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Trophoblast cells are able to regulate monocyte activity to control *Toxoplasma gondii* infection

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ABSTRACT

Introduction: Toxoplasma gondii is an intracellular parasite that causes severe disease when the infection occurs during pregnancy. Trophoblast cells constitute an important maternal–fetal barrier, with monocytes concentrating around them. Thus, interactions between trophoblasts and monocytes are important for maintaining a successful pregnancy, especially in cases of infection. This study aimed to evaluate the role of trophoblast cells (BeWo line) on monocyte (THP-1 line) activity in the presence or absence of *T. gondii* infection.
Methods: THP-1 cells were stimulated with supernatants of BeWo cells, previously infected or not with *T. gondii*, and then infected with parasites. The supernatant of both cells were collected and analyzed for cytokine production and *T. gondii* proliferation in THP-1 cells was determined.
Results: The results showed that after infection, the pattern of cytokines secreted by THP-1 and BeWo cells was characterized as a pro-inflammatory profile. Furthermore, supernatant of BeWo cells infected or not, was able to change the cytokine profile secreted by infected THP-1 cells, and this supernatant became THP-1 cells more able to control *T. gondii* proliferation than those that had not been stimulated.
Discussion: This effect was associated with secretion of interleukin (IL)-6 by the THP-1 cells and soluble factors secreted by BeWo cells, such as IL-6 and MIF.
Conclusion: Together, these results suggest that trophoblast cells are able to modulate monocyte activity, resulting in the control of *T. gondii* infection and subsequent maintenance of pregnancy.

1. Introduction

Toxoplasma gondii is the etiologic agent of toxoplasmosis, a disease that is usually asymptomatic in immunocompetent individuals, but can be severe in immunocompromised patients and cases of congenital toxoplasmosis [1,2]. The main defense mechanism against *T. gondii* is mediated by a T helper type 1 (Th1)-immune response characterized by secretion of pro-inflammatory cytokines [3]. During pregnancy, the maternal immune response shifts to a T helper type 2 (Th2) profile, with predominant secretion of anti-inflammatory cytokines, which allow fetal tolerance and maintenance of pregnancy [4,5]. However, this anti-inflammatory microenvironment becomes favorable to parasite replication, including *T. gondii* [6,7].

Several cellular components of the innate immune system are found at the site of embryo implantation. Monocytes constitute 20–30% of this population and remain present at high levels throughout pregnancy [8]. Previous studies demonstrated that trophoblast cells express pattern recognition receptors that recognize the presence of bacteria, viruses, parasites or dying cells, and then secrete cytokines and chemokines able to act on cells of the innate immune system present in the deciduas; for example, inducing the recruitment, differentiation and activity of monocytes [8,9]. Consequently, monocytes within the decidua accumulate around the invading trophoblast cells and acquire an activated phenotype that is important for the clearance of apoptotic cells and cellular debris, thus facilitating trophoblast

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migration during gestation [10–12]. This evidence suggests that the innate immune cells may play an important role in fine-tuning fetal–maternal immune responses and consequently in successful pregnancy.

Clinical studies, however, have established a strong association between pregnancy complications and intrauterine infections as a result of the interaction between trophoblast cells and innate immune cells [12,13]. Receptors present in trophoblast cells are able to recognize the presence of pathogens, followed by secretion of cytokines that act upon the innate immune cells within the decidua [14]. Therefore, the way that these cells interact with each other can be decisive to the tolerance of allogeneic fetus and the host defense against possible infections.

Monocytes are one of the major cells of the innate immune system responsible for the control as well as dissemination of *T. gondii* during the acute phase of infection, including at the maternal–fetal interface [15,16]. In this regard, our previous study verified that human villous explants from first trimester pregnancies expressed high levels of intercellular adhesion molecule 1 (ICAM-1) that favored increased adhesion of monocytes to trophoblast cells, favoring dissemination of the infection into the deep placental tissues [16]. Thus, this study aimed to investigate if factors secreted by trophoblasts influence cytokine production and *T. gondii* control by monocytes, through *in vitro* experiments using well established cell lines.

