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Polarisation of human macrophages towards an M1 subtype triggered by an atypical Brazilian strain of *Toxoplasma gondii* results in a reduction in parasite burden

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Abstract: *Toxoplasma gondii* Nicolle et Manceaux, 1909, the etiologic agent of toxoplasmosis, was considered a clonal population with three distinct genetic lineages (I, II and III); however, sequence analysis of different strains has revealed distinct atypical genotypes. Macrophages are essential for immunity against toxoplasmosis and differential cell regulation may affect the course of the disease. In this context, our study aims to investigate the infection by TgChBrUD2, a highly virulent atypical Brazilian strain of *T. gondii*, on the activation and polarisation of human macrophages. Human macrophage-like cells obtained from THP-1 cells were infected with TgChBrUD2, RH or ME49 strains of *T. gondii* to evaluate the impact of parasite infection on macrophage polarisation. Our results indicate that the TgChBrUD2 and ME49 strains of *T. gondii* induced a classic activation of human macrophages, which was confirmed by the high rate of spindle-shaped macrophages, low amount of urea and increase in the levels of nitrite, as well as the down-regulation of M2-markers. In contrast, RH strain promoted an alternative activation of macrophages. The polarisation of human macrophages towards an M1 subtype mediated by TgChBrUD2 and ME49 strains resulted in a low parasite burden, with high levels of IL-6 and MIF. Finally, the M2 subtype triggered by the RH strain culminated in a lower intracellular proliferation index. We concluded that the atypical (TgChBrUD2) and clonal (ME49) strains are able to elicit an M1 subtype, which results in parasitism control, partially explained by the high levels of IL-6 and MIF produced during the infection by these genotypes. In contrast, the clonal (RH) strain promoted a macrophage polarisation towards an M2 subtype, marked by a high parasite burden, with a weak modulation of pro-inflammatory cytokines. Thus, atypical strains can present different mechanisms of pathogenicity and transmissibility compared to clonal strains, as well as they can use distinct strategies to evade the host's immune response and ensure their survival.

Keywords: clonal strains, macrophage polarisation, toxoplasmosis, TgChBrUD2 strain

Toxoplasmosis is caused by the obligate intracellular protozoan parasite *Toxoplasma gondii* Nicolle et Manceaux, 1909, and it is one of the most common zoonotic food-borne infections (Dubey and Jones 2008). Epidemiological surveys show that *T. gondii* chronically infects approximately 30% of the global human population (Dubey et al. 2012, Jensen et al. 2015, Bigna and Tochie 2019). The prevalence of toxoplasmosis in humans is highly variable not only among countries, but also among regions within one country, since it is influenced by socioeconomic and environmental conditions, as well as cultural habits (De Barros et al. 2022, Stopić et al. 2022). Although, the seroprevalence has been decreasing globally over the past decades, is still very common in many countries (Stopić et al. 2022).

Primary infection with *T. gondii* usually results in mild or nonspecific symptoms in healthy individuals. However, as an opportunistic human pathogen, primary infection or

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reactivation of chronic infection can cause severe clinical manifestations in immunocompromised individuals such as toxoplasmic encephalitis, myocarditis and pneumonitis (Montoya and Liesenfeld 2004, Saadatnia and Golkar 2012). In addition, parasitic infection acquired during pregnancy or in the time around conception can result in the vertical transmission of *T. gondii* tachyzoites, and cause severe sequelae in infected foetuses and newborns, resulting in miscarriage, stillbirth, retinochoroiditis, intracranial calcification, hydrocephalus and cognitive disability, among others (Hampton 2015, Aguirre et al. 2019).

Early studies on the range of infection classified *T. gondii* to three genetic lineages (I, II and III), which are predominantly observed in Europe and North America (Yarovinsky 2008, Halonen and Weiss 2013). Regarding the virulence phenotypes of these lineages in murine models, the type I strains are uniformly lethal, whereas types II and III strains possess moderate or low virulence (Howe and Sibley 1995, Yarovinsky 2008). Interestingly, genetic analyses of *T. gondii* strains have revealed a fourth clonal lineage in North America, which displays intermediate or high levels of acute virulence in mice (Dubey et al. 2011, Khan et al. 2011). In contrast, *T. gondii* genotypes from other parts of the world, which do not fit the three dominant lineages, have been referred to as "atypical" strains (Pena et al. 2008, Su et al. 2012, Shwab et al. 2013).

Besides the lack of a clonal population structure and high genetic diversity, some atypical genotypes have the capacity to cause severe infection compared to typical strains (Ferreira et al. 2011, Jensen et al. 2015). This suggests that these genotypes can differ in pathogenicity and possibly in transmissibility (Grigg et al. 2001, Vallochi et al. 2005, Khan 2006, Campos et al. 2008, Sauer et al. 2011, Carneiro et al. 2013).

The results from several studies involving the analysis of *T. gondii* isolates from animal samples from different geographical areas of Brazil have shown the presence of four major genotypes, referred to as BrI, BrII, BrIII and BrIV, which are characterised by high genetic variability (Pena et al. 2008, Dubey et al. 2012). Our research group has recently isolated and characterised two atypical Brazilian strains of *T. gondii* obtained from heart tissue samples of free-range chickens (Lopes et al. 2016). These two isolates were named as TgChBrUD1 (genotype 11, type BrII) and TgChBrUD2 (genotype 6, type BrI and Africa 1) (Lopes et al. 2016). Our previous investigation suggests that both isolates are highly virulent in murine models, with TgCHBrUD2 being more virulent than the TgCHBrUD1 isolate (Franco et al. 2014, Lopes et al. 2016).

Regarding the host immune response, macrophages play an essential role in the early immune response against *T. gondii*, and are considered one of the most common cell line infected by the parasite *in vivo* (Wang et al. 2020). Macrophages have been considered essential effector cells during toxoplasmosis due to their ability to kill parasites and produce cytokines and chemokines essentials for a protective immune response (Dunay et al. 2008, Park and Hunter 2020). The infection of macrophages with different clonal or atypical strains of *T. gondii* can elicit two distinct profiles of polarisation: classically-activated macrophages (pro-inflammatory M1 macrophages) or alternatively-activated macrophages (anti-inflammatory M2 macrophages), which differ in expression of surface markers, cytokine production and effector functions (Jensen et al. 2011, Melo et al. 2011, Liu et al. 2013, Italiani and Boraschi 2014, Kong et al. 2015, Fox et al. 2019, Mukhopadhyay et al. 2020). The phenotypic characterisation of M1 macrophages involves the expression of the membrane marker CD64, whereas M2 macrophages can be characterised by the expression of CD206 and CD163 (Comalada et al. 2012, Orecchioni et al. 2019, Yao et al. 2019). In addition, M1 profile is marked by nitric oxide production and M2 profile is related with urea, a subproduct of the enzyme arginase (Mantovani et al. 2004, Yao et al. 2019).

