for macrophages activation upon *T. gondii* exposure during early phagocytosis. In this context, SCs binding and stimulatory effects on *T. gondii* infected and uninfected macrophages will be assessed during early phagocytosis.

Macrophage activation following *T. gondii* exposure will be determined during 22-24 hrs of phagocytosis. Macrophage cell viability, necrosis, and apoptosis during *T. gondii* exposure will be studied using fluorescence-activated cell sorting (FACS). Measuring production of NO will allow analysis of SC-mediated stimulatory effects on macrophages. NO release will indicate macrophage activation. We hypothesize that *T. gondii* will induce macrophage death (necrosis or apoptosis), whereas SCs will prevent macrophage cell death and promote resistance, viability, and activation of macrophages.

II. METHODS

A. Cell culture

According to the ATCC protocol, human foreskin fibroblasts were maintained in DMEM medium supplemented with 10% FBS, 1mg amphotericin B to 500 ml of DMEM medium and, 2% penicillin-streptomycin as follows. The old medium was aspirated when fibroblasts formed a confluent layer after 4-5 days and was replaced with 5 ml of 0.25% trypsin solution. Trypsin was gently distributed over the fibroblast's monolayer. The flask was placed at 37°C for 5 min in order to detach fibroblasts. Soon after, 15ml of DMEM growth media was added. Cells and medium were collected and aspirated into a 50-ml sterile tube. Tubes with cells were spun at 800-1000 rpm at 37°C for 5-10 minutes. After spinning, the supernatant was discarded. Fibroblasts (as pellets) were carefully re-suspended using 15ml of growth media. The fibroblasts were collected, then split into two small T-25 flasks and incubated in a CO₂ incubator in a 95% air/ 5% CO₂ / 37°C atmosphere. The exhausted growth media was changed twice a week.

B. Methods. *T. gondii* Propagation and Purification

T. gondii RH strain (Tg) was maintained as tachyzoites by serial passage on monolayers of HFFs at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin. The viability of the parasites was determined by trypan blue exclusion [28].

C. Preparation of Murine Peritoneal Macrophages

C57BL/6 mice were injected intraperitoneally with 1.0 ml of 3% thioglycolate broth. Five days after injection, mice were euthanized and peritoneal exudate cells were collected by lavage with 5.0 ml RPMI 1640. Macrophages were plated at 8×10⁵ macrophages per culture in 24-well plates for NO measurements. For FACS analysis, macrophages were plated in 6-well plates at 1.0-1.2×10⁶ macrophages per culture. After 1-2 hrs of incubation at 37°C (95% air, 5% CO₂), non-adherent macrophages were removed by washing using RPMI

1640 containing 10% fetal calf serum, 50 nM 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin.

D. NO Analysis

Cultures were infected using 0.8×10⁶ (1ml) *T. gondii* tachyzoites per well and treated with 3µg (33 µl) of SCs. Infected cultures were incubated for 22–24 hrs and macrophage activation was measured as NO production during phagocytosis of Tg. Culture supernatants (100 µl) were assayed for NO using the Griess assay. Briefly, 100 µl of cell culture supernatant was mixed with an equal volume of Griess reagent [0.1% (w/v) *N*-(1 naphthyl) ethylenediamine dihydrochloride and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid]. The samples were incubated at room temperature for 20 min and absorbance was measured at 490nm using a Bio-Tek Ex800 plate reader.

E. Fluorescence Activated Cell Sorting (FACS)

For FACS, cultures were infected with 3µg (33µl) of SCs and 0.8×10⁶(1ml) of tachyzoites per wells and incubated for 22–24 hrs. Effects of SCs and their impact on 1) SC-treated macrophages infected with tachyzoites, 2) SC-treated macrophages only, 3) macrophages infected with tachyzoites, and 4) macrophages only were investigated. After 20-24 hrs of phagocytosis, macrophages were harvested, washed in cold phosphate-buffered (PBS) saline for 15 min at 1,200 rpm and 37 °C, and the cell density was adjusted. After centrifugation, the supernatant was discarded and the cell pellets (100 µl) were re-suspended in 300 µl cold PBS and vortexed.