2. Materials and methods

2.1. Cell culture

BeWo and THP-1 cell lines obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium (Gibco, Paisley, UK), supplemented with 25 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin (all reagents from Sigma–Aldrich, St. Louis, MO, USA) and 10% heat-inactivated fetal calf serum (FCS) (Cultilab, Campinas, Brazil) – complete medium – in a humidified incubator at 37 °C with 5% CO_2 [17].

2.2. Parasites

Tachyzoites of the *T. gondii* 2F1 RH strain, which constitutively express cytoplasmic β-galactosidase, were a gift from Dr. Vern Carruthers, Medicine School of Michigan University (USA). The parasites were propagated in BeWo cells cultured in RPMI 1640 medium supplemented with penicillin, streptomycin and 2% FCS at 37 °C and 5% CO_2.

2.3. Treatment of THP-1 cells with supernatants of BeWo cells

BeWo cells were cultured in 96-well plates (1×10^5 cells/200 μL/well) in complete medium for 24 h at 37 °C and 5% CO_2. Cells were then infected with tachyzoites of *T. gondii* at a 3:1 (parasites: host cell) ratio or incubated only with medium (control). After incubation for 24 h at 37 °C and 5% CO_2, the plates were centrifuged (720 *g*, 10 min) and the cell-free supernatants were collected and stored at −80 °C until used for cytokine detection and treatment of THP-1 cells.

THP-1 cells were cultured in 96-well plates (1×110^5 cells/200 μL/well) in complete medium for 24 h at 37 °C and 5% CO_2 and then treated with the uninfected or infected BeWo supernatants (conditioned medium). As a control, THP-1 cells were cultured only with complete medium. After 24 h of treatment, the cell supernatants were removed and complete medium was added to the wells. THP-1 cells were then infected with tachyzoites (3:1; parasites: cells) and incubated for 24 h at 37 °C and 5% CO_2. The plates were centrifuged (720 *g*, 10 min) and the cell-free supernatants were collected and stored at −80 °C for cytokine detection.

2.4. Measurement of cytokines secreted by THP-1 and BeWo cells

2.4.1. Enzyme-linked immunosorbent assay (ELISA)

Human cytokines (IL-12, TGF-β1 and MIF) were measured in the supernatants of THP-1 and BeWo cells by sandwich ELISAs according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The sensitivity limits of these assays were 7.8 pg/mL for IL-12, and 25 pg/mL for TGF-β1 and MIF.

2.4.2. Cytometric bead array (CBA)

Human cytokines (IL-2, IFN-γ, TNF-α, IL-6, IL-4, IL-10 and IL-17A) were measured in the supernatants of THP-1 and BeWo cells by cytometric bead array™ (CBA, BD Biosciences, San Jose, CA, USA), using the Th1/Th2/Th17 kit (BD Bioscience), according to the manufacturer's instructions. The samples were analyzed under BD™ flow cytometry (FACSCalibur, BD Company, San Diego, CA, USA) and the data were calculated using a specialized software (BD™ Cell Quest and CBA software).

2.5. T. gondii proliferation assay in THP-1 cells

THP-1 cells were cultured in 96-well plates (2×10^4 cells/200 μL/well) at 37 °C and 5% CO_2 for 24 h and then stimulated with the uninfected or infected BeWo supernatants or with complete medium alone for 24 h. Subsequently, the supernatants were removed and the THP-1 cells were infected with tachyzoites of *T. gondii* (3:1; parasites: cell) and incubated for 24 h at 37 °C and 5% CO_2. The plates were centrifuged (720 *g*, 10 min), the supernatants removed and the cells analyzed for *T. gondii* intracellular proliferation using a colorimetric β-galactosidase assay as previously described [18].