The ability of *T. gondii* to modulate specific macrophage activation pathways has been associated with virulence, parasitic load and pathology during toxoplasmosis (Jensen et al. 2011, Kong et al. 2015). In addition, multiple reports in literature reinforce the concept that the severity of human toxoplasmosis is influenced by the parasite burden, parasite genotype, geographic location and host immune response (Campos et al. 2008, Dardé 2008, Carneiro et al. 2013, Jensen et al. 2015, Rico-Torres et al. 2016). In this sense, the present study aimed to investigate the infection by TgChBrUD2, a highly virulent atypical Brazilian strain of *T. gondii*, on the activation and polarisation of human macrophages, and offer new insights into the underlying mechanisms triggered by this atypical strain during the development of toxoplasmosis.

MATERIAL AND METHODS

Cell culture and parasite strains

Human choriocarcinoma-derived trophoblastic cells (BeWo lineage) and human monocyte-like cell line (THP-1 lineage) were commercially obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI 1640 medium (Cultilab, Campinas, SP, Brazil) supplemented with 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Sigma-Aldrich, St. Louis, MO, USA) and 10% fetal bovine serum (FBS) at 37 °C under a humidified atmosphere containing 5% CO_2 (Castro et al. 2013). *Toxoplasma gondii* tachyzoites from TgChBrUD2 (genotype 6, type BrI, and Africa 1), ME49 (type II) or RH (type I) strains were maintained by serial passages in BeWo cells cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2% FBS at 37 ºC and 5% CO_2 (Angeloni et al. 2013, Franco et al. 2015).

Differentiation of THP-1 cells into macrophage-like cells

Human macrophage-like cells were obtained by chemical stimulation and activation of THP-1 cells. Briefly, the culture supernatant containing THP-1 cells was centrifuged (400× *g*, 5 min) and the pellet with the cells was counted in Neubauer camera using Trypan blue viability exclusion. Next, cells were adjusted to 5×10^6 and added into 75 cm^2 flasks culture containing 15 ml of RPMI 1640 medium with 10% FBS at 37 ºC, 5% CO_2 ,and 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Biogen Com. Dist. Ltda, Sumarezinho, SP, Brazil) for 48 h (Park et al. 2007). The

criteria for differentiation of THP-1 cells into macrophages were cell adherence to the flasks and morphological changes (Reyes et al. 1999). Adherent cells obtained as described above were used for all further experiments.

Infection of macrophages by *Toxoplasma gondii* strains

TPH-1 differentiated macrophages were trypsinised and replated in 24-well culture plates (4×10^5 cells/210 µl/well) in complete medium for 24 h at 37 ºC and 5% CO_2. The cells were then infected with *T. gondii* tachyzoites from TgChBrUD2, RH, or ME49 strains at 2 : 1 ratio (parasites: host cells) for 24 h at 37 ºC and 5% CO_2. Non-infected cells were used as controls. Next, the plates were centrifuged (400 × *g*, 5 min) and the cell-free supernatants were collected and stored at -80 ºC for posterior cytokine analysis and nitrite measurement. In parallel, cells were collected for urea quantification, *T. gondii* intracellular proliferation or to analyse the expression of cell surface markers.

Cytokine, nitrite and urea determination

Cytokine release in the culture supernatants was accessed by using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). Assays for IL-10, IL-6, IL12p40, TNF-α, IFN-γ, TGF-β1 (OpTEIA, BD Bioscience, San Diego, CA, USA), and MIF (Duoset R&D Systems, Minneapolis, MN, USA) were performed according to the manufacturer's instructions. A microplate reader (Titertek Multiskan Plus, Flow Laboratories, McLean, VA, USA) was used for absobance measurements at 450 nm, and cytokine concentrations were expressed in pg/ml in comparison with a standard curve. The limits of detection of each cytokine were: MIF (62.5 pg/ml), IL-10 (7.8 pg/ml), IL-6 (4.7 pg/ml), TNF (7.8 pg/ml), IFN-γ (4.7 pg/ml); TGF-β1 (125 pg/ml), and IL12p40 (31.25 pg/ml).

Additionally, cell supernatants were subjected to the Griess method for nitrite measurement (Green et al. 1982). Briefly, cell supernatants were added into 96-well plates and mixed 1 : 1 with 1% sulfanilamide dihydrochloride and 0.1% naphthylenediamide dihydrochloride in 2.5% H_3PO_4. Optical density was measured at 570 nm with a microplate reader. Sample concentration was obtained by comparison with a standard curve of sodium nitrite with concentrations ranging from 5 to 200 µM/ml.

Detection of urea production in cell lysate was used as an indicator of arginase activity. Briefly, the macrophages were lysed in radioimmunoprecipitation assay buffer (RIPA) [50 mmol Tris, 150 mmol NaCl, Triton X-100 (1%), sodium deoxycholate (1%) and SDS (0.1%); pH 7.5] added cocktail of protease inhibitors (Complete®, Roche Diagnostic, Mannheim, Germany). The cell homogenate was centrifuged for 30 minutes at 15,000 × *g* at 4 °C. The supernatants were used for Urea UV Liquiform kit detection (Labtest, Lagoa Santa, Minas Gerais, Brazil). Replicate samples were processed in 96-well-plate format assays for the determination of urea concentration (mg/dl), according to the manufacturer's instructions. Absorbances were measured on a microplate reader at 340 nm and 37 ± 0.2 °C. The arginase activity was determined by the amount of detected urea.

***Toxoplasma gondii* intracellular proliferation assay: quantitative real-time PCR**

After 24 h of *T. gondii* infection, supernatants were removed and the total DNA was extracted from human macrophages using Wizard® Genomic DNA Purification Kit (Promega Co., Madison, WI, USA), according to the manufacturer's instructions. Total DNA was quantified by UV spectrophotometry (NanoDrop Lite Spectrophotometer; Thermo Scientific, Waltham, MA, USA). Real-time PCR was performed with a StepOnePlus® Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and Go Taq® quantitative real-time PCR (qPCR) Master Mix Kit (Promega Co.), according to the manufacturer's instructions. The reaction conditions followed the protocols of Wahab et al. (2009). The primers (forward, 5′-CACAGAAGGGACAGAAGT-3′ and reverse, 5′-TCGCCTTCATCTACAGTC-3′) were used for amplification of a repetitive area of 529 bp in *T. gondii* DNA. Positive and negative parasite controls were included in each assay. PCR reaction was performed with 200 ng of DNA target, and 100 ng of DNA standard curve was concomitantly included on each reaction. The threshold cycle (CT) value for each sample was compared to the standard control and the parasite quantity was analysed. The data were presented in *T. gondii* DNA (100 ng/µl).