Resuspended cells (500 µl) were transferred into BD-FACS tubes and placed in ice. Cells were labeled using 1.0 µl of the YO-PRO[®]-1 (Component A) and 1.0 µl of propidium iodide (PI) (Component B) stock solution. Cells were analyzed by FACS (BD FACS Calibur) using an excitation wavelength of 488 nm with green fluorescence emission for YO-PRO[®]-1 (i.e., 530/30 bandpass) and red fluorescence emission for the PI (i.e., 610/20 bandpass), gating on cells to exclude debris. Using single-color stained cells, the standard compensation was performed. The cell population was divided into three groups: 1) live cells with a low level of green fluorescence; 2) apoptotic cells with an incrementally higher level of green, and 3) necrotic cells with both red and green fluorescence.

III. STATISTICS

Results were considered to be statistically significant with *p*-values <0.05 using ANOVA. Tukey test was performed for post ANOVA to indicate which group was not statistically significant compare to others.

IV. RESULTS

In this study, SCs were used as surface immobilized molecules (ligands) to achieve *T. gondii* tachyzoites (Tg) recognition. SCs can interact with other species called targets [28]. We assessed eighteen different types of SPCs for their binding affinity toward Tg (targets) using binding assay (result not shown).

It was found that the studied SCs demonstrated: i) no effect), ii) lower binding affinity (LB), and iii) significantly higher binding affinity (HB) toward Tg.

We studied the effect of LB SCs (Fig. 5A and C) and HB SCs (Fig. 5B and D) on macrophages activation and NO production during a 24-hrs of Tg infection. Exposure of macrophages to Tg (M+Tg) increased NO production after 6 hours of infection. Macrophage exposure to Tg followed by SCs treatments (M+SC+Tg) demonstrated significant NO increase starting from 6-8 hours of Tg infection. Overall, no differences were observed between LB (Fig. 5A, C) and HB SCs (Fig. 5B, D) on NO production. Although, non-infected macrophages treated with SC4, 3 and 2 showed higher NO production compared to others (Fig. 5B, D).

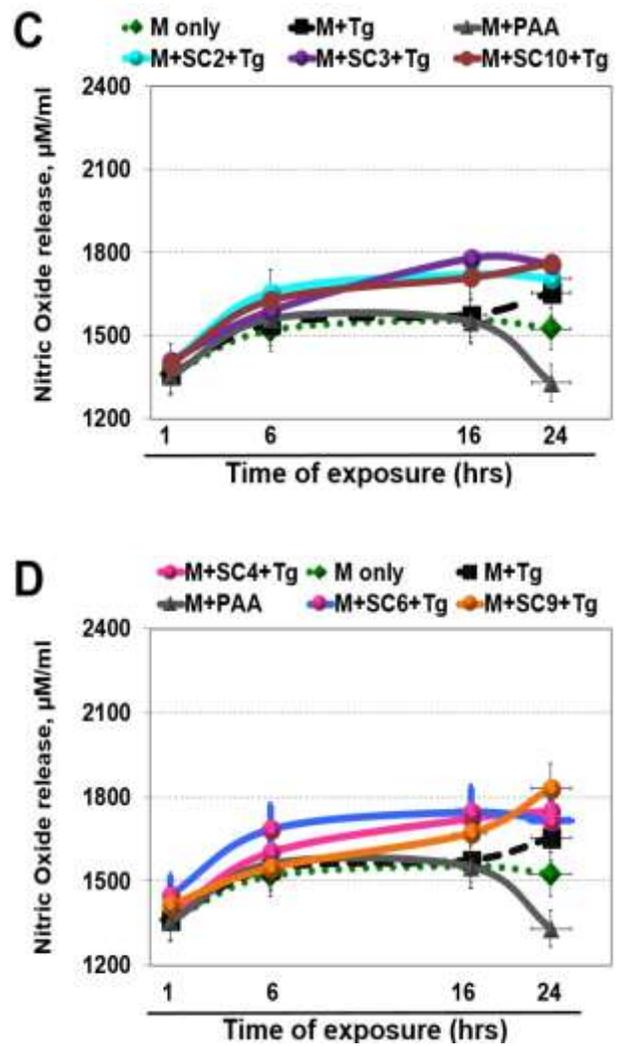
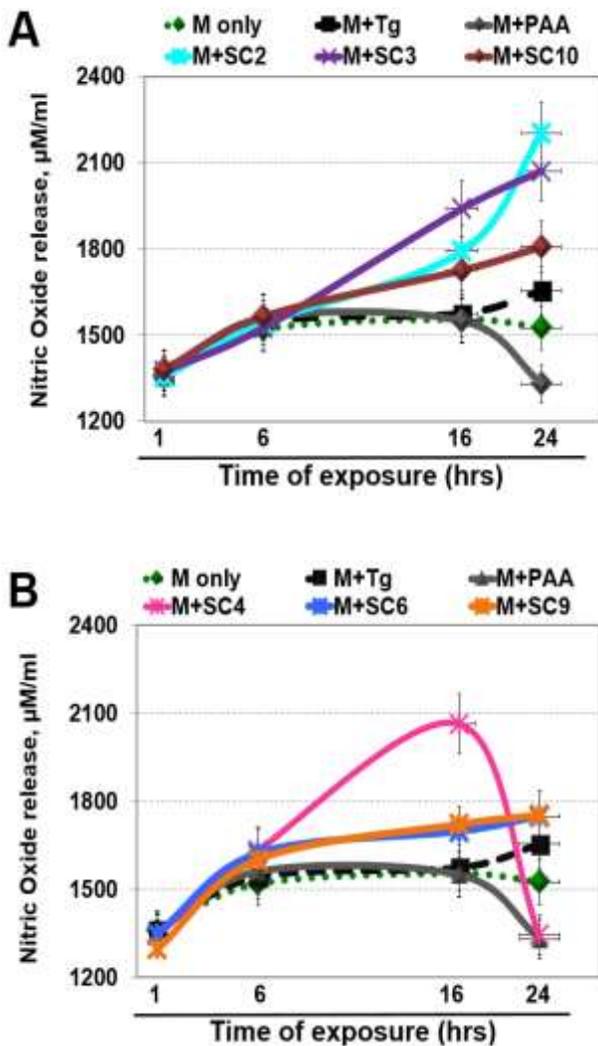