In a second set of experiments, THP-1 cells were cultured for 24 h as described above. Next, *T. gondii* tachyzoites were added to cells (3:1) and, after 4 h of infection, the cells were washed and treated with polyclonal rabbit anti-human IL-6 antibody (Peprotech Inc., RockyHill, USA) at 10 μg/mL. As controls, infected cells were treated with medium or 10 μg/mL of an irrelevant polyclonal rabbit IgG antibody (Jackson Immuno Research, West Grove, USA). After 24 h of treatment *T. gondii* intracellular proliferation was determined [18].

In another set of experiments, THP-1 cells were cultured into 96-well plates (2×10^4 cells/200 μL/well) for 24 h at 37 °C and 5% CO_2. The cells were then stimulated with supernatants of BeWo cells, infected or not, previously neutralized with 10 μg/mL of anti-IL-6 (Peprotech), anti-MIF (R&D Systems) or anti-TGF-β1 (R&D Systems) antibodies for 2 h at 37 °C and 5% CO_2. After 24 h of stimulation, THP-1 cells were infected with *T. gondii* tachyzoites (3:1) for additional 24 h. As controls, THP-1 cells were stimulated with non-neutralized BeWo cell supernatant or neutralized with 10 μg/mL of an irrelevant polyclonal rabbit IgG antibody (Jackson Immuno Research), and then infected with *T. gondii*. After 24 h of infection, *T. gondii* intracellular proliferation was determined [18].

Finally, THP-1 cells were cultured into 96-well plates (2×10^4 cells/200 μL/well) for 24 h at 37 °C and 5% CO_2. Cells were washed three times with in complete medium and then treated separately with human recombinant cytokines: rIL-6 (PeproTech) at 25, 50 and 100 pg/mL, rMIF (BDBioscience) at 2000, 4000 and 8000 pg/mL and rTGF-β1 (R&D Systems) at 500, 1000 and 2000 pg/mL for additional 24 h at 37 °C and 5% CO_2. Next, *T. gondii* tachyzoites were added to the cells (3:1) and, after 3 h of infection, the cells were washed with medium and again treated with the cytokines in the same concentrations. As controls, cells were infected and treated with medium only. After 24 h of treatment, parasite proliferation was measured [18].

T. gondii intracellular proliferation data were expressed as number of tachyzoites calculated in relation to the reference curve of 2F1 strain tachyzoites, ranging from 1×10^6 to 15.625×10^3 total parasites.

2.6. Analysis of cytokine signatures

Complementary "non-conventional" analysis of cytokine data was applied to evaluate the cytokine profile in the supernatant from each experimental condition, using the general concept of "low" and "high" cytokine levels as previously proposed by Luiza-Silva et al. [19]. For this purpose, the whole data universe of cytokine levels in the supernatants (pg/mL) recorded for all experimental groups was used to calculate the global median value for each cytokine. The global median values were used as the cut-off edge to tag each individual supernatant as it presents "low" or "high" levels of cytokines. Following, the frequency (%) of supernatant showing "high levels of cytokine" (above the global median cut-off) was calculated in order to determine the percentage of experimental conditions displaying high levels of cytokines. The "cytokine signature" for each experimental condition was then assembled as an ascendant curve of frequencies of high cytokine levels for each experimental condition. The comparative analysis of cytokine signatures was performed using the 50th percentile as a limit to identify the cytokines considered relevant on each experimental condition. In order to highlight the relevant differences between experimental groups, we have used gray rectangles to stretch the distinctive cytokines icon from each cytokine signature. This approach showed to be relevant to detect subtle changes in the cytokine signatures not detectable by conventional statistical approaches. The value of this non-conventional "qualitative-observational" method was emphasized during data analysis, as the patterns of cytokine signature were consistent with the parasite growth observed in parallel experiments.

2.7. Statistical analyses

Statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). Data were expressed as mean ± standard deviation (SD) of two to three independent experiments performed in triplicate. Data were compared using the Student's *t* test. Differences were considered statistically significant when $P < 0.05$.