Macrophage morphology

For examination of the macrophage morphology, the macrophages were cultured on round glass coverslips 13 mm diameter in 24-well plates (4×10^5 cells/210 µl/well) in complete medium for 24 h at 37 ºC and 5% CO_2. Then, the cells were infected or not with *T. gondii* tachyzoites from TgChBrUD2, RH or ME49 strains at 2 : 1 ratio for an additional 24 h. Next, we removed the supernatant, and the cells were fixed in 10% buffered formalin phosphate for 24 h. Subsequently, the coverslips with adhered macrophages were stained with 1% toluidine blue (Sigma) for 10 seconds. The coverslips were mounted with Entellan on glass slides. Cells were analysed in light microscopy using an Olympus BX51 and Olympus DP70 (Olympus, Shinjuku, Tokyo, Japan). The number of round and spindle-shaped cells was quantified in control (uninfected) and infected cells (TgChBrUD2-, RH- or ME49-infected macrophages). The coverslips with macrophages were divided into four quadrants. In a blind test, 100 cells were counted for each quadrant at a total magnification of 400×. The experiments were performed in triplicate.

CD64, CD163 and CD206 expression in macrophages by flow cytometry

Macrophages infected or not with *T. gondii* tachyzoites were incubated with allophycocyanin conjugated anti-CD64 antibody, phycoerythrin-conjugated anti-CD206 antibody, fluorescein and isothiocyanate anti-CD163 antibody all (Sigma-Aldrich), diluted in PBS 1x (1 : 100) for 30 min at 4 ºC. As control, an irrelevant isotype control mouse IgG was added (Milipore, São Paulo, SP, Brasil). Next, cells were rinsed in PBS 1x and fixed with paraformaldehyde (10%) and sodium cacodylate (0.1 M). Fluorescence of 20,000 cells in each experimental condition in triplicate was analysed using FACS Canto II, BD Company. The expression of cell surface markers was expressed as percentage of positive cells (%).

Statistical analysis

GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) was used for plotting the results and performing statistical analysis. All data are expressed as mean ± standard error of the mean (SEM), and the differences between groups were determined

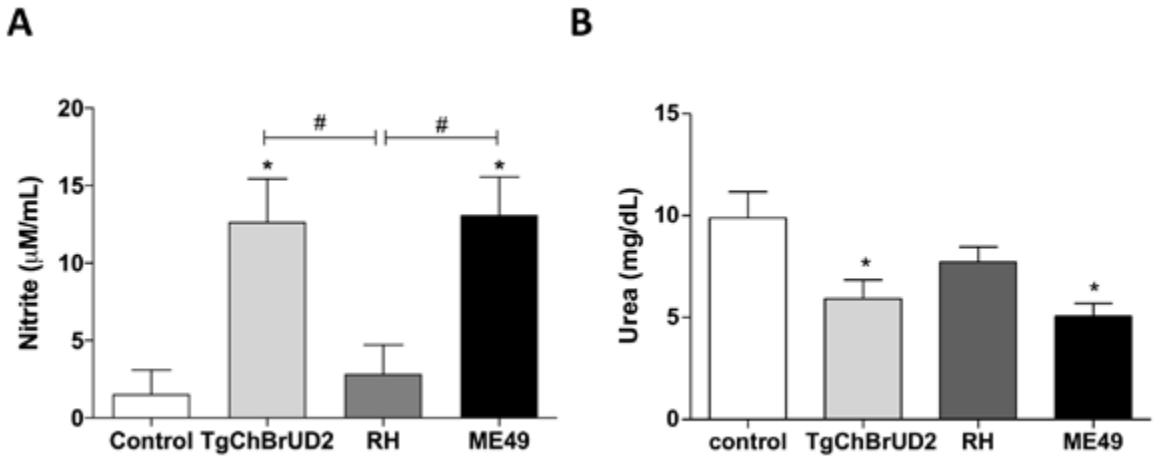

Fig. 1. Production of nitrite and urea by human macrophages infected or not with *Toxoplasma gondii* tachyzoites from TgChBrUD2, RH, or ME49 strains. THP-1 cells were differentiated into macrophages, cultured in 24-well plates for 24 h, infected or not by *T. gondii* tachyzoites (TgChBrUD2, RH or ME49 strains) for 24 h and the supernatant collected by posterior nitrite measurement for Griess assay (**A**). In parallel, cells were lysed by posterior urea measurement. The urea concentration was measured by the kit UREA UV Liquiform and results expressed in mg/dL (**B**). The results were expressed as mean ± SEM of three independent experiments performed in triplicate. *Comparison to uninfected control. #Comparison between TgChBrUD2 and ME49 with RH-infected macrophages. Significant differences were tested using Kruskal-Wallis and 'Dunn's multiple comparison test. Differences were considered significant when $P < 0.05$.

using ANOVA with multiple comparisons by the Bonferroni method in parametric tests. Non-parametric pairwise multiple comparisons were performed using the Kruskal-Wallis test and Dunn's test. Differences are considered statistically significant when $P < 0.05$.

RESULTS

Toxoplasma gondii strains specifically modulate nitrite and urea production in human macrophages

We measured nitrite production in the supernatant of macrophages infected or not with different strains of *Toxoplasma gondii*. Our results showed an increase in nitrite production in TgChBrUD2 and ME-49-infected macrophages in comparison to uninfected cells ($^{*}P < 0.05$) (Fig. 1A). Interestingly, the levels of nitrite produced by macrophages during TgChBrUD2 and ME49 infection were significantly higher than those induced by RH infection ($^{\#}P < 0.05$) (Fig. 1A). In addition, RH- or ME49-infected macrophages did not show any significant difference in nitrite release in relation to control, although a tendency of nitrite upregulation was observed during infection by ME49 strain (Fig. 1A).

The arginase activity was analysed by levels of urea released by macrophages infected or not by *T. gondii*. The results showed a reduction in the urea production by macrophages infected with TgChBrUD2 or ME49 strains compared to uninfected control ($^{*}P < 0.05$) (Fig. 1B). In addition, RH strain did not modulate the urea production by human macrophages (Fig. 1B).

Clonal and atypical *T. gondii* strains distinctly alter macrophage morphology

Morphology of adherent macrophages was assessed 24 h after infection by atypical and clonal *T. gondii* strains. Regarding the uninfected group, the number of round macrophages was significantly higher compared to the number of spindle-shaped ($^{*}P < 0.05$) (Fig. 2A). Furthermore, the number of uninfected macrophages with round morphology was higher when compared to round macrophages infected with TgChBrUD2 or RH ($^{\#}P < 0.05$) (Fig. 2A). TgChBrUD2 infection caused a high number of spindle-shaped macrophages and a low number of round cells ($^{*}P < 0.05$); in addition, the number of spindle-shaped macrophages infected with this strain was higher than in the uninfected group ($^{\&}P < 0.05$) (Fig. 2A). The infection by RH or ME49 strains did not show any statistical difference between the rounded or spindle-shaped morphology rates of macrophages (Fig. 2A). Illustrative photomicrographs of uninfected cells, macrophages infected with the TgChBrUD2, RH, or ME49 strains are shown in Fig. 2B-E, respectively.