Fig. 5. Nitric oxide (NO) released by macrophages only (M), macrophages exposed to *T. gondii* (M+Tg), and macrophages exposed to Tg and treated by LB SCs (B) and HB SPCs (M +SPCs+Tg) (D) compared to controls (A, C) including M only, M+Tg, M+PAA, M+SPC (LB or HB), $p < 0.05$.

The effect of SCs on macrophage viability (MV) and their resistance to *T. gondii* during phagocytosis were studied using FACS. The purpose of FACS was to assess whether LB or HB SCs will provide the higher protective effects on MV during *T. gondii* exposure. Figures 6A, B shows the percent of live (L), apoptotic (A), and necrotic (N) macrophages (M) upon *T. gondii* infection (M+Tg), and after SC treatment. FACS analysis demonstrated that SCs affected macrophage survival upon *T. gondii* exposure. *T. gondii* decreased MV from 99.4% (M) to 52.6% (M+Tg). LB (SC2, 3 and SC10+Tg) demonstrate noticeable rise level of live macrophage started from 69% to 71.5% (Fig. 6A) in comparison to the SCs only and M+Tg. The apoptotic macrophages slightly increase in infected macrophages after treated with LB SCs compared to non-treated infected macrophages.

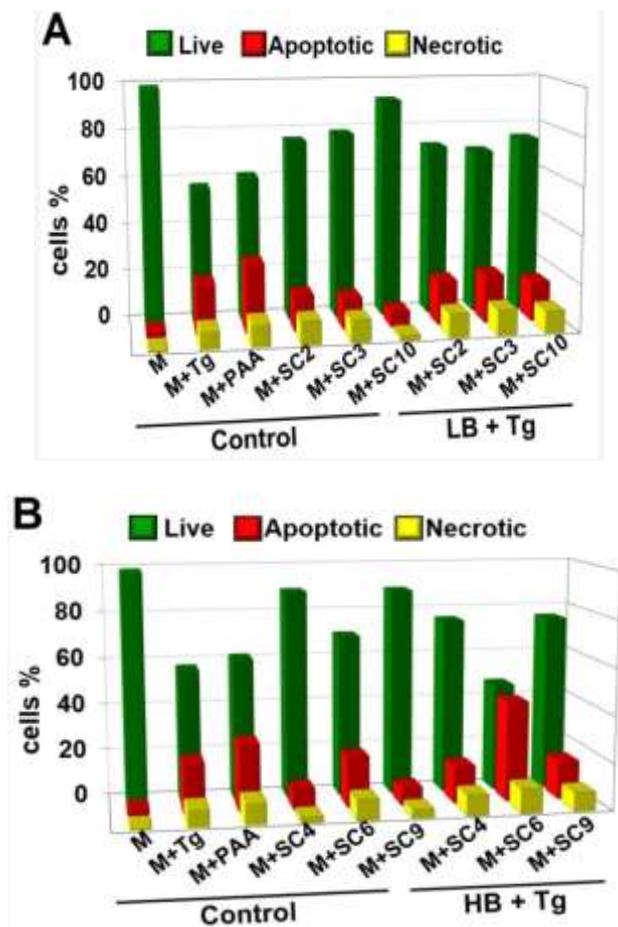


Fig. 6. FACS analysis of viable (green), apoptotic (blue) and necrotic (red) macrophages (M) exposed to *T. gondii* and A) LB SCs (M+SC2, SC3 and SC10+Tg) and B) HB SCs (M+SC4, SC6 and SC9+Tg) compared to untreated controls (M only, M+Tg, M+PAA, and M+SCs).

The effect of HB SC4, SC6, and SC9 on MV and their resistance during *T. gondii* infection were observed (Fig. 6B). FACS analysis demonstrated that SCs affected macrophage survival upon *T. gondii* exposure. *T. gondii* decreased MV from 99.4% (M) to 52.6% (M+Tg), SC4+Tg and SC9+Tg increased MV to 75.32%, 75.5% respectively. Whereas SC6 treatment of M exposed to Tg showed the lowest MV, 46.5%, it had highest apoptosis, 42.8%, and 10.4 % of necrosis. SC4 and SC9 decreased MV apoptosis from 21.7% to 15.8 and 16.9%, correspondingly.

V. DISCUSSION

SCs are implicated in many different biological events: intra- and intercellular transport, innate immunity, guidance and arrangement of cell-cell adhesion or movement and positive/negative growth control with repercussions for differentiation and malignancy [29]. A huge number of SCs structures have been identified in mammalian cells as well in parasites cell surfaces. These cell-surface molecules participate in cell physiology and play important roles in cell recognition, modulation of function of receptors and contribute in host-parasite interactions such as macrophage invasion [30].

The present research focused on exploiting different types of polymeric SCs that demonstrated binding affinity towards recognition of Tg.