TgChBrUD2- infected macrophages present a low cell surface expression of M2-markers

In order to corroborate our previous results, we also investigated the cell surface expression of CD64 (M1 macrophage marker), CD163 and CD206 (both M2 macrophage markers). Uninfected macrophages had a lower expression of both CD163 and CD206 compared to the percentage of CD64 ($^{*}P < 0.05$) (Fig. 3). Similarly, the infection by TgChBrUD2 and ME49 strains resulted in decreasing expression of CD163 and CD206 in comparison with CD64 levels ($^{*}P < 0.05$) (Fig. 3). In addition, RH-infected macrophages had a lower cell surface expression of CD163 compared to CD64 expression ($^{*}P < 0.05$) and presented higher expression of CD206 compared to the percentage of CD163 ($^{\&}P < 0.05$) (Fig. 3).

Human macrophages infected with both TgChBrUD2 and ME49 strains present a lower parasite burden

In order to evaluate possible differences between the parasite burden in macrophages infected with atypical

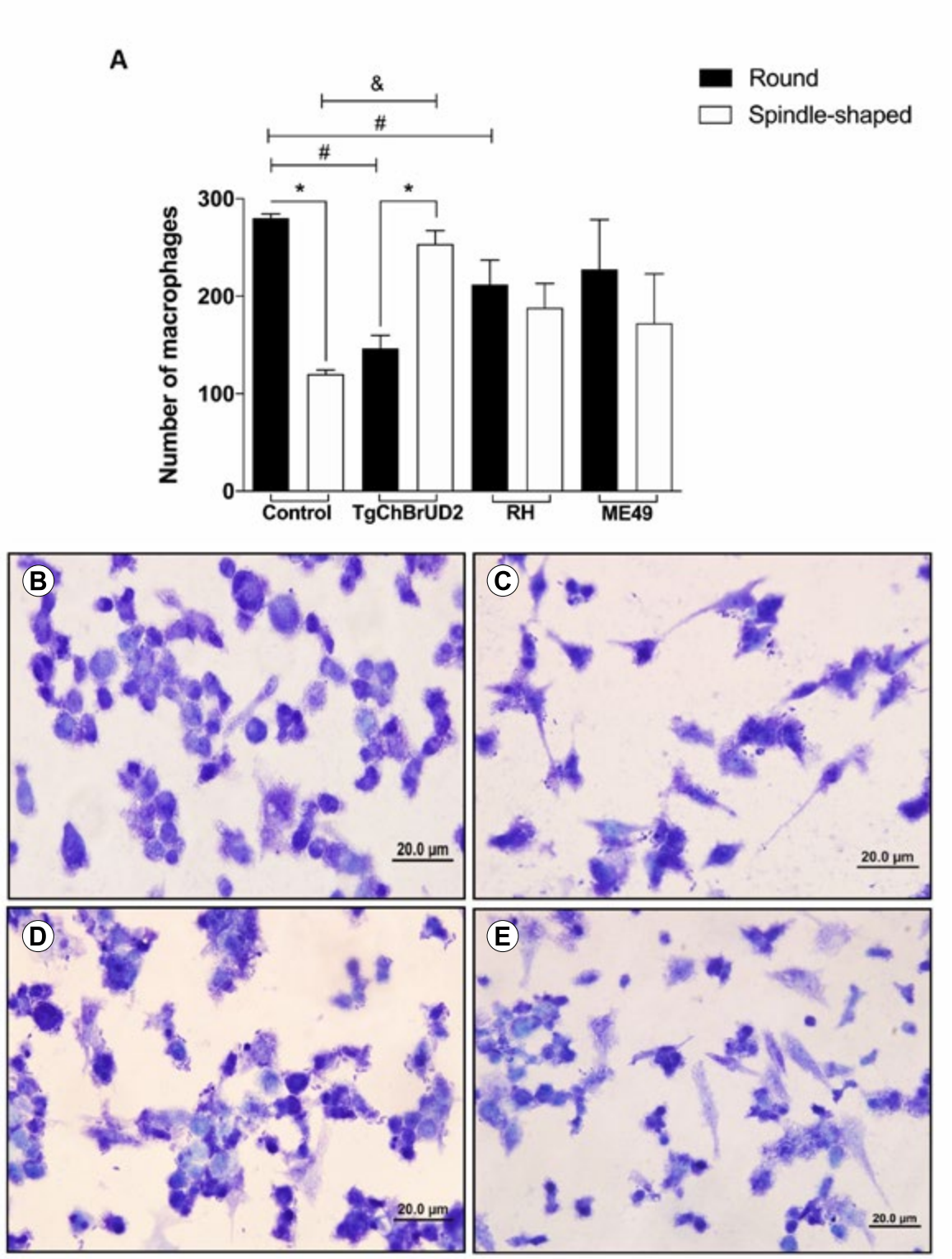

Fig. 2. Analysis of the morphology of human macrophages infected or not by *Toxoplasma gondii*. Human macrophages were cultured on 13-mm round glass coverslips into 24-well plates (4×10^5 cells/210µl/well), infected or nor with *T. gondii* tachyzoites from TgChBrUD2, RH, or ME49 strains and staining by toluidine blue. Quantification of uninfected and infected macrophages with round and spindle-shaped (**A**). Photomicrographs of uninfected macrophages (**B**) or infected by TgChBrUD2 (**C**), RH (**D**) or ME49 (**E**) strains. Total magnification of 400× is shown. Cells were analized by photomicroscope coupled to a camera (Olympus BX51 and Olympus DP70; Olympus, Shinjuku, Tokyo, Japan). The photomicrographs are representative of an experiment performed in triplicate. *Comparison between round and spindle-shaped macrophages. #,&Comparison between different experimental conditions indicated by connector lines. The experiment was performed in triplicate. Significant differences were analyzed using Kruskal-Wallis and Dunn's multiple comparison test. Differences were considered significant when $P < 0.05$. Data were obtained by analyzing four quadrants of coverslips. In a blind test, 100 cells were counted for each quadrant microscope.

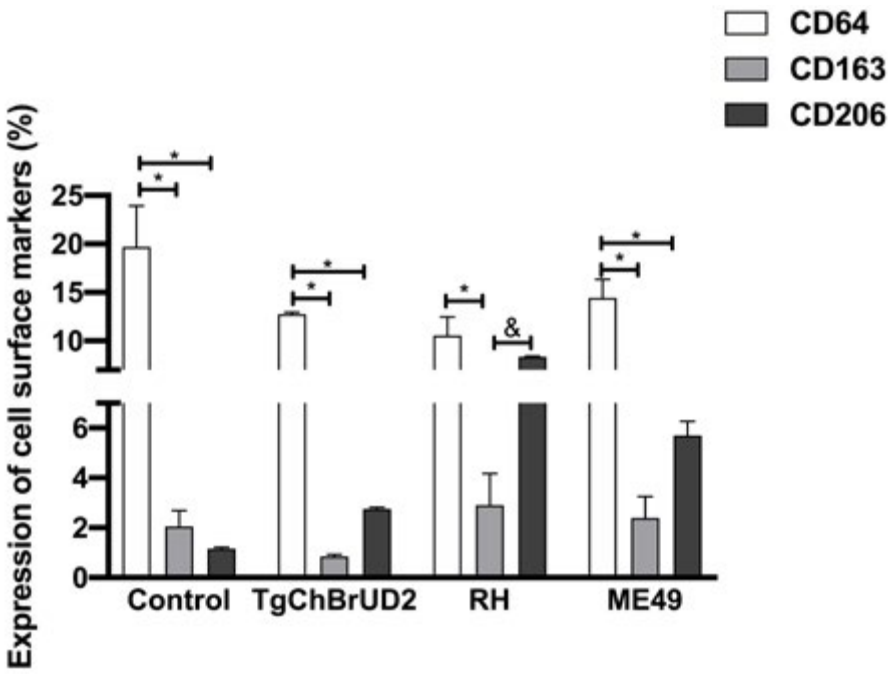

Fig. 3. Expression of the cell surface markers CD64, CD163 and CD206. The macrophages from each experimental condition were incubated with allophycocyanin-conjugated anti-CD64 antibody, phycoerythrin-conjugated anti-CD206 antibody, fluorescein and isothiocyanate anti-CD163 antibody (all Sigma-Aldrich). Irrelevant isotype Mouse IgG was used as control. The analysis was performed by flow cytometry. Data were expressed as mean ± SEM of two independent experiments performed in triplicate. *Comparison to the percentage of CD64. &Comparison to the percentage of CD163. Significant differences were analyzed using two-way ANOVA and Tukey's multiple comparisons test. Differences were considered significant when $P < 0.05$.

and clonal strains, *T. gondii* intracellular proliferation was quantified by real-time PCR. Our results showed that RH strain promoted higher parasite intracellular proliferation in macrophages in comparison to cells infected by TgChBrUD2 or ME49 strains ($^{\#}P < 0.05$) (Fig. 4A). Representative photomicrographs show the parasite burden in macrophages infected with TgChBrUD2 (Fig. 4B), RH (Fig. 4C) and ME49 (Fig. 4D).

Atypical and clonal *T. gondii* strains upregulate MIF and downregulate IL-10, while TgChBrUD2 strain induces a strong IL-6 response in human macrophages

Data analysis demonstrated that macrophages infected with *T. gondii* tachyzoites from TgChBrUD2, RH or ME49 strains significantly secreted low levels of IL-10 than the uninfected group ($^{*}P < 0.05$) (Fig. 5A). In contrast, infection with all investigated strains promoted an increase of MIF production when compared to the control group (uninfected cells) ($^{*}P < 0.05$) (Fig. 5B). ME49-infected macrophages produced higher levels of MIF in comparison with TgChBrUD2- or RH-infected macrophages ($^{\#}P < 0.05$) (Fig. 5B). Upregulation of IL-6 is seen in macrophages infected with TgChBrUD2 strain compared to the uninfected cells ($^{*}P < 0.05$) (Fig. 5C). In addition, TgChBrUD2-infected macrophages secreted higher amounts for IL-6 than RH or ME49 strains ($^{\#}P < 0.05$) (Fig. 5C). Furthermore, we also analyse the levels of TGF-β1 (Fig. 5D), TNF-α (Fig. 5E) and IFN-γ (Fig. 5F), and found no significant differences under any experimental condition. IL12p40 cytokine showed no detectable level (data not shown).

DISCUSSION

Due to significant advances in molecular tools, we can better conduct characterisation of different strain types and their distribution worldwide (Shwab et al. 2013). Thus, in addition to the strains belonging to the three classic dominant lineages, many distinct genotype patterns of *Toxoplasma gondii* strains have been described, especially in Central and South America (Sibley and Ajioka 2008, Rajendran et al. 2012, Shwab et al. 2013).

Literature reports show that atypical strains in Brazil, Europe and North America may be associated with the most severe cases of human toxoplasmosis, evidencing the existence of a broad spectrum of variations in virulence, host immune response, pathogenicity, and transmissibility mediated by atypical isolated strains (Grigg et al. 2001, Vallochi et al. 2005, Khan 2006, Campos et al. 2008, Sauer et al. 2011, Carneiro et al. 2013). The exact mechanism triggered by atypical strains in the course of infection and their interaction with the host's immune components is complex and remains unclear. The present study investigated the impacts of *T. gondii* infection of TgChBrUD2 strain, a highly virulent atypical Brazilian strain, on the activation and polarisation of human macrophages.

Initially, to characterise the macrophages profile induced by *T. gondii* infection, we measured the levels of nitrite and urea. Our data demonstrated that TgChBrUD2- and ME49-infected macrophages had an increase of nitrite and decrease of urea production; in contrast, the infection by RH strain resulted in low levels of nitrite and tended to produce large amounts of urea. Based on the literature,