Since NO is an important signaling molecule that acts in many tissues to regulate a variety of physiological procedures and plays vital role in the immune response against pathogen infection [31], the role of SCs and their effects on macrophage NO production was examined. Infected and non-infected cells which treated with SCs increased the level of NO production after six-eight hrs of incubation were compared to controls.

It was reported that NO lead to a complete death for *S. aureus*, MRSA, *E. coli*, Group B *Streptococcus*, *P. aeruginosa*, and *Candida albicans* within 4 hrs of exposure [32],[33]. Furthermore, it found that apoptosis in non-infected bystander host cells is because of the secretion of NO released by *T. gondii* infected cells [34]. In addition, NO has also been verified to be involved in the regulation of apoptosis [35] and being noticed in many different primary or immortalized murine or human cells [36], [37]

Therefore, SCs influences on cell death including apoptosis and necrosis induced by *T. gondii* were studied. *T. gondii* infection of macrophages induced macrophage cell death, while HB as well LB SCs promoted the viability of the infected macrophages, decreasing the necrosis by up to 10.6 and 8.07%.

Overall the results showed that SCs help to activate and promote cells viability against *T. gondii* and stimulated NO production while killing tachyzoites. SCs increased macrophage cell viability up to 75.5% and decreased apoptosis while the necrosis level showed $\leq 10\%$.

Intercellular communication may engage by particular carbohydrate motility binding to receptors on cell surface. Desired interactions could be promoted by specific types of intercellular interaction achieved by sugars [38].

The mechanism of *T. gondii*'s entry into the macrophages play very important role to the tachyzoites destiny. Live tachyzoites that actively invade the host cell develop a conventional a protective non-fusogenic parasitophorous vacuole (PV) [39]. Conversely, host cell phagocytosed the dead or opsonized parasites and targeted to lysosomal compartments for degradation [40]. Toxoplasma invasion occurs much more rapidly than phagocytosis [41]

The capability of *T. gondii* to live in the majority of nucleated cell types reduced when parasites are opsonized with specific antibody. In addition the respiratory burst raised when the mouse macrophage phagocyte antibody-coated parasites [42], rapid acidification, and fusion with endosomes/lysosomes [43].

Our data showed that despite their differences, both HB and LB exhibit a significant activate phagocytic cells (Fig. 5, 6). Prospective research foresees the analysis of structurally different SCs and their effects on macrophage, cyto-protection, and killing *T. gondii*.

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