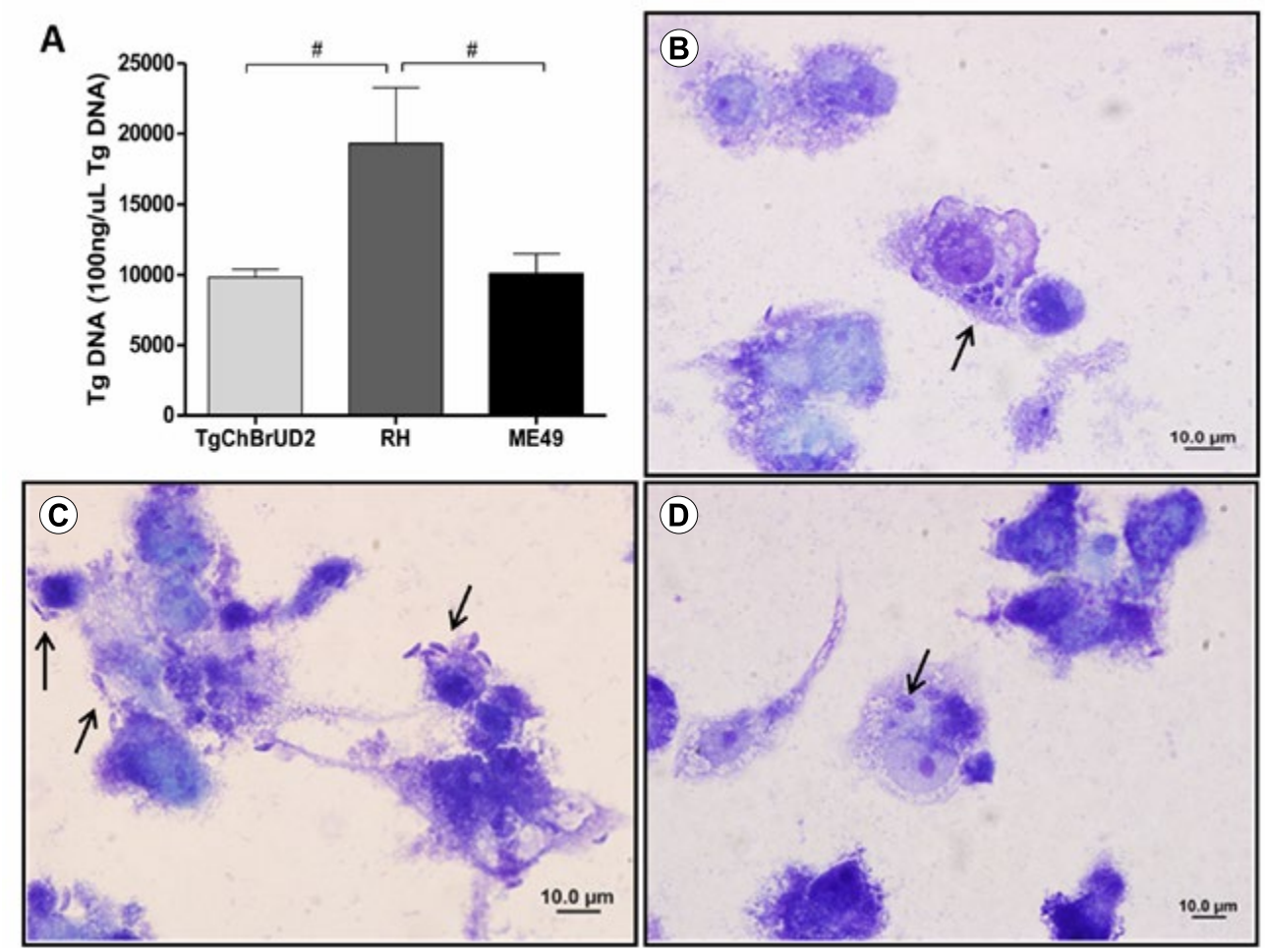

Fig. 4. *Toxoplasma gondii* intracellular proliferation in human macrophages infected with TgChBrUD2, RH, or ME49 strains. THP-1 cells were differentiated into macrophages, cultured in 24-well plates for 24 h, infected or not by *T. gondii* (TgChBrUD2, RH or ME49 strains) for 24 h and the cells collected by posterior analisis of the parasite intracellular proliferation by real-time PCR (**A**). In parallel, the macrophages were cultured on 13-mm round glass coverslips into 24-well plates (4×10^5 cells/210µl/well), infected or not with *T. gondii* tachyzoites from TgChBrUD2, RH, or ME49 strains at a 2 : 1 (parasites: host cell) and staining by toluidine blue. Illustrative photomicrographs of TgChBrUD2- (**B**), RH- (**C**) and ME49-infected macrophages (**D**) of one experiment performed in triplicate. The results were expressed as mean ± SEM of three independent experiments performed in triplicate. #Comparison to RH-infected macrophages. Significant differences were analyzed using one-way ANOVA and 'Bonferroni's multiple comparisons test. Differences were considered significant when $P < 0.05$.

it is widely known that urea, a subproduct of the enzyme arginase, is one of the most common markers of M2-type polarisation, while nitric oxide and subproducts are characteristics of the M1 macrophage profile (Murray and Wynn 2011, Mills 2012). Interestingly, it has been reported that macrophages can be alternatively activated by an independent manner of arginase metabolism (Ishikawa et al. 2007).

Moreover, our results demonstrated that the infection by TgChBrUD2 strain-induced changes in cell morphology, resulting in a high number of spindle-shaped macrophages, which is a hallmark of M1 polarisation (Porcheray et al. 2005, Aldridge et al. 2009, Vogel et al. 2014). Curiously, the infection by both clonal strains culminated in a similar rate of round and spindle-shaped macrophages. However, although the literature considers the macrophage morphology as a parameter to classify M1/M2 subsets (Vogel et al. 2014), some previous studies demonstrate that an anti-inflammatory microenvironment can result in the spindle and/or round macrophages, which could partially explain our results observed in infected macrophages with clonal lineages (Verreck et al. 2004, Waldo et al. 2008, Jaguin et al. 2013).

Corroborating with our previous data, we also assessed the cell surface expression of CD64 (M1-marker), and CD163 and CD206 (both M2-markers) (Vogel et al. 2014, Akinrinmade et al. 2017). We observed that TgChBrUD2- and ME49-infected macrophages had a low expression of M2-markers and high expression of M1-marker. In addition, despite the high levels of CD64, the infection by RH strain also resulted in an augmented percentage of CD206 (M2-marker). Based on the literature and our data, we suggest that infected macrophages with TgChBrUD2 and ME49 strains assumed a M1 subtype, and the infection by RH strain resulted in M2 subtype. These findings are partially supported by Jensen et al. (2011), who working

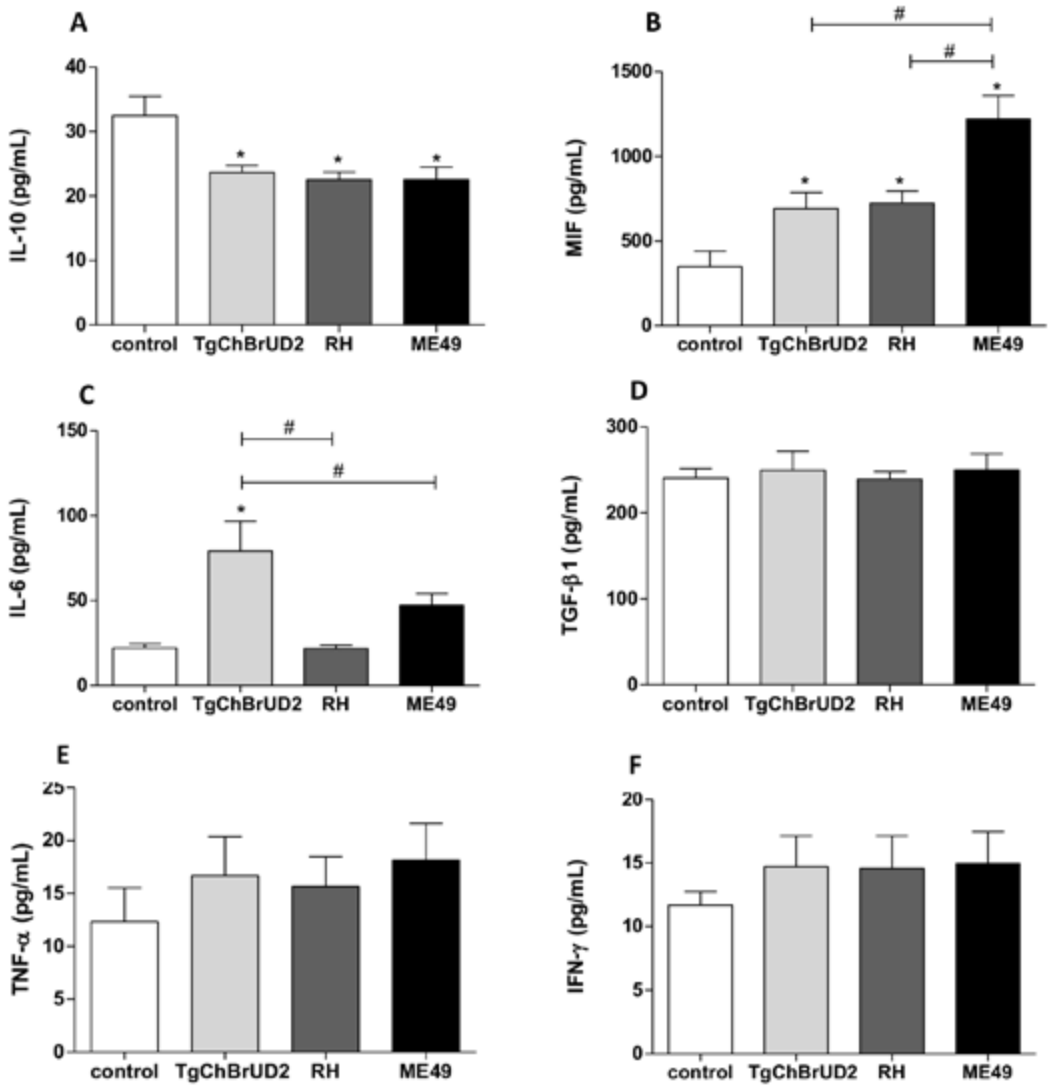

Fig. 5. Cytokine production in human macrophages infected or not by *Toxoplasma gondii* tachyzoites from TgChBrUD2, RH, or ME49 strains. THP-1 cells were differentiated into macrophages, cultured in 24-well plates for 24 h, infected or not by *T. gondii* (TgChBrUD2, RH, or ME49 strains) for 24 h and the supernatant was collected by posterior cytokine measurement. The IL-10 (**A**), MIF (**B**), IL-6 (**C**), TGF-β1 (**D**), TNF-α (**E**), and IFN-γ (**F**) cytokines levels were measured by ELISA and the data expressed in pg/ml. The results were expressed as mean ± SEM of three independent experiments performed in triplicate. *Comparison to uninfected control. #Comparison between different experimental conditions indicated by connector lines. Significant differences were analyzed using one-way ANOVA and Bonferroni's multiple comparisons test. Differences were considered significant when $P < 0.05$.

with clonal strains of *T. gondii* demonstrated that infected macrophages with type I (i.e., RH) and III strains are polarised towards an M2 activation state, whereas type II (i.e., ME49) strains are related with aspects of M1 polarisation (Jensen et al. 2011, Murray 2011).

Our research group has recently demonstrated that the atypical strain TgChBrUD2 possesses a highly virulent phenotype in animal models (Franco et al. 2014, Lopes et al. 2016). Interestingly, Franco et al. (2015) revealed that non-pregnant females of the rodent *Calomys callosus* (Rengger) chronically infected with ME49 and reinfected with TgChBrUD2 strain were more susceptible to infection during pregnancy, with a low survival rate and high morbidity score, as well as had a large number of pregnant animals with fetal reabsorption and a high faecal loss rate (Franco et al. 2015). Thus, we speculate that these severe manifestations triggered by TgChBrUD2 strain, especially in congenital toxoplasmosis, may be related, in part, to the pro-inflammatory M1 macrophage polarisation mediated by this strain. This is hypothesised by independent studies that have shown that M1 macrophages are essential players in eliciting and maintaining an exacerbated Th1 response at the maternal-faetal interface, thus contributing to inflammatory responses and abnormal pregnancy during *T. gondii* infection (Wang et al. 2011, Kong et al. 2015, Ning et al. 2016, Li et al. 2017, Park and Hunter 2020). To confirm this hypothesis further studies need to be thoroughly evaluated.

Macrophages play an important role during the immune response against *T. gondii* and the parasitic infection of

host macrophages affects their polarisation state, which can directly interfere with parasite control (Jensen et al. 2011, Kong et al. 2015). In agreement with the literature, our study demonstrated that the genotypes analysed were able to cause a distinct polarisation of human macrophages. In this scenario, an intriguing question is raised: What would be the impact of the activation state of the host macrophages on parasite control? Our results revealed that TgChBrUD2- and ME49-infected macrophages had a similar parasite burden and both presented a lower parasite intracellular proliferation compared to RH strain.

Previous studies using distinct experimental models have demonstrated a different infection rate for clonal and atypical strains. For example, it was shown that the infection of BeWo cells with TgChBrUD2 strain presented a lower replication index in comparison with TgChBrUD1 strain (Ribeiro et al. 2017). Similarly, Franco et al. (2019), using human villous explants from the third trimester of pregnancy, reported that TgChBrUD1-infected villous had a higher parasite burden compared to the infection caused by clonal (RH and ME49) or atypical (TgChBrUD2) strains (Franco et al. 2019). In contrast, experimentation with *C. callosus* demonstrated distinct susceptibility to both mentioned Brazilian atypical strains of *T. gondii*, where TgChBrUD2-infected animals had significantly higher parasite load in both liver and spleen than TgChBrUD1-infected animals (Franco et al. 2014). Thus, clonal and atypical strains can possess different behaviours in different experimental models.

Combining our data with the current literature, we propose that different *T. gondii* genotypes have specific virulence factors, which can modulate, in different ways, the host immune response. This modulation directly affects the parasite's ability to replicate within the macrophages, thus allowing the establishment of infection (Lüder et al. 2009, Hunter and Sibley 2012, Reese et al. 2014). To unveil the role of the host immune response on the control of infection, we measured the levels of pro- and anti-inflammatory cytokines released by human macrophages infected or not by different *T. gondii* strains. We observed that all investigated strains promoted an augmentation of MIF levels, but only the TgChBrUD2 strain was able to cause a strong up-regulation of IL-6. On the other hand, infection mediated by all strains culminated in a decrease of the IL-10 levels.

Several investigators have shown that IL-6 and MIF are essentials cytokines to impair *T. gondii* infection. It was shown that IL-6, a multifaceted cytokine, can control *T. gondii* parasitism in human monocytes and human trophoblastic cells (Castro et al. 2013, Barbosa et al. 2015); however, IL-6 has been associated with pathological conditions in some cases (Händel et al. 2012). Moreover, the literature reports that MIF production can be upregulated by parasite factors and this cytokine has a critical function in reducing *T. gondii* infection (Ferro et al. 2008, Flores et al. 2008, De Oliveira Gomes et al. 2011, Franco et al. 2019, Teixeira et al. 2020). Thus, we suggest that the low rate of parasite proliferation of TgChBrUD2 strain, reported in this study, is likely associated with the high levels of IL-6 and MIF produced by TgChBrUD2-infected M1 macrophages. In contrast, the reduced parasite burden in ME49-infected macrophages may be associated with other mechanisms, since the levels of IL-6 were lower in these cells than in macrophages infected by the atypical strain.

An additional and non-exclusive hypothesis to explain the lower parasite burden demonstrated in TgChBrUD2- and ME49-infected macrophages compared to the RH strain may be related to the time of parasite growth and host cell lysis. Literature findings have demonstrated that one tachyzoite of a type I strain is sufficient to generate high parasite loads *in vivo*; in contrast, a higher amount of type II tachyzoites is demanded to culminate in a similar parasite load rate (Saeij et al. 2005). In order to explain this phenomenon, some studies have reported that type I strains grow faster than type II or III strains. In addition, it has been shown that parasites belloging to the type I strain (e.g., RH strain) are able to lyses cultured cells much faster than the type II or III strains (Radke 2001, Sibley et al. 2002). Taken together, these parasite's abilites can result in a higher reinvasion rate of type I parasites. Supporting this hypothesis, the current literature reports that extracellular type I parasites remain infectious for a longer time compared with the type II or III strains, resulting in a higher infection rate, as well as higher parasite burden (Saeij et al. 2005).

Also, in agreement with Angeloni et al. (2009), it is possible to hypothesise that TgChBrUD2- and ME49-infected macrophages could suffer high levels of apoptosis in comparison to cells infected with RH strain, which trigger significant control of infection (Angeloni et al. 2009). Finally, human neuroblast cell line (SH SY5Y), microglial (CMH5) and endothelial cells (Hbmec) produced high levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) when infected with ME49 strain in relation to RH-infected cells, demonstrating that GM-CSF can contribute to a increase pro-inflammatory profile that control tachyzoites growth from type II strain in comparison to type I (Hakimi et al. 2014). Although we did not verify the GM-CSF levels in our study, it is possible to suggest that GM-CSF can influence in the difference on the parasite growth rate. However, to confirm these hypotheses, further studies need to be carried out.

In the present study, we observed that the infection of human macrophages with the highly (TgChBrUD2) or moderately virulent (ME49) strains showed similar results in parasite burden, as well as the ability to induce M1 macrophage polarisation. The polarisation is marked by an elevated production of pro-inflammatory cytokines; in contrast, the highly virulent (RH) strain, caused a higher parasite index and modulated the macrophage polarisation towards a M2 subtype. This hypothesis is corroborated by lower levels of pro-inflammatory mediators. The ability of *T. gondii* genotypes to modulate host cells in strain-specific ways has been previously demonstrated by our research group. Angeloni et al. (2009) showed that the infection by ME49 strain culminated in a predominant pro-inflammatory response, with the high secretion of MIF, IL-6, TNF-α and IL-12 by BeWo cells. In contrast, RH-infected cells re-

sulted in an anti-inflammatory microenvironment by high levels of TGF-β and IL-10 (Angeloni et al. 2009). Thus, different parasite strains of *T. gondii* can evolve in distinct manners to modulate important defence mechanisms and the host immune response maximise their intracellular survival (Angeloni et al. 2009, Jensen et al. 2011). Illustrating that, it was demonstrated that a distinctive unregulated proinflammatory immune response might favour vertical transmission of *T. gondii* and the development of severe clinical manifestations in human congenitally infected newborns (Gómez-Chávez et al. 2020).

Various *T. gondii* genotypes can induce opposite immune responses in infected human macrophages. Based on our data and the current literature, we hypothesised that the macrophage activation mediated by the RH strain might be related to modulation of the host's immune system to avoid immunopathological events caused by the excessive Th1 response during parasite infection (Mordue et al. 2001, Jensen et al. 2011). In contrast, the classic activation triggered by TgChBrUD2 and ME49 strains is likely associated with the maintenance of a Th1 response required for parasite control and, at the same time, paradoxically promoting an attempt to establish a chronic infection (Mordue et al. 2001, Nguyen et al. 2003, Jensen et al. 2011). However, we also have to consider that the TgChBrUD2 strain fails to establish a life-long chronic infection since this strain caused 100% of mortality of infected animals up to 10 days post-infection (Franco et al. 2014). Therefore, in agreement with our data, we suggest that the premature death of TgChBrUD2-infected animals may be due to hyper inflammation and/or heavy parasite burden in the tested experimental models.

In conclusion, this study demonstrated that the Brazilian atypical strain (TgChBrUD2) and clonal strains of *T. gondii* (ME49 and RH) elicit a distinct macrophage polarisation. Atypical (TgChBrUD2) and clonal (ME49) strains were able to elicit an M1 subtype, which results in parasitism control; in contrast, the clonal (RH) strain promoted a macrophage polarisation towards an M2 subtype, marked by a high parasite burden. Furthermore, atypical strains can present different behaviours than the classic clonal strains, thus highlighting the urgency for new studies regarding the underlying mechanisms triggered by atypical parasite genotypes and their interaction with multiple host types.

List of abbreviations

BeWo: human choriocarcinoma-derived trophoblast cell; CD: cluster of differentiation; DNA: deoxyribonucleic acid; FBS: fetal bovine serum; IL-: interleukin; iNOS: inducible nitric oxide synthase; MIF: macrophage migration inhibitory factor; PMA: phorbol 12-myristate 13-acetate; qPCR: quantitative real-time PCR; RIPA: radioimmunoprecipitation assay buffer; SDS: sodium dodecyl sulfate; TGF-β1: transforming growth factor-beta; THP-1: human monocyte-like cell line; TNF-α: tumor necrosis factor.

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Authors' contributions. PSGG and PSF conceived and designed the study, carried out the lab data analysis, interpreted the results, and drafted the manuscript. SCT and SF wrote, revised and edited the manuscript. PMG, TEA and DWFB contributed to the experimental manipulations. GLSF, AOG, JRM and BFB designed biological experiments, analysed the data, and participated in the data interpretation. EAVF coordinated the biological experiments, interpreted the data and edited the manuscript. All authors read and approved the final manuscript.

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